Evaluation of feed additives Nutrifen® and NutrifenPLUS® on broiler performance

by

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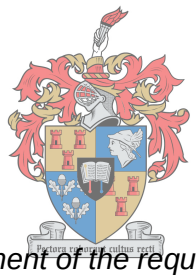

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

The main aim of this study was to determine the effects of feed additives Nutrifen® and NutrifenPLUS® on broiler performance over a period of 32 days. Two separate experiments were conducted; one to determine toxicity/safety of the additives, and another to measure a number of performance parameters relevant to the industry that may be affected by different concentrations of additive. In the case of the toxicity trial, a total of 60 Cobb500 mixed gender broilers were fed treatment diets containing 0% additive, 0.4% Nutrifen®, 0.4% NutrifenPLUS®, 0.2% Nutrifen®, 0.2% NutrifenPLUS® and 0.015% Zinc Bacitracin as the positive control. Birds were subsequently slaughtered at 14 days of age and analysed for gizzard erosion using a four point scoring system. No significant differences between treatments were reported in terms of gizzard erosion, implicating that both additives are non-toxic in this regard and safe to use at the specified levels. The main study was conducted using 360 mixed gender Cobb500 broilers with four treatment diets and a positive and negative control. Each treatment consisted of six replications and diets contained the following concentrations of additives: 0.2% Nutrifen®, 0.2% NutrifenPLUS®, 0.1% Nutrifen®, 0.1% NutrifenPLUS®, 150g/ton zinc bacitracin, and a negative control. All diets during both trials were maize and soya based, and formulated according to commercial specifications. Similarly, all birds were housed in the same facility and under the same environmental conditions according to Cobb500 guidelines, which were monitored closely throughout the house. Performance was determined as a function of three main areas of commercial significance, namely production parameters (live weight, feed intake, feed conversion ratio, European production efficiency factor, protein efficiency ratio, average daily gain and mortality), organ and tibia bone characteristics (absolute and relative organ weights, liver colour CIE-Lab colour meter, intestinal pH, tibia bone breaking strength), as well as meat quality and carcass characteristics (carcass weight, dressing percentage, commercial cut proportions, proportions of breast components, muscle colour using a CIE-Lab colour meter, and pH and chemical composition of breast muscle). No significant differences were observed with regard to any production parameters and in terms of meat quality and carcass characteristics, very few parameters differed significantly between treatments. Only redness (a*) of the breast muscle and meat fat percentage showed any statistical differences, with supplementation of 0.2% NutrifenPLUS® and 0.2% Nutrifen® reducing the values of each parameter respectively, relative to the negative control. Similarly, no significant differences were reported in terms of organ weights or liver colour, and tibia bone characteristics showed few statistically significant differences. Only one tibia bone parameter was affected significantly by treatment; this being the calcium:phosphorus ratio measured from the bone ash. Supplementation with 0.1%

NutrifenPLUS® differed significantly from both control diets, and 0.2% NutrifenPLUS® produced a significantly lower ratio relative to all other treatments.

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Notes

The language and style used in this thesis are in accordance with the requirements of the *South African Journal of Animal Science*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters is therefore unavoidable.

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List of abbreviations

a*	Redness
ADG	Average daily gain
AGP	Antibiotic growth promoter
Al	Aluminium
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
b*	Yellowness
B	Boron
BWG	Body weight gain
°C	Degrees Celsius
Ca	Calcium
Cu	Copper
DAFF	Department of Agriculture, Forestry and Fisheries
EO	Essential oil
EPEF	European production efficiency factor
FCR	Feed conversion ratio
Fe	Iron
FI	Feed intake
g	Grams
GH	Growth hormone
GIT	Gastro-intestinal tract
GI Tract	Gastro-intestinal tract
GLM	General linear model
HDL	High density lipoprotein
IBD	Infectious bursal disease
K	Potassium
kg	Kilogram
L*	Lightness
LD	*Longissimus Dorsi*
LDL	Low density lipoprotein
LW	Live weight
m	Meter
Mg	Magnesium
ml	Millilitre
mm	Millimetre
Mn	Manganese
N	Newton
Na	Sodium
ND	Newcastle disease
P	Phosphorus

PER	Protein efficiency ratio
PFA	Phytogenic feed additive
pHi	Initial pH
pHu	Ultimate pH
PSE	Pale soft exudative
SAPA	South African Poultry Association
SI	Small intestine
TD	Tibial dyschondroplasia
WHO	World Health Organization
Zn	Zinc
%DM	Percentage dry matter

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Chapter 1

General Introduction

With a rapidly expanding human population (FAO, 2016) and broiler meat being the staple protein source of so many (SAPA, 2012), maintenance of modern day high growth and feed efficiencies is becoming more of a necessity. This coupled with a rising concern over antibiotic resistance and product residues are placing the poultry industry under immense pressure to find alternative sources of growth promotion (Alloui *et al.*, 2014). Considering that orally administered antibiotics perform their function primarily by altering gut microbe content, composition and metabolic activity as well as destruction of pathogens (Dibner & Richards, 2005), it can be inferred that any supplement or combination of supplements resulting in similar changes to the microbial community may yield comparable growth responses.

Historically, a variety of phytogenic substances such as herbs, spices and aromatic plants have been used as traditional healing remedies, exhibiting antimicrobial, anti-inflammatory (Yang *et al.*, 2015), antidiabetic (Ar *et al.*, 2013) and hypocholesterolemic effects among numerous other properties (Paravar *et al.*, 2013). The modes of action concerning these aspects are well-documented in many plants *in vitro* (Kamel, 2001) and are believed to be brought about by the presence of specific bioactive compounds (Brenes & Roura, 2010). The effects of such substances and their modes of action are however less consistent *in vivo* and require further research (Windisch *et al.*, 2008), hence the current study. Variable performance results may also arise from differences in quality and quantity of bioactive substances present in many plant based additives (Burt, 2004). With such a variety of different animal and plant species and the inevitable variability between and within those species, it has proven difficult to standardise the use of phytogenic additives in modern agriculture (Burt, 2004); especially with such a limited understanding of their *in vivo* activity (Windisch *et al.*, 2008).

Further argument in favour of phytogenic additives as growth enhancer is that they may present solutions to other adverse effects, that have to a certain extent been induced by intense selection for fast growth. In past times broiler carcass fat has typically ranged between eight and fifteen percent, however recent studies commonly show carcass fat levels in excess of 18% (Nikolova *et al.*, 2007). These elevated fat concentrations tend to create an extra processing cost, and discourage health conscious consumers as well as reduce shelf-life through increased lipid oxidation (Fasseas *et al.*, 2008), which have been some of the major driving forces behind the search for effective methods of fat regulation (Paravar *et al.*, 2013).

These regulation strategies have presented themselves in the form of a number of plant extracts that have been reported to hold antioxidative (Brenes & Roura, 2010) and hypocholesterolemic properties (Paravar *et al.*, 2013), as well as the ability of certain bioactive compounds to influence glucose metabolism through various hormonal pathways (Pearson, 2009). Furthermore, improved mineral uptake especially calcium and phosphorus as a result of phytogenic supplementation, may lead to more efficient bone mineralization (Ziaie *et al.*, 2011) and in turn reduce the likelihood of bone breakage during slaughter and processing as well as fewer metabolic diseases during production (Whitehead, 2007), which is a big issue faced by poultry producers today. In addition, other meat quality characteristics such as colour may be enhanced (Pirmohamammadi *et al.*, 2016; Warris, 2000).

The aim of the current study was to test the effects of fenugreek supplementation against those of a commonly used antibiotic in the poultry industry, with regard to production and certain meat quality aspects. Fenugreek was administered in the form of two commercially available products; Nutrifen® and NutrifenPLUS® in the diet. With a closed system, controlled environment, and strict biosecurity measures in place, it was expected that few differences would be observed between treatment groups and that fenugreek supplementation would result in production performance values similar or comparable to those of the antibiotic treatment. If any differences were to be observed, it was suspected that they would be with regard to parameters that are not typically influenced by degree of pathogenic exposure. Pathogenic impact was determined in this case by relative lymphoid organ weights i.e. spleen and bursa, where enlargement would reveal some level of exposure. Intestinal pH was also considered as a form of indication as to any direct buffering effects, or possible shifts in microbial population dynamics. In addition, a toxicity study was included to expose any ill effects that may be associated with fenugreek supplementation on the gizzard, and also to assess feed quality; mainly with regard to the presence of possible toxins.

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Chapter 2

Literature Review

2.1 Introduction

A range of non-nutritive feed additives are used in modern poultry production, the most well-known being antibiotics. Antibiotics have been added in sub-therapeutic amounts to poultry diets for more than 50 years (Alloui *et al.*, 2014), in order to promote growth through various pathways in the gastrointestinal tract (Dibner & Richards, 2005) . With the recent awareness of antibiotic resistance and proposed restrictions (Allen *et al.*, 2013), coupled with a rapidly expanding human population and the consequent need for intensification (FAO, 2016); the importance of finding alternative sources of growth and health promotion has been emphasized (Cowan, 1999).

Although exact mechanisms of antibiotic growth promoters (AGP's) action are not completely understood, it is clear that their benefits stem from changes made to the gastro-intestinal environment and the resident microbial population (Dibner & Richards, 2005). These alterations may include reductions in pH, competitive exclusion of pathogens and shifts in commensal microflora dynamics, direct reduction of overall microbial load, stimulation and development of the intestinal immune system, as well as direct pathogenic destruction (Visek, 1978; Gaskins *et al.*, 1997; Dibner & Richards, 2005). It is highly likely that a number of these benefits occur simultaneously in

the gastro-intestinal tract of antibiotic-fed birds (Gaskins *et al.*, 1997), and are linked to improved nutrient digestibility and absorption from the digestive tract as well as enhanced overall gut health (Kamel, 2001). Where high growth rates and low feed conversion ratios are a priority as they are in monogastric species like poultry and pigs, it is important to note that these two factors are often a reflection of the dynamic balance of the intestinal environment (Gaskins *et al.*, 1997).

Numerous other feed additives such as pro- and pre-biotics, as well as organic acids have also been employed in attempts to alter the state of the GIT environment advantageously (Hume, 2011; Costa *et al.*, 2013). Their modes of action however appear slightly different to those of antibiotics (Khan & Iqbal, 2016), and results with regard to performance enhancement have been largely inconsistent (Fascina *et al.*, 2012; FAO, 2016). Similar can be said for plants and various plant-derived substances, although many are known to possess potentially beneficial properties such as antimicrobial, anticoccidial and anti-oxidative potencies in a number of animals and humans (Lahucky *et al.*, 2010; Laila & Murtaza, 2015). Certain herbs

and spices have also been found to improve aspects of product quality such as meat colour and fat content in broilers (Paravar *et al.*, 2013). Advantages associated with phytogenic supplementation are thought to be instigated by the presence of specific bioactive compounds found in a variety of aromatic plants (Kamel, 2001) including fenugreek; a spice most well-known for its diverse medicinal attributes (Ar *et al.*, 2013).

2.2 The modern broiler and repercussions of intensification

Bone status is a commonly used indicator of mineral adequacy in poultry diets, especially calcium and phosphorus (Ziaie *et al.*, 2011). Although there is a poor consensus on what the actual requirements are for Ca and P regardless of growth rate (Angel, 2011a), it is clear that these minerals are essential for proper bone formation and development, as dietary deficiencies often lead to skeletal abnormalities and related leg deformities (Thorp & Waddington, 1997). Development can also be influenced by a number of other factors however, including nutrition, genetics, gender, age and absolute growth rate (Ziaie *et al.*, 2011).

Sufficient bone formation is most commonly determined by its breaking strength and ash content, which in turn is influenced by the level of mineralization (Thorp & Waddington, 1997). Thus, variation in the Ca/P ratio is likely to cause changes in the mineral crystal structure, meaning that an abnormal ratio could result in weaker bones (Thorp & Waddington, 1997). Modern broiler strains genetically selected for fast growth often show a predisposition for highly porous cortical bones and related leg abnormalities (Shim, *et al.*, 2012). These bones generally have a low ash content (Williams *et al.*, 2000) and inadequate breaking strength, indicating an insufficient level of mineralization; often leading to a higher risk of fracturing under the gravitational stress of increased muscle mass (Ziaie *et al.*, 2011). Higher incidence of fracturing/splintering during slaughter and processing is also common amongst broilers with faster muscle deposition, resulting in possible carcass and meat downgrades (Whitehead, 2007). Such osteoporotic fractures during the course of production can incur substantial economic loss as well; usually a consequence of higher mortality rates, decreased feed intake, and an ensuing reduction in feed efficiency and body weight gain (Shim, *et al.*, 2012). Furthermore, they pose a serious animal welfare concern as birds often suffer through considerable pain and discomfort (Whitehead, 2007).

Appearance is an important aspect of meat and meat products that determines consumer preference in most markets (Ponsano *et al.*, 2004), which is no less true for poultry products, especially egg yolk, meat and skin colour (Breithaupt, 2007). Colour is commonly associated with age, animal health status and overall quality of the product (Breithaupt, 2007), which tend

to influence the decision of the consumer to buy the product and the decision to re-purchase respectively.

In more extensive farming systems where birds are raised on maize and grass based diets; adequate xanthophyll intake ensures greater pigmentation and more visually appealing meat (Ponsano *et al.*, 2004). With a shift towards intensive farming practices however, faster growing modern broilers fed primarily on nutrient dense diets do not ingest sufficient xanthophylls; resulting in less carcass pigmentation (Castañeda *et al.*, 2005). To counteract this phenomenon and conform to consumer acceptability, many producers add colorants to the birds' diet in the form of natural or synthetic oxycarotenoids (xanthophylls), which provide pigmentation by selective deposition or accumulation in different animal tissues (Breithaupt, 2007). Nowadays there has been a tendency for producers to use natural colorants which are derived primarily from plants, algae and specific microbes (Ponsano *et al.*, 2002) as they may include a number of benefits, without any of the possible carcinogenic effects of synthetic colour additives (van Esch, 1986).

Certain plants are also thought to influence the rate and extent of muscle acidification *post-mortem*, which in turn affects colour changes that occur in a muscle when it is converted to meat (Pirmohamammadi *et al.*, 2016). During the development of *rigor*, muscle proteins tend to denature, and myofibrillar proteins get closer to their isoelectric point as a result of lactic acid build-up (Warris, 2000). The result is a reduction in water-binding capacity and increased light scattering properties of the contractile elements of the muscle fibres, which leads to increased exudation of fluid and a paler colouration (Warris, 2000). This occurs in all muscles *post-mortem*, however if pH levels fall too rapidly or too low, a condition known as PSE can develop; a condition which comes about from a rapid initial decline in pH at higher temperatures, and a low ultimate pH, causing meat to appear abnormally pale and display excess exudation when packaged (Alvarado & Owens, n.d.). Fortunately this is not a common occurrence in broilers, as small carcasses tend to cool relatively quickly before muscles become too acidic (Warris, 2000).

The chemical composition of broiler meat is equally as important for health-driven and gourmet markets, due to its fat content (Erener *et al.*, 2011). With a shift in consumer preference toward organic and natural poultry products (Paravar *et al.*, 2013), the food industry has been obliged to include natural antioxidants as a way of improving the quality and nutritional value of food products (Velasco & Williams, 2011), and increasing the oxidative stability of poultry meat (Paravar *et al.*, 2013). This has encouraged nutritionists to consider the use of phytogenic products as a way of manipulating poultry fat composition, as many of these additives are known to possess hypocholesterolemic effects and antioxidative potencies (Paravar *et al.*,

2013). These attributes may allow phytogenics to serve as alternatives to synthetic antioxidants, extending the shelf-life of poultry meat and being more acceptable to the health-conscious consumer (Fasseas *et al.*, 2008).

Fat load has become a major problem in modern broiler production, where intense selection for high growth rates and final body weight, have induced excess fat deposition (Nikolova *et al.*, 2007). Although other factors such as nutrition, sex and age also have a strong influence on broiler carcass fat, a clear trend has emerged over an extended period with regard to growth rate (Tumova & Teimouri, 2010). In the past, carcass fat of broilers generally ranged between 8- and 15%, whereas modern fast-growing lines regularly exhibit in excess of 18% (Nikolova *et al.*, 2007). Selective breeding for lower carcass fat content may seem like the obvious solution, however this would almost certainly lead to a reduction in fat-free body weight, as fat and live body weight (LBW) are genetically correlated to some extent (Becker *et al.*, 1979). Again, this has encouraged the use of phytogenic supplements to manipulate fat deposition and composition, and potentially reduce overall fat content (Paravar *et al.*, 2013). It has also proven difficult to reduce fat in any specific portion of the carcass, since fat deposition in one part of the body is related to the total body fat percentage of the individual bird (Becker *et al.*, 1979).

2.3 Antibiotics

2.3.1 Role in agriculture

For more than 50 years antibiotics have been included in poultry feeds (Alloui *et al.*, 2014) as therapeutic agents, prophylactics and growth promoters, but in the last decade their inclusion has raised growing concern amongst the global agricultural community (van Vuuren, 2001). Their prolonged use in modern agriculture has been a key contributor to the development of antibiotic resistant strains of bacteria, the accumulation of antibiotic residues in both animal products and the environment in less developed countries and also to the gradual destruction of symbiosis between the birds and their desirable flora (Alloui *et al.*, 2014).

Some bacteria have always been intrinsically insensitive to antibiotics regardless of their previous exposure, but most resistant strains have come about as a result of genetic change (van Vuuren, 2001). These genetic alterations are acquired via genetic mutations or the transfer of genetic material from resistant bacteria to more sensitive strains (Courvalin, 1994). In essence, the administration of sub-therapeutic antibiotic doses creates an intense selection pressure in favour of resistant or resilient bacteria; allowing them to amplify even in the presence of therapeutic doses of the same antibiotic (Aarestrup, 1999). These resistant bacteria can remain part of the animal's intestinal flora right up until the time of slaughter where

contamination of the carcass could occur during processing (Rasschaert *et al.*, 2007). Such strains are relatively common in food animals, with chickens often harbouring food-borne pathogens such as *Campylobacter* and *Salmonella*; bacteria which could then be passed on to humans via the food chain (van Vuuren, 2001). These bacteria could pose more of a threat if resistant to a number of commonly used antibiotics in the field of medicine.

The use of antibiotics can also result in the deposition of residues in the final animal product (Livingston, 1985). These residues are of concern for two reasons; they could have direct toxic effects on humans, or they could lead to the gradual alteration of microflora in the human gut which could promote the development of antibiotic resistant strains of bacteria, and consequently the failure of antibiotic therapy for clinical purposes (Nisha, 2008). According to Nisha (2008), this situation requires that stringent restrictions be placed on the use of AGP's in animal feed worldwide, with both the EU and the USA having already taken action (Allen *et al.*, 2013).

2.3.2 Intestinal microbiota of the broiler

Young animals, from the time they are born are exposed to a succession of different microbial communities in the gut, which have a massive influence on a variety of physiological, immunological, nutritional and protective functions of the GIT (Dibner & Richards, 2005). These factors in turn, play a crucial part in the development, overall health and performance of the animal (Richards *et al.*, 2005). It has also been proven in a number of experiments, that various commensal bacteria play a pivotal role in the development of certain organs, tissues and the immune system (McCracken & Gaskins, 1999), as well as providing the animal with a variety of important nutritional components (Wostmann, 1996). Although these microbes are of vital importance to the overall development of the animal and provide a multitude of benefits, they also compete with the host for valuable nutrients, produce toxic compounds that may limit nutrient absorption, as well as inducing a permanent immune response in the GIT (Richards *et al.*, 2005). These all come as an energy expense to the host and depress production performance thereof (Dibner & Richards, 2005).

Before birth/hatching, the gut of a monogastric animal is generally sterile, with microorganisms beginning to colonize the GIT immediately post-partum (Yin *et al.*, 2010). These microbes originate from a number of sources, namely the mother, the diet and exposure to the surrounding environment (Dibner & Richards, 2005). Initially, aerobic bacteria and facultative anaerobes such as *E. coli* and *Lactobacilli* appear (Mackie *et al.*, 1999) in the proximal portion of the gut (Anderson *et al.*, 2000), multiplying rapidly to form a reduced environment, which later allows the colonization and establishment of the obligate anaerobes that form the predominant community of microflora in the small intestine (Dibner & Richards, 2005). It is

also important to note that although the environmental influence on the extent of colonization and the composition of microflora is significant, the animal itself seems to possess internal selection mechanisms that ensure the correct progression of colonization (Dibner & Richards, 2005).

Different areas of the GI tract are also preferentially colonized by different species of microbes with some not being as densely populated as others (Dibner & Richards, 2005). For instance, because of a high digesta flow/throughput rate and low pH fewer, more acid-tolerant bacteria inhabit the proximal area of the small intestine as opposed to the ileum, colon and caeca. The ileum exhibits a much greater number of bacterial cells and a wider diversity compared to the duodenum, and the large intestine due to its slower passage rate is even more heavily colonized (Gaskins *et al.*, 2002).

These microflora provide a number of benefits to the host animal, most notably, the competitive exclusion of pathogens or non-indigenous bacteria (Snel *et al*., 2002), production of nutritional compounds in the form of fermentation products (Dibner & Richards, 2005), and the development of the hosts' intestinal defences (Gabriel *et al.*, 2006).

Intestinal bacteria play a vital role in the development of the GIT immune system in monogastric animals (Gabriel *et al.*, 2006). This is illustrated by the fact that bacteria-free animals tend to show a considerably less mature gut immune system, with underdeveloped lymphoid organs corresponding to lower B- and T-cell counts and antigen concentrations (Wostmann, 1996). It has also been established that certain species or bacterial colonies stimulate the GIT immune system to varying degrees, making them more or less important to the maturation of the hosts' defences (McCracken & Gaskins, 1999). It is clear therefore, that certain bacteria are required for immunogenic stimulation and development (Snel *et al*., 2002), although it is thought that some growth-promoting antibiotics may act by decreasing the concentration of immunogenic microorganisms that inhabit the small intestine (Anderson *et al.*, 2000). This may limit the amount of inflammation and energetic costs associated with the elicited immune response, thereby promoting growth efficiency. Under such circumstances, the housing conditions of the animal become a factor to consider, where the trade-off between immune competence and reduced local inflammation associated with lower energy costs, can determine production potential (Anderson *et al.*, 2000).

Although the intestinal microflora provides a number of advantages, they also come at great cost to the animal (Richards *et al.*, 2005). It is generally accepted that GIT microorganisms compete with the host for nutrients (Pan & Yu, 2014), however they also increase mucous production and epithelial cell turnover rate, reduce fat digestibility and produce toxic

compounds such as amino acid catabolites that may have a significant impact on growth performance and overall health status of the animal (Dibner & Richards, 2005). Furthermore, considerable competition exists between microbial activity and the host in the small intestine for energy (Gaskins *et al.*, 2002) and amino acids (Apajalahti & Vienola, 2016). This has been observed in pigs where available glucose is used by bacteria to produce lactic acid, depriving the host of potential energy for growth. Furthermore, the presence of lactic acid in the gut stimulates greater peristaltic activity and higher digesta passage rates, reducing overall digestibility and nutrient absorption (Saunders & Sillary, 1982). According to work by Reeds *et al.*, 1993 the skeletal muscles and GI tract of young, rapidly growing animals also compete for the same limited supply of nutrients, with gut microbiota using up to 6% of total dietary amino acids (Apajalahti & Vienola, 2016). This would imply that a reduction in overall microbial load brought about by the use of AGP's may be a primary mechanism by which improved growth performance is often achieved.

There are two major compounds produced by intestinal bacteria that are toxic or harmful to monogastric animals, namely phenolic/aromatic compounds and ammonia (Anderson *et al.*, 2000). Phenolic compounds such as 4-methylphenol and 3-methylindole (skatole) are highly toxic; produced by bacterial degradation of tryptophan and tyrosine in the distal portion of the gut and excreted via the urine (Deichmann & Witherup, 1943). It is suggested that these compounds may have a substantial growth depressing effects, since negative correlations exist between the urine concentration of 4-methylphenol and body weight gain observed in weanling pigs (Yokoyama *et al*., 1982); a postulate which is supported by the fact that these compounds are not produced or excreted by bacteria-free rats (Bakke & Midtvelt, 1970). In addition, it has also been demonstrated that the inverse relationship between weight gain and volatile phenol excretion in rats is reversed by the oral administration of the antibiotic, chlortetracycline (Bernhart & Zilliken, 1959), indicating that inhibition of the production of phenolic compounds by certain GIT bacteria may be the main mechanism by which antibiotics promote growth (Anderson *et al.*, 2000).

Apart from microbially-produced toxic compounds, the inhibition of microbial bile acid transformation in the gut is also thought to depress growth performance in monogastrics (Anderson *et al.*, 2000). It has been suggested that the deconjugation and dehydroxylation of bile by microbes present in the GIT may limit the absorption of lipids by the host animal and result in the production of toxic by-products (Eyssen, 1973). Very few studies have been done to support this theory, however one such study by Madsen *et al.*, 1976 showed that bile acids are not deconjugated in the gut of bacteria-free animals.

Different types of bacteria may lead to the production of one or more of the abovementioned toxins; however it is important to note that Gram positive facultative anaerobes that dominate the small intestine often produce all three metabolites and can be considered a primary target for AGP's or their alternatives (Anderson *et al.*, 2000).

Most microorganisms present in the gastrointestinal tract are considered commensal or symbiotic, but some varieties can also have detrimental effects on the health status of the bird (Dumonceaux *et al.*, 2006). This relationship between a bird and its gut microbiota can therefore be viewed as a dynamic balance between mutualism and pathogenicity (Farthing, 2004) which may be manipulated through the feeding of non-nutritive additives such as antibiotics to promote growth and feed efficiency (Anderson *et al.*, 2000; Gaskins *et al.*, 2002). With this being said, microbial activity in the large intestine seems more beneficial to the host than harmful (Gaskins *et al.*, 2002), producing a number of useful fermentation products; however the primary energy absorption site is that of the small intestine (Thomke & Elwinger, 1998); Anderson *et al.*, 2000). This suggests that microbial activity in this area is likely to have a greater impact on growth performance (Anderson *et al.*, 2000).

2.3.3 Modes of action

Antibiotics have proven to be highly effective growth promoters over many decades and have been linked to increased body weight gain, independent of feed intake; responses which have been partially associated with improved protein metabolism (Anderson *et al.*, 2000) as they are observed regardless of the protein concentration in the diet (Gaskins *et al.*, 2002). Their exact pathways and mechanisms of action however are not completely understood, although their action is unquestionably focused on the gut, since most orally-administered antibiotics are not absorbed (Dibner & Richards, 2005). With this knowledge considered, at least four different primary mechanisms have been proposed. In addition, the effectiveness of antibiotics most likely lies with more than one, if not all of these modes of action (Gaskins *et al.*, 1997) and all four mechanisms share a common postulate that intestinal bacteria, whether they be pathogenic or commensal, inhibit or depress animal growth in some way (Gaskins *et al.*, 1997). This is strongly supported by the fact that antibiotics are only effective under sub-optimal conditions (Ferket, 2004). Furthermore, the inoculation of germ-free animals with commensal GI bacteria tends to reduce growth rate (Coates, 1980) suggesting that they too have a negative effect on production.

It is generally accepted that antibiotics reduce the overall microbial load of the GIT by direct interaction with the intestinal bacterial community. This forms the base of the four different mechanisms that have been established thus far, and provides an explanation as to the

reduction in competition for nutrients and the reduction of growth-inhibiting secondary microbial metabolites (Visek, 1978).

Firstly, reducing sub-clinical infection would relieve the animal of some immune obligation and ultimately leave more energy available for growth (Allen *et al.*, 2013). Furthermore, lowering overall microbial load would reduce the presence of growth-depressing metabolites and nutrient utilization by GIT bacteria, again increasing the amount of dietary energy available to the host (Anderson *et al.*, 2000). The use of antibiotics has also been associated with a thinner intestinal wall, thereby enhancing nutrient uptake into the bloodstream and resulting in better feed efficiency (Visek, 1978). This reduction in intestinal (small intestine) wall thickness comes from a lower concentration of toxins and secondary metabolites produced by gut microflora, which generally irritate the lining of the small intestine (SI) and depress the efficiency of nutrient uptake by the animal (Ibraheim *et al.*, 2004). There is also considerable evidence to suggest that AGP's modify the composition, ratios and activities of the gut microbial populations (Visek, 1978). This indicates that the most likely mechanism is the one which entails microbially-induced growth depression of the host being reversed by metabolic inhibition, or the complete elimination of responsible microorganisms via the dietary inclusion of antibiotics (Coates, 1980).

2.4 Phytogenic additives

Growing public awareness with regard to bacterial resistance in particular, has led to a greater urgency in the quest for sustainable AGP alternatives, with plant extracts/ phytogenic compounds starting to show potential as adequate replacements (Alloui *et al.*, 2014). Phytochemicals are defined as plant-derived natural bioactive compounds that have positive effects on animal growth and health (Yang *et al.*, 2015). These are generally contained within a relatively narrow range of specialised plants; serving as interaction mechanisms between the plants and surrounding environments, as well as protection against herbivores and/or pathogens and, physiological and environmental stresses (Wenk, 2003).

Phytogenic feed additives can be classified into four major groups, based primarily on their origin and required processing techniques (Van der Klis & Vinyeta, 2014). Herbs are flowering, non-woody and non-persistent plants (Windisch *et al.*, 2008) which have been found in many cases to improve nutrient digestibility and uptake, by direct stimulation of both the immune and endocrine systems (Wenk, 2003). Spices are the second major class of phytogenics; a group of plants commonly used in human foods due to their aromatic nature and intense flavours (Windisch *et al.*, 2008). The third and possibly the most important group known as essential oils (EO's), are volatile lipophilic compounds derived by cold expression and/or

steam and alcohol distillation (Windisch *et al.*, 2008) from a variety of herbs and spices (Brenes & Roura, 2010). These along with various other active compounds, are thought to be responsible for the majority of benefits associated with phytogenic supplementation.

For decades, many different varieties have been incorporated into animal feeds in order to improve productivity and maintain animal health, however the industrialisation of poultry husbandry has placed greater emphasis on their inclusion in modern broiler diets (Alloui *et al.*, 2014). In fact many phytogenic feed additives (PFA's) have exhibited similar effects on the GIT to those of some organic acids and antibiotics. Some of these effects include reduced microbial load, fewer fermentation products, less activity associated with the lymphatic system of the gut and improved digestion and nutrient utilisation, without incurring the collateral effects of antibiotic growth promoters (Windisch *et al.*, 2008). These outcomes tend to reflect a better overall gut equilibrium (Windisch *et al.*, 2008), consequently leading to positive reverberations on growth performance and/or feed conversion in many cases (Yatoo *et al.*, 2012; Weerasingha & Atapattu, 2013; Mamoun *et al.*, 2014; Al-Beitawi & El-Ghousein, 2015). Improvements in growth performance have also been linked to morphological changes in the jejunum, where increased villi height and crypt depths lead to greater surface area for the absorption of a variety of nutrients (Jamroz *et al*., 2005).

Several benefits have been documented in a number of animal species as a consequence of phytogenic supplementation thus far, including improved growth rates in broilers (Alloui *et al.*, 2012), better egg quality parameters in layers (Awadein *et al.*, 2010) and enhanced lactation performance in both goats and dairy cows (Kholif & El-Gawad, 2001). Observations such as these have largely been attributed to the anti-oxidative, antimicrobial and anti-inflammatory properties of many herbs, spices, essential oils and other secondary metabolites found in plant extracts (Yang *et al.*, 2015); equating to responses such as improved digestibility, nutrient absorption and the destruction of pathogens in the animal gut (Kamel, 2001; Balunas & Kinghorn, 2005; Athanasiadou *et al.*, 2007).

With regard to such acclimation, phytogenic feed additives and their *in vitro* effects have been well-documented; however their modes of action are still relatively unclear (Kamel, 2001). Furthermore, similar to other additives, results concerning growth and feed efficiency have been inconsistent and rather limited (Windisch *et al.*, 2008). Other limitations with using phytogenic additives may include things such as side effects, regulatory obstacles and economic viability, which could also challenge their implementation in the near future (Yang *et al.*, 2015). In addition to this, botanical origin, type of plant, harvest season, transformation and composition of plants and their extracts, geographical origin and processing technique

could all influence phytochemical efficacy (Windisch *et al.*, 2008), further complicating the relationship between mode of action and aspects of their application (Alloui *et al.*, 2014).

2.4.1 Properties and modes of action

A whole host of different molecules are contained within various parts of certain plants and their extracts; many of which possess intrinsic bio-activities on animal physiology and metabolism as well as the gut microbial population (Kamel, 2001). It is well-known that most desirable performance effects observed in poultry are due to essential oils and other secondary metabolites, which commonly exhibit an impact on digestive secretions, immune response, gut pathogens, blood circulation and exert antioxidant properties (Brenes & Roura, 2010).

According to Yitbarek, 2015, overall gut function is influenced primarily by digesta passage rates, digestive enzyme activity and digestive secretions. An optimal balance between these factors would then theoretically lead to improved nutrient utilization and ultimately better growth performance. It has been observed on numerous occasions, where essential oils and other phytogenic substances have resulted in enhanced digestive enzyme activity and nutrient absorption from the from the small intestine, which could potentially improve feed and growth efficiency in broilers (Rao *et al.*, 2003). Furthermore, essential oils have been observed to have a stimulatory influence on intestinal mucous secretion, which is thought to obstruct the adhesion of pathogens to the epithelial lining, thereby stabilizing the microbial equilibrium in the GIT (Jamroz *et al*., 2005). Performance enhancements in such cases were also attributed to morphological alterations in the jejunum, including increased villi height and greater crypt depth induced by the supplements.

Many phytogenic compounds are best known for their antimicrobial capabilities against specific bacteria and some fungal species *in vitro* (Allen *et al.*, 1997). It is apparent that many bioactive plant compounds have the ability to influence the cell wall characteristics of certain microbial cells, thereby altering their putative virulence properties and ultimately killing them (Kamel, 2001). Essential oils perform this action by increasing the permeability of bacterial cell walls, causing leakage of intracellular compounds and death as a result (Burt, 2004). It is also important to note however that essential oils are far more effective against gram positive bacterial species, as 90-95% of a gram positive bacterial cell wall consists of peptidoglycan, which is easily penetrated by hydrophobic molecules (Yang *et al.*, 2015). Gram negative bacteria however, tend to have a much thicker outer membrane making them less permeable and more resistant to the entry of such molecules (Trombetta *et al.*, 2005). This being said, gram negative bacterial membranes can still be penetrated by essential oils, although much higher doses are required (Alloui *et al.*, 2014). Organic acids are however generally accepted

to be more effective against such gram negative bacteria, meaning that a combination of the two treatments may provide a more comprehensive intestinal equilibrium shift (Zhou *et al.*, 2007).

Furthermore, the activity and effectiveness of supplementation can be highly variable depending on physico-chemical characteristics of the bioactive compounds and the bacterial strains being targeted (Sari *et al.*, 2006). Effects may also vary according to the location of the functional alkyl or hydroxyl group of the essential oil; this generally being the main determinant of the level of activity that a compound has on different microbial species (Yang *et al.*, 2015). Another important consideration is the fact that, like antibiotics essential oils do not distinguish between pathogenic and commensal bacteria (Alloui *et al.*, 2014), however they generally have little effect on *Bifidobacteria* and *Lactobacilli* which form the bulk of the GIT bacterial population (Dibner & Richards, 2005; Alloui *et al.*, 2014; Oakley *et al.*, 2014). In addition, these microbes are included in most probiotic formulas, meaning that it would be possible to use probiotics in conjunction with these additives (Alloui *et al.*, 2014).

Another point of interest is the anti-oxidative properties possessed by many plant derived substances, especially those of phenolic terpenes and flavenoids (Cuppett & Hall, 1998). Dietary supplementation with plants, or extracts rich in these compounds could contribute to dietary lipid protection from oxidation, reducing the chances of spoilage as well as providing the final products with a certain degree of oxidative stability (Brenes & Roura, 2010). In this way, both feed quality could be improved and meat shelf-life could be extended. The use of phytogenic substances for this purpose is however less cost effective than currently used antioxidants, although further development of processing techniques and intensification of specific plant species could alleviate the economic impact somewhat (Alloui *et al.*, 2014).

2.4.2 Fenugreek

Fenugreek (*Trigonella foenum-graecum*) is an annual legume crop (Thomas *et al.*, 2011) native to North Africa and countries that border the eastern Mediterranean (Ar *et al.*, 2013). It is cultivated all over the world (Alloui *et al.*, 2014) for its multifunctional characteristics, but is used mainly as a spice for its intense flavour and aroma in many countries (Thomas *et al.*, 2011). The plant is grown primarily in India, Pakistan and China, however as mentioned before, it is widely distributed (Alloui *et al.*, 2012). The seeds in particular are known for their bitter, pleasantly sweet taste and are used in both ground and whole forms to add flavours to teas, curry powders and spice blends (Wani & Kumar, 2016). Aside from its potential as a flavourant it has become well-known throughout the world for its excellent medicinal and nutritional properties, with the seeds being the most valuable component (Ahmad *et al.*, 2015). Its therapeutic prospects include effects such as anti-oxidative, antidiabetic (Ar *et al.*, 2013),

hypoglycaemic, anti-inflammatory (Mandegary *et al.*, 2012), antibacterial, and antimicrobial properties (Moradi kor & Moradi, 2013). Furthermore, fenugreek seeds are known to possess immunological activity (Wani & Kumar, 2016). Nutritional properties of the seeds are equally as impressive; containing high levels of copper, manganese and potassium (Nour & Magboul, 1986), saponins (Rao & Sharma, 1987), protein, fibre, oleic, linolenic and linoleic acids and vitamins A, B_1, B_2 and C (Ahmad *et al.*, 2015). According to literature protein levels range from 20-30% (Rao & Sharma, 1987, Faiza *et al.*, 2015, Nour & Magboul, 1986) with high proportions of lysine, leucine and tryptophan being reported. Fenugreek proteins unfortunately also have low methionine content, making them comparable to those of other commonly used legumes (Rao & Sharma, 1987) such as soybean (Elmahdy & Elsebaiy, 1985).

According to Khan *et al.*, (2009) the most important components contained in fenugreek seeds are saponins, fenugreekine, nicotinic acid, phytic acid, trigonelline, scopoletin and coumarin, which are presumed to account for some its numerous therapeutic effects (Ar *et al.*, 2013). For example, fenugreekine has been known to increase peripheral glucose utilization in humans, leading to improved pancreatic function (Ar *et al.*, 2013). Poultry meat is also renowned as a source of unhealthy fat and cholesterol (Mallika *et al.*, 2009), in fact it contains two to three times the amount of polyunsaturated fat as a weight percentage than most red meats (Simopoulos, 2002). The WHO, 1999 stipulates that dietary fat should make up 15-30% of total calorie intake of which saturated fat should make up roughly 0-10%. Modern broiler meat has consistently been found to contain approximately 15-20% fat of which 85% is not required for normal physiological function (Choct *et al.*, 2000). Excess broiler carcass fat is therefore considered as a waste of dietary energy by producers and is seen as a waste product by consumers, ultimately making it an economic loss to the poultry industry (Fouad & El-Senousey, 2014). Limiting the presence of this fat in the meat would therefore add great value to the broiler industry as a whole.

Phytochemicals known as steroidal saponins, and more specifically diosgenin, have been linked to significant reductions in serum cholesterol in mice and humans (Cayen & Dvornik, 1979; Ar *et al.*, 2013). This is believed to be the result of lower plasma cholesterol concentrations and increased overall cholesterol excretion. It has been suggested that diosgenin increases faecal cholesterol excretion by the stimulation of biliary cholesterol secretion and by reducing absorption in the intestine, which ultimately reduces deposition in the meat tissue (Temel *et al.*, 2009). With fenugreek seeds containing approximately 4.8% saponins (Laila & Murtaza, 2015), it is possible that their product derivatives may hold a solution to fat restriction in the modern high-fat broiler strains.

2.4.3 Polyphenolic compounds of fenugreek

2.4.3.1 Saponins

Fenugreek seeds have long been known to stimulate human appetite (Singletary, 2017); a property which is thought to arise from the presence of a steroidal saponin component known as diosgenin (Cantox, 2008). Saponins are naturally occurring surface-active glycosides derived mainly from plants (Das *et al.*, 2012), but also from lower marine animals and some rhizo bacteria (Yoshiki *et al.*, 1998). These can have a wide variety of biological properties depending on modifications to the ring structure of the aglycone moieties, and the number of sugars attached to them (Das *et al.*, 2012). Dietary saponins are however poorly absorbed through the intestinal membrane, which means that their biological functions are performed primarily in the GIT (Cheeke, 1996), much like antibiotics (Dibner & Richards, 2005).

Compounds such as diosgenin have been associated with increases in insulin sensitivity (Gupta *et al.*, 2001) and lower concentrations of serum low-density lipoproteins (LDL) (Hannan *et al.*, 2003), which both play a major role in the regulation of the appetite-regulating hormone, ghrelin (Cantox, 2008). Experimental obesity models indicate that this is brought about, by the promotion of adipocyte differentiation and the inhibition of adipose tissue inflammation (Raju & Rao, 2012). Heightened insulin sensitivity seems to stimulate the release of ghrelin from ghrelinergic cells in the GIT into the bloodstream, where it has the potential to trigger feed intake, or inhibit appetite depending on its octanoylation status (Pearson, 2009).

Enzymes associated with serum lipoproteins, especially LDL, are involved with the breakdown of ghrelin from its active octanoylated form to its degradation/non-active form, desacyl ghrelin which inhibits appetite (De Vriese *et al.*, 2007); thus any compounds such as diosgenin that result in a greater HDL:LDL ratio would favour the active form, thereby stimulating feed intake rather than inhibiting it (Pearson, 2009). Furthermore, ghrelin has been found to regulate the release of growth hormone (GH) from the pituitary gland of rats (Lee *et al.*, 2007), as well as stimulate immune cell activation and inflammation (Klok *et al.*, 2007) which could lead to improved growth efficiency in poultry. Saponin-based adjuvants themselves also possess the unique ability to stimulate cell-mediated responses and antibody production in the GIT (Barr *et al.*, 1998), which could reduce pathogenic stress on the animal under sub-optimal conditions. The exact mechanisms by which saponins stimulate the immune system are not completely understood, but evidence suggests that they act by increasing the uptake of antigens from the gut and through other membranous surfaces, thereby initiating a more intense response (Das *et al.*, 2012). In addition, many saponins found in fenugreek seeds also have antimicrobial properties, forming complexes with sterols present in bacterial cell membranes, thereby causing the cells to lyse (Morissey & Osbourn, 1999).

It is also common belief that certain saponins are responsible for a reduction in intestinal ammonia production, thereby reducing air pollution in the housing environment and relieving health stress on the animals (Windisch *et al.*, 2008). In support of this notion is the fact that the feeding of the active saponin components extracted from *Yucca schidigera* to broilers has been observed to reduce intestinal and faecal urease activity (Nazeer *et al.*, 2002).

2.4.3.2 4-Hydroxisoleucine

4-Hydroxyisoleucine is a unique non-proteinogenic amino acid found in fenugreek seeds (Laila & Murtaza, 2015), and similar to sapogenin has proven influential on blood glucose and insulin release (Sauvaire *et al.*, 1998; Avalos-Soriano *et al.*, 2016). This amino acid possesses insulinotropic biological activity, allowing it to increase glucose-induced insulin secretion via direct stimulation of the islets of Langerhans (Sauvaire *et al.*, 1998), which could in turn have an overall effect on energy metabolism (Laila & Murtaza, 2015).

2.4.3.3 Alkaloids

Trigonelline is a major alkaloid component present in fenugreek seeds, having been known to exhibit hypoglycemic, hypolipidemic, antibacterial, antiviral and anti-tumour effects (Zhou *et al.*, 2012). Furthermore, there is evidence to suggest that it too, has an influence on glucose metabolism by altering the activity of related enzymes and stimulating insulin secretion (National Center for Biotechnology Information, 2017).

2.4.3.4 Flavenoids

A number of flavonoids have been isolated from fenugreek seeds, most notably vitexin, tricin, naringenin, quercetin and tricin-7-O-beta-D-glucopyranoside (Shang, *et al*., 1998). The most bioactive of these being quercetin, which is a strong antioxidant (Laila & Murtaza, 2015). Studies suggest that quercetin has a number of beneficial properties such as anti-inflammatory, antioxidant, anti-tumour, antidiabetic and immunomodulatory effects, making it a compound of interest when considering phytogenic extracts (Laila & Murtaza, 2015).

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Chapter 3

Evaluation of Nutrifen® and NutrifenPLUS® as non-nutritive feed additives and their impact on selected production parameters in broilers

Abstract

In this study, Nutrifen® and NutrifenPLUS® were investigated as possible antibiotic growth promoter (AGP) alternatives. Their effects on production parameters including live weight and body weight gain, feed intake, feed conversion ratio (FCR), average daily gain (ADG, European production efficiency factor (EPEF), protein efficiency ratio (PER) and liveability were determined. For the purpose of the trial, 360 day-old Cobb500 mixed gender broilers were raised on a total of six different treatment diets over a period of 32 days. Ten birds were randomly placed into each of the 36 cages to be used, with each cage being assigned one of the six treatment diets. Each treatment diet shared the same basal formulation, with only the type or concentration of feed additive being altered. Treatments consisted of six replications and contained the following non-nutritive feed additives: negative control (no additive), positive control (150g/ton zinc bacitracin antibiotic), 0.1% Nutrifen®, 0.2% Nutrifen®, 0.1% NutrifenPLUS® and 0.2% NutrifenPLUS®. Birds were fed *ad libitum* throughout the trial and feed intake and body weight data was collected weekly. All data was collected in the early morning, before the birds were fed. Results showed no significant differences between treatments and growth rate (live weight and cumulative live weight gains), and feed intake (weekly and cumulative). Similarly FCR, ADG, EPEF, PER and liveability all showed no significant differences between treatments.

3.1 Introduction

Antibiotic growth promoters are antibacterial compounds that can be added to the feed or water of a commercial production animal in sub-therapeutic amounts; done over an extended period in order to enhance feed efficiency (Allen *et al.*, 2013). The quest for antibiotic alternatives has been emphasized in recent times however, mainly due to the inadvertent emergence of antibiotic resistance (van Vuuren, 2001). A major problem with the development of resistance, is the crossover of certain antibiotics between the agricultural industry and the treatment of human disease (Hume, 2011), which has influenced the call for restrictions in the use of human health related antibiotics for animal production (Allen *et al.*, 2013). By the beginning of 2006, the use of all antibiotics for growth promotion purposes was banned in the European Union (EU), with the USA also having proposed restrictions (Franz *et al.*, 2010). For the sake of animal well-being however, both still allow the administration of therapeutic doses over shorter timescales, or to treat specific bacterial infections (Allen *et al.*, 2013). These restrictions have led to a greater need for alternative sources that result in comparatively reliable performance enhancements, without the collateral effect of antibiotics (Windisch *et al.*, 2008). Possible alternatives could be narrowed down, if a better understanding of the mechanisms of antibiotic action is established (Allen *et al.*, 2013).

In animals such as pigs and poultry where high growth rates are a primary objective, the hosts' response to gut bacteria and the implications of this require special attention (Gaskins *et al.*, 2002). The numerically dense and metabolically active microbiota of the gastrointestinal tract (GIT) are typically viewed as a beneficial entity to the host (Gaskins *et al.*, 1997; Gaskins *et al.*, 2002), as they perform both nutritional and defensive functions (Dibner & Richards, 2005); although the host animal must first invest substantially in sequestering the gut microbes away from the epithelial surface and mounting rapid immune responses against organisms that do manage to breach initial defences (Gaskins *et al.*, 2002). Antibiotic inhibition of normal microbiota may therefore reduce maintenance costs of the gastro-intestinal system, and improve nutrient utilization, ultimately leading to enhanced growth performance (Gaskins *et al.*, 1997; Dibner & Richards, 2005). Improved growth efficiency could also be acquired by targeting specific pathogens or altering the hosts' immune response to harmful bacteria over time, thereby reducing pathogenic burden on the animal (Looft & Allen, 2012). These theories share a common postulate that both pathogenic and commensal bacteria depress growth of the animal, either directly or indirectly though their metabolic activities (Anderson *et al.*, 2000; Gaskins *et al.*, 2002; Dibner & Richards, 2005). In support of this, the growth performance of pathogen-free broilers is not enhanced by the oral administration of antibiotics (Visek, 1978), whereas pathogen-free animals inoculated with gastro-intestinal (GI) bacteria, results in

depressed growth rates (Coates, 1980). It is also well-known that antimicrobial growth promotors (AGP's) are only effective growth promoters under suboptimal conditions (Ferket, 2004). Furthermore, pathogen-free animals have a thinner small intestinal wall, less lymphoid components, thinner *lamina propria* and more slender villus structure, and a slower epithelial cell turnover time indicating a lesser immune response (Coates, 1980). Seeing as skeletal muscles and the GIT draw from the same limited nutrient supply i.e. compete for resources (Reeds *et al.*, 1993), an extended renewal time may reduce basal energy expenditure and improve the efficiency of nutrient utilization for overall growth (Coates, 1980).

Attention has been drawn to certain plant species/extracts as potential AGP replacements (Ferket, 2004; Brenes & Roura, 2010) for their various beneficial properties. Fenugreek in particular is well-known for its nutritional and medicinal attributes, with the seeds being well-documented for having anti-oxidative, antidiabetic (Ar *et al.*, 2013), hypoglycaemic, antimicrobial (Moradi kor & Moradi, 2013), hypocholesterolemic, anti-carcinogenic, (Wani & Kumar, 2016) and anti-inflammatory (Yang *et al.*, 2015) effects, as well as immunological activity (Wani & Kumar, 2016). Most of these benefits have been attributed to the abundance of polyphenolic compounds such as galactomannan (fibre), diosgenin (saponin), quercetin (flavonoid), trigonelline (alkaloid) and 4-hydroxyisoleucine (amino acid) (Laila & Murtaza, 2015). With this being said, numerous studies have been performed on fenugreek, as well as various other herbs and spices to determine whether a plant or plant extract/s could have comparable benefits to those of antibiotics in the poultry industry. The most commonly used phytogenic compounds in poultry production are derived from plants such as oregano, cayenne, pepper, mint, cinnamon, anise, garlic, thyme, chilli, sage and rosemary among others (Yitbarek, 2015).

Many of today's extracts contain a variety of specific bio-active compounds from which their multifunctional benefits are derived (Kamel, 2001; Costa *et al.*, 2013). Most of these compounds have intrinsic bio-activities on animal physiology and metabolism (Kamel, 2001) of which not all are advantageous (Raju & Rao, 2012); however a vast number of the molecules which account for these actions are still unknown (Kamel, 2001). More information regarding dose response, toxicity, metabolism, maximum residue level (MRL) and response under different conditions is therefore required in the field of phytogenics, with the intent of establishing an international database for the purpose of all commercial animal production (Kamel, 2001).

3.2 Materials and Methods

3.2.1 Birds and housing

For the purpose of this study, 360 day-old unsexed Cobb 500 broiler chicks were raised to an age of 32 days. Chicks were obtained on the morning of hatching from County Fair hatchery five in Klapmuts, Western Cape and placed into poultry house C, at Mariendahl experimental faculty of Agrisciences and is located in the Western Cape, roughly 14 km from the main Stellenbosch campus. The experimental facility was equipped with raised (1.07m above floor) wire cages that measured 0.9m x 0.6m, each housing 10 birds. Chicks were obtained from the same parental group to reduce genetic variation, and were all vaccinated against Newcastle disease (ND) and Infectious bursal disease (IBD) before leaving the hatchery.

When chicks arrived at the farm, they were separated into groups of 10. These groups were weighed to get the initial mass, and placed at random into the experimental pens/cages. Treatments had already been randomly assigned to the cages before the birds were placed. In total 36 pens were used, with 10 birds being placed into each pen. Six treatments were applied from day one until the end of the trial with each treatment consisting of six replications. The mass of all 10 birds in each pen was recorded weekly throughout the trial, so that an average individual bird live weight could be calculated for each treatment. On weighing days during the trial, feed was removed before the lights came on in the house so that birds could be weighed before feeding and an accurate mass could be recorded. Feed weights were also recorded initially, after which remaining feed weights were recorded weekly throughout the trial using the same scale that was used to weigh the birds. The remaining feed weights were then used to calculate average weekly and cumulative feed intake per bird. The data gathered above was then used to calculate feed conversion ratio (FCR), European production efficiency factor (EPEF) and average daily gain (ADG) using Equation 3.1,**Error! Reference source not found.** and a linear regression respectively. Mortalities were recorded daily and accounted for, before any calculations were performed.

Initially, small feed trays and bell drinkers were provided but as the chicks grew larger, larger feed troughs were used. From week two, bell drinkers were also removed for an hour after feeding to stimulate the use of the automated nipple drinkers in the cages. Once the birds could use the nipple drinkers effectively, the drinkers were raised at regular intervals as the birds grew and bell drinkers were removed permanently.

3.2.2 Management and experimental conditions

A temperature controlled housing system was used and the facility was completely sealed so that no draughts could enter and the temperature could be maintained effectively. All aspects of the poultry house such as temperature, humidity and ventilation were tested over a period of four days, seven days prior to the chick's arrival, to ensure that all components of the facility were functioning at optimal level. The house was also run at suitable conditions 24 hours prior to the beginning of the trial to ensure that the correct conditions were reached and maintained before the chicks were placed in the facility. Feed trays and drinkers were all washed thoroughly and disinfected before the commencement of the trial. A footbath containing disinfectant was placed in the entrance to the house so that everyone entering the facility could immediately disinfect their shoes, and the number of people entering the experimental facility was also kept to a minimum for biosecurity reasons.

Conditions inside the experimental facility were strictly monitored and regulated according to the commercial Cobb500 management guidelines throughout the trial period. Light hours and temperature inside the facility was reduced accordingly over the course of the experiment. For the first seven days, the facility was checked at least once every two hours to ensure that the temperature, ventilation, humidity and lighting were being maintained within certain specified boundaries so as to limit mortalities. Birds were checked constantly for abnormal behaviour, which could have been an indication of one of the aforementioned aspects being incorrect, or a sign of potential disease. Feed and water levels were also monitored constantly to ensure that all birds were eating and drinking normally. Equations for determination of feed conversion ratio (FCR) and European production efficiency factor (EPEF) is shown in Equation 3.1 and 3.2 below.

Equation 3.1 $$\text{FCR} = \frac{\text{Cumulative feed intake per bird (g)}}{\text{Average liveweight gain per bird (g)}}$$

Equation 3.2 $$\text{EPEF} = \frac{\text{Liveability (\%)} \times \text{Liveweight gain (kg)}}{\text{Age (days)} \times \text{feed conversion ratio}} \times 100$$

3.2.3 Treatments and feed formulation

As mentioned, six treatments diets were applied in this trial with each treatment consisting of six replications. These treatments were assigned randomly to each of the 36 cages to avoid bias. All feed was mixed in the feed mixing facility at the Mariendahl experimental farm in the small scale commercial ribbon mixer. Birds were allocated 900g, 1200g, 1200g of starter, grower and finisher diets per bird respectively over the duration of the trial, with each diet being

formulated according to commercial nutrient specifications. The diets and nutrient specifications can be seen below in

and Table 3. respectively. Different levels of supplementation were included in the treatment diets, with each containing the following concentrations of additive: no additive, 150g/ton Zinc bacitracin antibiotic, 0.1% Nutrifen®, 0.2% Nutrifen®, 0.1% NutrifenPLUS®, 0.2% NutrifenPLUS®. Feed was provided in a mash form, since the Nutrifen® diets could not be pelleted, as only a relatively small quantity of feed was being mixed. All birds were fed on an *ad libitum* basis and provided with clean drinking water throughout the trial.

Constituents of the treatment additives can be seen below:

Nutrifen®

Fenugreek cotyledon concentrate (*Trigonella foenum-graecum*)

NutrifenPLUS®

Fenugreek cotyledon concentrate (*Trigonella foenum-graecum*) - 72%
Fennel seed powder (*Foeniculum vulgare*)
Saw Palmetto berry powder (*Serenoa repens*)
Brown Kelp powder (*Laminariales*)
MSM (naturally-sourced methylsulfonylmethane)
Apple cider vinegar powder

Table 3.1 Table of ingredients used for the three-phase basal treatment diets

Ingredient	Starter (%)	Grower (%)	Finisher (%)
Maize	45.09	61.62	66.06
Soybean full fat	11.53	18.00	21.61
Soybean 47	27.78	13.96	8.82
Fish meal 65	5.36	3.00	0.00
L-lysine HCl	0.18	0.11	0.13
DL-methionine	0.40	0.17	0.15
L-threonine	0.11	0.00	0.04
Vitamin + mineral premix	0.20	0.25	0.25
Filler	0.20	0.20	0.20
Limestone	1.45	1.14	1.15
Salt	0.12	0.23	0.29
Monocalcium phosphate	1.22	1.15	1.22
Sodium bicarbonate	0.16	0.17	0.08
Sunflower oil	6.20	0.00	0.00
Total	**100**	**100**	**100**

Table 3.2 Nutrient compositions of the basal treatment diets used as formulated and as mixed

		Starter		Grower		Finisher	
Nutrient	**Units**	**Formulated**	**Actual**	**Formulated**	**Actual**	**Formulated**	**Actual**
AME	MJ/kg	12.65	-	13.00	-	13.40	-
CP	%	25.00	24.22	20.00	18.41	17.39	16.25
Fat	%	10.94	10.94	6.18	6.70	6.67	6.46
Moisture	%	10.64	10.03	-	10.89	-	10.90
Ash	%	5.03	6.76	-	6.05	-	6.23
Dry Matter	%	89.36	89.97	-	89.11	-	89.10
Crude Fibre	%	3.12	2.30	3.22	2.87	3.24	2.29
Arginine	%	1.66	-	1.31	-	1.13	-
Avl. Lysine	%	1.43	-	0.68	-	0.65	-
Lysine	%	1.60	-	1.19	-	1.00	-
Isoleucine	%	1.14	-	0.90	-	0.77	-
Leucine	%	2.09	-	-	-	-	-
Methionine	%	0.80	-	0.51	-	0.43	-
T.S.A.A.	%	1.19	-	0.83	-	0.72	-
Threonine	%	1.07	-	0.78	-	0.71	-
Tryptophan	%	0.29	-	0.24	-	0.20	-
Valine	%	1.26	-	0.99	-	0.84	-
Linoleic Acid	%	3.57	-	3.04	-	3.43	-
Avl. Phosphorus	%	0.50	-	0.42	-	0.38	-
Calcium	%	1.05	-	0.84	-	0.76	-
Chloride	%	0.23	-	0.22	-	0.22	-
Potassium	%	-	-	0.79	-	0.73	-
Sodium	%	0.16	-	0.18	-	0.15	-
Total Phosphorus	%	-	-	0.67	-	0.62	-

AME = apparent metabolisable energy; CP = crude protein; Avl. = available; T.S.A.A. = total sulphur containing amino acids

3.2.4 Proximate analysis of treatment diets

Diets were analysed by proximate analysis, to ensure that protein, fat and energy as well as ash, moisture and fibre were present in concentrations similar to those of the formulated treatment feeds. Before undergoing analysis, 200-300g of each feed sample was homogenized using a 1.5mm hammer mill.

3.2.4.1 Moisture

Moisture content was determined according to the AOAC Official Method 934.01 as advised by the Association of Official Analytical Chemists as part of the AOAC International (2002). Briefly, 2g of each feed sample was weighed accurately into a moisture-free crucible before being dried at 100°C for 24 hours. Samples were removed from the oven, allowed to cool for 30 minutes in a desiccator and weighed.

3.2.4.2 Ash

Ash content was analysed according to the AOAC Official Method 942.05, as advised by the Association of Official Analytical Chemists as part of the AOAC International (2002). Moisture-free samples, previously used to determine moisture content were placed in a furnace at a temperature of 500°C for 6 hours. Samples were placed in a desiccator for 30 minutes before being weighed.

3.2.4.3 Crude fat

Crude fat determination was done by means of acid hydrolysis as described by the AOAC Official Method 954.02, advised by the Association of Official Analytical Chemists as part of the AOAC International (2002). Briefly, 2g of each sample was weighed accurately to 0.001g into test tubes. After the addition of ethanol (2ml) and HCL (10ml), test tubes were placed in a water bath for 30-40 minutes. Test tubes were then allowed to cool to room temperature, and the contents poured into separating funnels. The following steps were then performed: addition of 25ml diethyl ether, shake for 1 minute, addition of 25ml of petroleum ether and shake for 1 minute. The upper portion of the solution was then poured into a fat cup. This process was then repeated twice more using 15ml and 25ml of diethyl ether and petroleum ether respectively. Fat cups were then placed in a sand bath, to allow the ether to evaporate. Once all ether had evaporated, fat cups were allowed to cool in a desiccator for 30 minutes and then weighed to determine the fat percentage.

3.2.4.4 Crude protein

Crude protein was determined by means of the official AOAC Dumas combustion method 992.15 as described by the Association of Official Analytical Chemists (2002). The homogenized samples (0.1000g) were analysed for quantitative nitrogen content using the LECO FP528 which was calibrated with EDTA before use. Nitrogen content could then be used to calculate the crude protein percentage by multiplying by a factor of 6.25.

3.2.4.5 Crude fibre

Crude fibre analysis was performed by means of an ANKOM machine, as described by the Association of Official Analytical Chemists, Official Method 962.09 as part of the AOAC International (2002). In summary, 0.95-1.00g of sample was weighed into marked filter bags. Bags were heat-sealed and allowed to soak in petroleum ether for 10 minutes to extract fat from the samples. It should be noted that blank bags of known weight were included in all processes so as to correct for moisture. Bags were then placed on a dry surface to air-dry and subsequently placed on the bag suspender in the ANKOM. Ambient temperature acid solution (0.255N H_2SO_4) was then poured into the fibre analyser vessel and the samples heated and agitated for 40 minutes. The vessel was then rinsed with water (50-90°C) twice, for a total of 10 minutes before the addition of an ambient temperature base (0.313N NaOH). The same agitating, heating and rinsing process was then followed as with the acid solution. Bags were then gently squeezed to remove excess water and placed in acetone for 3-5 minutes. The bags were removed from the acetone and the acetone left in the bags was allowed to evaporate for approximately 10 minutes. Bags were placed in an oven for 2-4 hours at 100°C to extract all remaining moisture and subsequently weighed. Bags were ashed in a furnace at 600°C for 2 hours to calculate the loss of organic matter.

3.2.5 Statistical analysis

The following hypotheses were proposed:

H_0: There is no association between production parameters and the treatment diets applied.

H_a: There is evidence to suggest that there is an association between the specified production parameters and the treatment diets applied.

The assumptions of normality and homoscedasticity were tested for each data set using Statistica GLM (general linear models), before any further analysis was performed. Data that satisfied these assumptions was analysed by means of a one-way ANOVA (Least Square

Means) and a Fisher's LSD *post hoc* test. In cases where data did not satisfy the assumptions, Games-Howell non-parametric *post hoc* tests were performed.

A significance level of 5% (p-value $\leq$0.05) was used to declare statistical significance in all analysis, whereas a p-value $\leq$0.01 (1%) was considered highly significant. Furthermore, the one-way ANOVA that was performed can be explained by the following model: $Y_{ij} = \mu_i + \alpha_j + \varepsilon_{ij}$, where Y_{ij} *is* the response variable, μ_i the overall mean, α_j the treatment effect and ε_{ij} the unexplained error.

Average daily gain (ADG) was analysed in a different manner to the other data sets. Initially a simple linear regression line was fitted to the production data obtained from each cage, in order to determine the relationship between age (X) and live weight (Y). From the linear regression, mean live weight gain over time was then established. A mean for each treatment was subsequently drawn from the data for further analysis. Following this procedure, a one-way ANOVA and Fisher's LSD *post hoc* test was performed on the ADG means, to reveal any statistical significance.

The simple linear regression fitted to the live weight data in order to interpret ADG can be explained by the following model: $Y_i = \beta_0 + \beta_1 X_i + \varepsilon_i$, where Y_i is the response/dependent variable, β_0 the intercept of the best-fitting line, β_1 the slope of the best-fitting line, X_i the treatment value corresponding to the independent variable, and ε_i the unexplained error associated with the treatment effect that could not be explained by the regression line. The gradient of the line of best fit was used to find average values, which were analysed by means of a one-way ANOVA.

3.3 Results and Discussion

3.3.1 Live weight

The live body weight (LBW) of Cobb500 broiler chickens was not affected to any significant extent by the dietary inclusion of Nutrifen® or NutrifenPLUS® (p>0.05). This was found to be the case throughout the trial. Refer to Table 3. for LBW results. The same can be said for cumulative live weight gains throughout the entire 32 day period (p>0.05), as indicated by Table 3..

Looking at available literature, phytogenic supplementation in general has shown varying results depending on factors such as genetics, plant and animal species, additive concentration, stage of production and stress factors. Most studies involving fenugreek supplementation in broiler diets report significant increases in LBW body weight at some point

during their experiments (Alloui *et al.*, 2012; Khan *et al.*, 2013; Sahoo *et al.*, 2013; Weerasingha & Atapattu, 2013; Mamoun *et al.*, 2014; Abed & Kadhim, 2014), which was also found to be true for weaned piglets (Kumar *et al.*, 2014) as well as meat rabbits (Zeweil *et al.*, 2015). Although this trend was common to all of the studies mentioned, results were highly inconsistent. In some cases, LBW was increased in a linear fashion as the concentration of dietary fenugreek increased (Khan *et al.*, 2013), whereas in others, smaller amounts of fenugreek proved to be more effective than higher concentrations (Weerasingha & Atapattu, 2013). Also, the LBW and body weight gain (BWG) parameters showed significant variation at different stages of production. For example, Abbas (2010) determined that fenugreek had no significant effect on LBW at 42 days but reduced LBW compared to the control group at 21 days of age. Similarly, Duru *et al.* (2013) reported significantly lower live body weights and body weight gains at days 21 and 42. At day 21, this was especially true for fenugreek supplemented at higher concentrations (4%), however by day 42 the concentration was found to be less influencial. In agreement with Duru *et al.* (2013), Khadr & Abdel-Fattah (2007) observed a linear decrease in LBW corresponding to increasing dietary fenugreek supplementation. These results are in complete contrast to Alloui *et al.* (2012) and Al-Beitawi & El-Ghousein (2015) where weight gain was significantly improved by 0.3% (fenugreek) and a 0.2% (aniseed, cumin, thyme mixture) supplementation respectively, at both 21 and 42 days.

In another study by Sahoo *et al.* (2013) LBW was measured only at day 42, but significant differences between fenugreek treatment and control diets were observed. In this case, the treatment diet containing the highest level of herbal supplementation produced a 7% increase in LBW compared with the control group. This could however, be partly due to the presence of two other herbs that were included in the supplement as well as fenugreek. The two other components of the herbal mixture were *Commiphora mukul* and *Allium sativum*, which coincidently share many common properties with fenugreek. In a similar study by Abdel-Rahman *et al.* (2014), a herbal supplement containing tumeric and fenugreek was used as a form of treatment, but LBW was determined at regular intervals in this case. Improvements were only observed during weeks four and five however, and not at day 42 as with Sahoo *et al.* (2013). Again, the inclusion of more than one phytogenic source in every treatment makes it difficult to attribute improved performance to either one of the herbs/spices.

Abed & Kadhim (2014) reported that fenugreek supplemented at a concentration of 1% led to lower LBW values in the first two weeks of treatment, but significantly higher LBW values in weeks three, four and five of the study. In this case however, replications were insufficient and evidence should not be regarded as conclusive. With this being said, a similar and more conventional study by Elagib & Elamin (2013) supported these findings to a certain extent.

Lower live weights were observed when birds were fed a diet containing 2% fenugreek in the starter phase (days 1-21), but were considerably higher during the finisher phase (21-42 days). In fact, fenugreek supplementation out-performed all other treatments during the latter half of this experiment.

Prabhudas (2015) tested fenugreek as a partial soya replacement and found that LBW gain was improved significantly in comparison to the control group between the age of 15 and 28 days. These superior live weight gains were attributed to the fact that the fenugreek seeds used in the experimental feeds were roasted. According to Prabhudas (2015), roasting the seeds reduces their bitterness, making them more palatable. This probably accounts for the greater feed intake during this period of enhanced growth performance. Increased growth rates were also attributed to the possible presence of antimicrobial phenolic acids which could have sterilized the gastrointestinal tract, resulting in enhanced growth performance. There is however little evidence to support this theory, but further investigation on the subject may be of value to the broiler industry. Khan *et al.* (2009) stated that any increases in muscle weight are due to the antioxidant properties of fenugreek seeds, which increase the concentration of digestive enzymes and reduce bacterial activity in the gut. There is also a lack of substantial evidence to support this theory, and reasons for improved growth performance in certain cases are still largely unknown.

Looking at available literature as a whole, trends with regard to improved growth performance with fenugreek supplementation are typically observed towards the end of the trial periods. Most notably around the 42 day mark, at which point birds are under considerable stress from their exceedingly high muscle mass and generally insufficient skeletal support. The application of dietary fenugreek has also proven effective during periods of prolonged heat stress. Prajapat (2016) explored the potential of fenugreek as a performance enhancer under tropical conditions, where the average ambient temperature throughout the study was 36 °C and reached extremes of 40 °C inside the pens during the day. In this case, fenugreek supplementation enhanced growth performance considerably throughout the entire treatment period. Furthermore, improvements in growth rate were also observed by Elagib & Elamin (2013) in a similar study, but only during the latter stages.

The current trial somewhat supports a theory of fenugreek being more effective as a growth promoter under stressful conditions, as does Kassu *et al.* (2016). No external stressors were present in these cases and no discernable differences were observed in LBW or BWG; birds were handled constantly and raised by the same people throughout the trial, conditions were strictly monitored and maintained according to Cobb500 commercial broiler guidelines in a controlled environment, and birds were allowed adequate space and access to feed and water

at all times. In both of these studies, fenugreek supplementation was included in the treatment diets at the same concentrations of 0.1- and 0.2%. Unlike the current trial however, Kassu *et al.* (2016) extended the study until day 49, which still yielded a lack of evidence to suggest that fenugreek is an effective growth promoter under non-stressful conditions. Another study which seems to support this theory, is that of (Weerasingha & Atapattu, 2013). In this study it was mentioned that the birds were moved from a floor system to a raised wire cage system midway through the trial. It was also stated that blood samples were taken from birds on the same day. This process and complete change of environment is likely to have caused considerable stress, and could explain some of the variation observed between treatments. Two pieces of available literature do however contradict this theory, where similar observations were made with no mention of any possible stress factors (Elbushra, 2012; Mamoun *et al.*, 2014). The improved growth performance observed by Mamoun *et al.* (2014) can most likely be attributed to the substantially higher feed intake's, however the results of Elbushra (2012) are more complex and difficult to explain. Further research is therefore warranted in this area.

Table 3.3 Mean live weight (±SE) as influenced by the inclusion of Nutrifen® and NutrifenPLUS® in the diet of Cobb500 broiler chickens over a period of 32 days

Treatment	Day 0 (g)	Day 7 (g)	Day 14 (g)	Day 21 (g)	Day 28 (g)	Day 32 (g)
Neg. CON	44 ± 1.2	160 ± 4.2	441 ± 15.8	883 ± 43.5	1476 ± 38.1	1950 ± 53.8
Antibiotic	44 ± 1.1	163 ± 5.6	441 ± 16.6	883 ± 36.2	1478 ± 51.2	1968 ± 52.5
0.1%N	44 ± 1.0	166 ± 5.4	452 ± 18.2	886 ± 38.9	1465 ± 51.9	1940 ± 60.4
0.2%N	44 ± 1.0	159 ± 8.8	435 ± 21.8	866 ± 41.7	1437 ± 63.2	1902 ± 65.1
0.1%N+	44 ± 1.2	162 ± 2.7	447 ± 20.7	873 ± 54.0	1458 ± 85.3	1934 ± 83.5
0.2%N+	44 ± 1.2	161 ± 6.0	449 ± 22.9	897 ± 30.6	1472 ± 44.5	1979 ± 54.6
p-value	0.946	0.412	0.693	0.848	0.830	0.361

[a,b]Means with different superscripts differ significantly from each other ($P<0.05$)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bacitracin antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

Table 3.4 Mean cumulative weight gain (±SE) as influenced by the inclusion of Nutrifen® and NutrifenPLUS® in the diet of Cobb500 broiler chickens over a period of 32 days

Treatment	Day 0-7 (g)	Day 0-14 (g)	Day 0-21 (g)	Day 0-28 (g)	Day 0-32 (g)
Neg. CON	115 ± 3.6	397 ± 15.9	840 ± 43.2	1433 ± 37.8	1906 ± 53.6
Antibiotic	118 ± 5.1	397 ± 15.9	839 ± 35.6	1435 ± 50.7	1924 ± 51.8
0.1%N	121 ± 4.5	408 ± 17.8	842 ± 38.2	1421 ± 51.2	1896 ± 59.9
0.2%N	115 ± 8.4	392 ± 21.3	823 ± 41.2	1394 ± 62.9	1858 ± 65.2
0.1%N+	118 ± 1.9	403 ± 20.5	829 ± 53.4	1415 ± 84.4	1890 ± 82.8
0.2%N+	117 ± 5.1	404 ± 22.6	853 ± 30.5	1428 ± 43.9	1935 ± 54.5
p-value	0.288	0.689	0.851	0.828	0.361

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bacitracin antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

3.3.2 Feed intake

Both weekly and cumulative feed intakes were not affected significantly by the dietary inclusion of Nutrifen® and NutrifenPLUS® in broiler chickens. This observation could be made throughout the 32 day trial, as can be seen in Table 3. and Table 3. respectively.

Other studies involving the dietary inclusion of fenugreek in broiler feeds showed great variation with regard to feed intake (FI). In some cases, higher feed intake values were observed when fenugreek treatment was applied (Alloui *et al.*, 2012; Sahoo *et al.*, 2013; Mamoun *et al.*, 2014), whereas in other cases it was concluded that fenugreek supplementation had no significant effect (Khan *et al.*, 2013; Kassu *et al.*, 2016). Abbas (2010) even reported a decrease in broiler feed intake compared to control treatments at 42 days as a result of fenugreek supplementation. Most studies showing results for higher FI, also produced better feed conversion ratios (FCR), which will be discussed later in the chapter; however this was also the case in some instances where intake was reduced by dietary fenugreek (Abaza, 2007; Abbas, 2010). Similar to Abbas (2010) and Abaza (2007), a study by Duru *et al.* (2013) revealed lower feed intake values as a result of treatment, but no significant improvement in FCR was found. This makes differences in feed intake difficult to interpret, since stress does not seem to explain this variation between treatments, as was theorised with regard to growth performance. Furthermore, no significant effects on intake were apparent in many cases (Awadein *et al.*, 2010; Khan *et al.*, 2013; Weerasingha & Atapattu, 2013). Alloui *et al.* (2012) also reported no significant changes in average intake values at 21 days, but as mentioned, did show higher feed intake at 42 days. In the case of this study, increases were attributed to the presence of galactomannans and neurin, which are compounds known to have beneficial effects on gut microflora, thus stimulating appetite and improving feed conversion ratio. A comprehensive study by Kassu *et al.* (2016) seems to contradict these findings, as fenugreek was tested alongside black cumin and turmeric. Both of the other spices reduced feed intake during the latter stages of the study (21-49 days) without affecting LBW or BWG, whereas fenugreek supplementation resulted in intake values comparable to those of the control diet.

Variation in feed intake also seems to be a factor with regard to other livestock when fenugreek supplementation is involved. Petrus & Smit (2014) and Pearson (n.d.) observed higher FI's in goats and dairy cows respectively when treated with fenugreek-based supplements (Nutrifen® and NutrifenPLUS® respectively), whereas Alemu & Doepel (2011) reported a decrease in dry matter intake (DMI) when fenugreek haylage was included in the dairy cow diet. A herbal

mixture containing fenugreek, also showed no significant influence on intake for weaned piglets between the ages of two and three months (Kumar *et al.*, 2014).

Although for the most part these differences are difficult to explain without further research, results do exhibit a noteworthy trend, which seems supported by the current trial. Supplementing broiler diets with fenugreek only led to greater feed intake at the age of 42 days, in general. Before this stage, few increases were witnessed in comparison to control groups. In cases where the feed intake was unaffected by fenugreek supplementation, as in the current trial, the trials did not reach the 42 day stage. It is also important to take p-values over the duration of the current trial into account. One can see that the p-values of both weekly and cumulative feed intake's tend towards the 5% significance level as time passes. It can therefore be speculated, that if the trial period had been extended to the 42 day stage or beyond, significant differences in both parameters may have been observed.

Concentration of fenugreek supplementation also seems to play a role in poultry intake regulation, as can be seen in the study by Awadein *et al.* (2010), where over the same period, 0.1% supplementation had no significant influence on feed intake but 0.5% produced lower values relative to control diets. This trial was performed on layer hens over a period of 12 weeks, where again the length of the trial seems to be of significance. The importance of concentration is also exhibited in a study by Mamoun *et al.* (2014), where the highest intake values were recorded for 2% fenugreek supplementation at 42 days. More emphasis will be placed on this study however later in the chapter when discussing FCR.

Contradictory to most literature mentioned, Elbushra (2012) reported higher feed intake's for birds treated with 1.5% fenugreek at 14 days, but significantly lower intake's for treatment groups at the age of 42 days. This still however defines the importance of supplement concentration with regard to feed intake, although no clear conclusions can be drawn from the individual study.

Table 3.5 Mean weekly feed intake (±SE) as influenced by the inclusion of Nutrifen® and NutrifenPLUS® in the diet of Cobb500 broiler chickens

Treatment	Day 0-7 (g)	Day 7-14 (g)	Day 14-21 (g)	Day 21-28 (g)	Day 28-32 (g)
Neg. CON	141 ± 7.5	410 ± 24.5	656 ± 20.9	1016 ± 31.5	928 ± 48.9
Antibiotic	144 ± 5.4	422 ± 33.8	646 ± 30.7	982 ± 28.6	912 ± 33.0
0.1%N	140 ± 7.0	413 ± 26.4	647 ± 42.3	1006 ± 32.2	908 ± 29.7
0.2%N	141 ± 6.2	412 ± 16.2	643 ± 27.7	1009 ± 28.0	904 ± 46.5
0.1%N+	139 ± 6.2	417 ± 10.7	650 ± 26.3	1039 ± 57.6	937 ± 51.3
0.2%N+	143 ± 9.8	422 ± 13.2	655 ± 27.1	998 ± 39.5	880 ± 24.2
p-value	0.891	0.904	0.973	0.219	0.241

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bacitracin antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

Table 3.6 Mean cumulative feed intake (±SE) as influenced by the inclusion of Nutrifen® and NutrifenPLUS® in the diet of Cobb500 broiler chickens

Treatment	Day 0-7 (g)	Day 0-14 (g)	Day 0-21 (g)	Day 0-28 (g)	Day 0-32 (g)
Neg. CON	141 ± 7.5	551 ± 26.1	1207 ± 35.4	2223 ± 61.7	3151 ± 87.3
Antibiotic	144 ± 5.4	566 ± 35.8	1212 ± 49.0	2195 ± 72.4	3107 ± 97.8
0.1%N	140 ± 7.0	554 ± 31.7	1200 ± 69.9	2206 ± 100.1	3114 ± 126.4
0.2%N	141 ± 6.2	552 ± 17.7	1196 ± 30.6	2205 ± 54.4	3109 ± 95.6
0.1%N+	139 ± 6.2	556 ± 16.3	1206 ± 33.1	2245 ± 73.1	3181 ± 114.8
0.2%N+	143 ± 9.8	564 ± 12.1	1219 ± 31.2	2217 ± 41.4	3097 ± 63.4
p-value	0.891	0.851	0.954	0.855	0.670

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bacitracin antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

3.3.3 Feed conversion ratio

The feed conversion ratio (FCR) as expected, showed an increasing trend throughout the trial, but did not differ significantly between treatments at any given point during the 32 day period ($p>0.05$). Refer to Table 3. for p-values and FCR trends observed during the course of the study.

In general phytogenic supplementation has yielded better feed conversion ratios in poultry and other farmed animals. Diets containing black cumin seeds for example have led to better FCR values in many cases (Al-Beitawi & El-Ghousein, 2008; Yatoo *et al.*, 2012; Al-Beitawi & El-Ghousein, 2015). (Yatoo *et al.*, 2012) even compared the effects of cumin and fenugreek on the FCR of broilers and found that black cumin alone was the most effective treatment. Statistically similar FCR values were yielded by a treatment combining the two spices, but most of the positive effects were attributed to cumin since as an individual spice, fenugreek supplementation did not produce any significant changes in FCR. Similarly, Kassu *et al.* (2016) compared the individual effects of fenugreek, black cumin and turmeric and found fenugreek to be statistically non-influential. The other two spices however, both resulted in improved feed conversion ratios. Another study (Kirubakaran *et al.*, 2016) involving dietary combinations of garlic, black pepper and fenugreek led to the same conclusions. No treatments containing fenugreek showed any significant or even numerical effect on FCR. A combination of garlic and pepper however, statistically out-performed all other treatments, producing the best FCR values. Ibraheim *et al.* (2004) also fed crushed garlic and onion to Muscovy ducks, which resulted in improved FCR at the age of 10 weeks. This was attributed to a thinner intestinal wall of the treatment birds, which enhanced nutrient absorption efficiency from the gut. Greater body weight gains and reduced feed intake observed in this study were said to be a result of the sulphur components of onion and garlic that are considered to be active antimicrobial agents.

In most cases a positive correlation has been discovered between FCR and fenugreek supplementation (Abbas, 2010; Alloui *et al.*, 2012; Sahoo *et al.*, 2013; Weerasingha & Atapattu, 2013; Mamoun *et al.*, 2014). FCR is generally not significantly affected within the first 21 days (Alloui *et al.*, 2012, Abbas, 2010), but after 38 (Weerasingha & Atapattu, 2013) and 42 days (Abbas, 2010; Alloui *et al.*, 2012; Sahoo *et al.*, 2013) considerable improvements have been observed. This logic is supported by the current trial, where p-values tend nearer to the 5% significance level at the end of the 32 day period. This suggests that FCR may have been influenced by treatment had the current experiment continued over a longer period of time, however current broiler producton cycles are generally closer to 32 days. It is also important to note that in studies by Weerasingha & Atapattu (2013) and Mamoun *et al.* (2014)

it was determined that a lower (1%) inclusion level showed the most significant improvements compared to control groups. Anise (Yazdi *et al.*, 2014) and marjoram (Osman *et al.*, 2010) supplementation exhibited similar trends, where only lower concentrations (1g/kg) during the starter and finisher phases were influential. On the contrary, other studies (Sahoo *et al.*, 2013) showed improvements in FCR to be proportional to fenugreek supplementation. Toaha *et al.* (2016) found 2% fenugreek to be the most effective, but also found significant improvements in FCR when birds were fed concentrations of 1- and 3%. Abaza (2007) and Awadein *et al.* (2010) also observed better FCR values in treated layer hens, although it should also be noted that both of these studies were carried out over extended periods of time (12 weeks).

Contrary to other available literature, results obtained by Hernández *et al.* (2004) suggest that phytogenic supplementation has little influence on the FCR of broiler chickens. In this case FCR was unaffected throughout the study, by two herbal extracts containing mixtures of oregano, cinnamon and thyme, and sage, thyme and rosemary. This seems to be an exception, although it does need to be taken into account when considering these herbs as antibiotic replacements.

Table 3.7 Feed conversion ratio (±SE) as influenced by the inclusion of Nutrifen® and NutrifenPLUS® in the diet of Cobb500 broilers over a period of 32 days

Treatment	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-32
Neg. CON	1.22 ± 0.07	1.39 ± 0.09	1.44 ± 0.06	1.55 ± 0.04	1.65 ± 0.07
Antibiotic	1.21 ± 0.07	1.43 ± 0.10	1.45 ± 0.06	1.53 ± 0.05	1.62 ±0.04
0.1%N	1.15 ± 0.05	1.36 ± 0.07	1.43 ± 0.04	1.55 ± 0.05	1.64 ± 0.05
0.2%N	1.23 ± 0.13	1.42 ± 0.12	1.46 ± 0.08	1.58 ± 0.07	1.67 ± 0.06
0.1%N+	1.18 ± 0.06	1.38 ± 0.09	1.46 ± 0.08	1.59 ± 0.08	1.69 ± 0.08
0.2%N+	1.22 ± 0.12	1.40 ± 0.09	1.43 ± 0.03	1.55 ± 0.03	1.60 ± 0.04
p-value	0.596	0.817	0.919	0.403	0.133

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bacitracin antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

3.3.4 EPEF, PER, ADG and Mortality

Nutrifen® and NutrifenPLUS® were found to have no significant impact on the average daily gain (ADG), European production efficiency factor (EPEF), liveability or the protein efficiency ratio (PER), shown by $p>0.05$ in Table 3..

European production efficiency factor (EPEF) takes multiple factors into account, including age (days), liveability (%), FCR and live weight of the birds; making it a good indicator of overall performance and flock health. Higher values tend to suggest better technical performance (ROSS, 2007), and values in excess of 300 are considered excellent indicators of a healthy broiler flock (Basson, 2011). No significant variation between treatments was observed with regard to EPEF of the current study, apart from an apparent numerical increase due to antibiotic treatment. Similar trends were observed by Teuchert (2014) who fed oregano to broilers under the same conditions; finding supplementation to have little influence on EPEF. This could be due to low mortality rates observed across all treatments, stemming from a lack of exposure to pathogenic or environmental challenges (Teuchert, 2014), which is indicated by the high average EPEF value for the overall study (363.05). The same numerical increase was observed in protein efficiency ratio (PER) where birds were fed either antibiotics, 0.2% NutrifenPLUS® (current study) or basil (Ahmed *et al.*, 2015). Increases indicate that these treatments may have reduced the microbial load of the gut, thus making more dietary protein available to the bird. Although this theory is not supported statistically by these studies, Elbushra (2012) did find a statistical increase in PER when broilers were fed diets supplemented with fenugreek at various concentrations. Furthermore, Mona Osman *et al.* (2010) found that the feeding of rosemary, marjoram or sweet basil to broilers also resulted in statistically improved PER values. In all studies, no apparent stress was noted. These results therefore lend a certain support to the proposed theory.

Average daily gain (ADG) was linearly increased by fenugreek supplementation in weanling pigs over a period of 42 days. During days 14-42 however, only 0.2% fenugreek inclusion had an effect, whereas 0.1- and 0.2% both improved ADG during the first two weeks (Begum *et al.*, 2016). Numerically the ADG of broilers was increased by basil supplementation (Ahmed *et al.*, 2015), but was not influenced by black cumin, turmeric or fenugreek (Kassu *et al.*, 2016), which agrees with results of the current study. The same lack of significance was observed by Hossian *et al.* (2015) when garlic powder was fed to meat rabbits. Contradicting results of this study, Cardinali *et al.* (2015) found that feeding rabbits oregano led to statistically higher ADG values.

Table 3.8 Effects of including Nutrifen® and NutrifenPLUS® at varying concentrations on the mean ADG, EPEF, liveability and PER (±SE) in broiler feed

Treatment	ADG	EPEF	Liveability (%)	PER
Neg. CON	59.09 ± 0.53	357.29 ± 34.30	96.67 ± 5.16	2.96 ± 0.11
Antibiotic	59.05 ± 0.54	381.02 ± 15.88	100.00 ± 0.00	3.03 ± 0.07
0.1%N	60.07 ± 0.54	369.17 ± 16.01	100.00 ± 0.00	2.98 ± 0.07
0.2%N	59.07 ± 0.54	349.93 ± 29.76	98.33 ± 4.08	2.92 ± 0.11
0.1%N+	59.48 ± 0.53	353.94 ± 34.80	98.33 ± 4.08	2.91 ± 0.13
0.2%N+	59.46 ± 0.54	366.95 ± 21.03	95.00 ± 5.48	3.05 ± 0.07
p-value	0.741	0.365	0.214	0.117

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bacitracin antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

3.4 Conclusion

Growth rate, feed intake and FCR are all of great value to the commercial poultry producer, as feed costs make up roughly 70% of total expenses. Under a variety of conditions these production parameters may be negatively influenced, and feed additives such as antibiotics are currently being implemented to maintain profitability. With recent emphasis being placed on antibiotic resistance, the use of antibiotic growth promoters is being phased out, leaving a gap in the modern broiler production chain. Urgency for alternative production enhancers is therefore increasing, and phytochemicals such as Nutrifen® and NutrifenPLUS® may form a partial solution.

No aspects relating to any production parameters mentioned in the chapter were affected by the addition of Nutrifen® or NutrifenPLUS® to broiler diets in the current study. With this observation in mind, these treatments were also comparable to the antibiotic treatment, suggesting that birds were not challenged in any way. For studies of this nature to yield significant results, birds need to be maintained in sub-optimal conditions or at least be exposed to some sort of pathogenic challenge. It is also worth mentioning that PER, although not statistically improved compared to the control, did show a slight numerical increase with 0.2% NutrifenPLUS® supplementation; as did antibiotic treatment. This could be an indication that NutrifenPLUS® has an influence on protein digestion, absorption or utilization by the bird. No evidence can substantiate this however, but further research could be beneficial.

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Chapter 4

Investigation of Nutrifen® and NutrifenPLUS® as broiler feed additives and their impact on bone, organ and digestive tract parameters

Abstract

Nutrifen® and NutrifenPLUS® were investigated as potential broiler feed additives with regard to their effects on tibia bone parameters, gastro-intestinal and gizzard pH, liver colour, gizzard erosion and organ weight. Three-hundred and sixty, day-old, mixed gender Cobb500 broilers were assigned to six different treatment diets over a period of 32 days. Each treatment consisted of six replications containing the following non-nutritive feed additives: negative control (no additive), positive control (150g/ton zinc bacitracin antibiotic), 0.1% Nutrifen®, 0.2% Nutrifen®, 0.1% NutrifenPLUS® and 0.2% NutrifenPLUS®. Birds were fed on an *ad libitum* basis throughout the duration of the trial. At the age of 32 days, six birds that closely represented the average weight in the pen were selected from each treatment and slaughtered for analysis. Organs were excised and weighed, liver colour readings were taken and gizzards were cut open longitudinally, washed and scored by the same person throughout. The pH of the duodenum, jejunum, ileum and caecum were determined using a calibrated probe. Tibia bones were analysed for ash and dry matter content, breaking strength and mineral composition. Organ weights (actual and relative to body weight) showed no significant difference between treatments. Similarly, treatment did not affect the pH of any of the gastrointestinal components or the gizzard. Breaking strength, dry matter, ash content and mineral composition were all consistent across treatments; however the calcium:phosphorus ratio showed highly significant differences. NutrifenPLUS® at both 0.1- and 0.2% concentrations exhibited significantly lower ratios than the control, with a 0.1% concentration being significantly lower than all other treatments.

4.1 Introduction

Skeletal abnormalities as well as bone breakages during slaughter and processing, have become a growing concern amongst modern poultry producers and processing plants (Sanotra *et al*., 2001). Combined with a relatively poor understanding of the actual calcium and phosphorus requirements of a broiler chicken (Angel, 2011a) and intense genetic selection for fast growth (Świątkiewicz *et al*., 2014), intensively raised birds are more susceptible to poor calcification (Williams *et al*., 2000), highly porous cortical bones and weaker skeletal systems in general (Shim *et al.*, 2012). As a result, broilers often suffer from leg abnormalities that may cause considerable discomfort (Sanotra *et al*., 2001) and higher mortality rates, leading to substantial economic loss (Świątkiewicz *et al*., 2014). Not only do mortality rates increase, but birds suffering from leg deformities such as tibial dyschondroplasia (TD) or rickets, show a tendency for reduced feed intake, and reduced feed efficiency and growth performance as a consequence (Świątkiewicz *et al*., 2014). In addition, skeletal weaknesses often equate to higher incidences of fracturing or splintering during slaughter and/or processing; increasing the probability of carcass and meat downgrades and in turn, resulting in further economic losses as well as animal welfare issues (Whitehead, 2007).

Since bone strength is thought to be a function of the level of mineralization (Thorp & Waddington, 1997), it can be inferred that any dietary constituent influencing mineral metabolism may have an impact on skeletal development. Fenugreek and three of its extracts in particular, have exhibited positive effects on osseous tissue development and function (Folwarczna *et al.*, 2014), as well as dietary supplementation of the plant itself having shown an influence on intestinal conditions in pigs (Zentek *et al.*, 2013).

The pH level of specific areas of the gastro-intestinal tract (GIT) plays a vital role in establishing specific microbial populations, which are generally viewed as beneficial entities to the host (Gaskins *et al.*, 1997). Most of these favourable bacteria thrive at a relatively acidic pH of between 5.8 and 6.2, whereas pathogens tend to prefer higher pH levels of 7 or above (Ferd, 1974). Furthermore, the GIT pH plays a crucial role in determining the digestibility and absorption of nutrients, where lowering the pH by the addition of organic acids tends to increase absorption rates (Boling *et al*., 2001). Lower intestinal pH has also been linked to an increase in calcium absorption and femur calcium content in rats (Kishi *et al.*, 1999), and has positive effects on mineral retention and bone ash percentage in pigs (Radcliffe *et al.*, 1998; Jongbloed *et al.*, 2000). The relatively acidic pH of avian species is dependent on numerous variables, including health or immune status of the bird (Rahmani *et al.*, 2005). In turn, development of the avian gut immune system is also partly dependent on the content and

variety of intestinal commensal bacteria, which can be altered with the use antibiotic growth promoters (AGP's) (Lee *et al.*, 2012) and possibly certain plant extracts (Cherian *et al*., 2013). Relative spleen, thymus and bursal weights can be used to partially exhibit this intricate relationship that exists between the gut and the immune system (Toghyani *et al*., 2010). Larger lymphoid organs tend to indicate a stronger disease resistance (Jaffe, 1966), which is considered to be mainly as a result of greater immunoglobulin production (Yamamoto and Glick, 1982).

Higher hepatic and overall lipid concentrations of the liver in most species can indicate an abnormal physiological state, with unusually light livers often leading to broiler carcass condemnation (Trampel *et al.*, 2005). Pre-slaughter feed restriction has also been shown to have a major impact on lightness, where longer withdrawal periods, up to a point, generally yield lower and more conventional values (Trampel *et al.*, 2005). Typical pre-harvest withdrawal periods will last approximately 12 hours in modern commercial production (Willis *et al.*, 1996), although optimal timescales are subject to change depending on various production factors (Thompson & Applegate, 1988). Little is known however, regarding the effects of phytogenics on liver colour in poultry.

Gizzard erosion manifests itself as lesions, or the extensive sloughing, thickening or loosening of the koilin lining (Itakura *et al*., 1982; Fossum *et al*., 1988). This can lead to a reduction in feed intake and consequent slow growth rate, as well as higher mortalities and substantial economic loss (Fossum *et al*., 1988). Erosion is commonly associated with a number of factors such as feed mould/mycotoxins (Dorner *et al*., 1983), dietary copper sulphate levels (Fisher *et al*., 1973), stress (Dzaja *et al*., 1996) and/or most notably gizzerosine, which acts on histamine (H_2) receptors in the proventriculus to stimulate gastric acid secretion (Masumura *et al.*, 1985). The effect of gizzerosine however is almost 10 times stronger than that of histamine, leading to excessive acid secretion and severe damage to the gizzard lining (Masumura *et al.*, 1985). Very little research has been done concerning the influence of plant derived compounds on the gizzard, hence the current experiment.

4.2 Materials and Methods for Toxicity Study

4.2.1 Animals and experimental facility

For the purpose of this study, 60 Cobb500 day-old, unsexed broiler chicks were used. Housing and management practices were the same as those specified in Chapter 3. However initially, due to space limitations, the cages to be used for this trial were split in two. These "half cages" measured roughly 0.5m x 0.6m. Ten birds were therefore placed in each "half cage". When

the birds reached six days of age, more space was available in the house and each treatment was spilt into one full cage. Before placement, the wire floors of the cages were covered with multiple layers of newspaper to prevent injury. This newspaper was removed and replaced on day six when treatments were split. Each cage was equipped with a feed tray and a bell drinker. Birds were fed *ad libitum* and provided with clean water daily, during the entire trial.

All chicks were from the same parental group, thereby reducing genetic variation. Chicks also underwent the standard commercial procedure of being vaccinated against Newcastle disease and infectious bursal disease (IBD) before leaving the hatchery. In the case of this trial, birds were randomly allocated to their different cages and were not weighed or sexed before being placed, since no production parameters were being investigated. Birds were also checked at regular intervals during the first eight to ten days when they were most sensitive to environmental fluctuations and/or infection.

4.2.2 Diet formulation and treatments

A commercial broiler starter diet was formulated using Winfeed feed formulation software as shown in Table 4.1 and Table 4.2. This formulation was used to mix 60kg of feed, which was intended to be used for the entire trial. The mixed feed was then split into six smaller bags, each bag weighing 10kg. These bags were labelled and placed next to the experimental cages corresponding to the trial design. Feed was mixed in the feed mixing facility on the experimental farm. The basal starter diet was mixed using a small scale ribbon commercial mixer, and mixing of the additives on day six was done by hand. The chicks were fed this diet *ad libitum* in a mash form for the first seven days, after which the treatments were applied. Generally, a commercial broiler diet would be fed in pellet form to maintain a more complete profile but in this case, pelleting of the Nutrifen® diets was not possible due to the small quantities being used.

The treatments used were formulated as follows: no additive, 150g/ton Zinc bacitracin antibiotic, 0.2% Nutrifen®, 0.4% Nutrifen®, 0.2% NutrifenPLUS® and 0.4% NutrifenPLUS®. Six treatment diets were formulated and applied from day seven up until slaughter on day 14, with each treatment consisting of 10 repetitions/birds. The additives were added to the remaining starter feed from each cage on the night of day six according to the concentrations mentioned, and fed for the remainder of the trial. For the purpose of this study, concentrations of Nutrifen® additive were exaggerated to a certain extent, in order to determine if the Nutrifen® additives would cause gizzard erosion at levels higher than those recommended. In the case of no significant differences being observed between the Nutrifen® treatments and the control, it would suggest that the Nutrifen® product is safe to use.

Constituents of the treatment additives can be seen below:

Nutrifen®

Fenugreek cotyledon concentrate (*Trigonella foenum-graecum*)

NutrifenPLUS®

Fenugreek cotyledon concentrate (*Trigonella foenum-graecum*) - 72%

Fennel seed powder (*Foeniculum vulgare*)

Saw Palmetto berry powder (*Serenoa repens*)

Brown Kelp powder (*Laminariales*)

MSM (naturally-sourced methylsulfonylmethane)

Apple cider vinegar powder

Table 4.1 Basal starter diet composition for gizzard erosion trial

Ingredient	(%)
Maize	45.09
Soybean full fat	11.53
Soybean 46	27.78
Fish meal 65	5.36
L-lysine HCl	0.18
DL-methionine	0.40
L-threonine	0.11
Vitamin + mineral premix	0.20
Bentonite (filler)	0.20
Limestone	1.45
Salt	0.12
Monocalcium phosphate	1.22
Sodium bicarbonate	0.16
Oil - sunflower	6.20
Total	**100**

Table 4.2 Nutrient composition of basal starter treatment diet as formulated according to Cobb500 nutrient specifications

Nutrient	Units	Formulated
AME	MJ/kg	12.65
CP	%	25.00
Fat	%	10.94
Moisture	%	10.64
Ash	%	5.03
Dry Matter	%	89.36
Crude Fiber	%	3.12
Arginine	%	1.66
Avl Lysine	%	1.43
Lysine	%	1.60
Isoleucine	%	1.14
Leucine	%	2.09
Methionine	%	0.80
T.S.A.A.	%	1.19
Threonine	%	1.07
Tryptophan	%	0.29
Valine	%	1.26
Linoleic Acid	%	3.57
Avl Phosphorus	%	0.50
Calcium	%	1.05
Chloride	%	0.23
Sodium	%	0.16

AME = Apparent metabolisable energy; CP = Crude protein; T.S.A.A. = total sulphur containing amino acids; Avl = available

4.2.3 Slaughter and data collection

Birds were sacrificed on day 14 of the trial by cervical dislocation at the on-site abattoir, in a sanitary environment. Immediately after slaughter, the birds were dissected and gizzards were removed. The gizzards were then cut open longitudinally, and washed under clean, running water before being placed in labelled bags according to treatment. These were taken back to the lab, where they were immediately scored and data was recorded. Gizzards were scored according to a four-point ordinal scoring system described in Table 4.3, by a single person to ensure consistency in the results.

Table 4.3 Four-point gizzard erosion scoring system

Score	Description
0	No erosion
1	Light erosion (roughness of epithelia)
2	Modest erosion (roughness and gaps)
3	Severe erosion (roughness, gaps and ulcers on stomach wall showing slight haemorrhaging)
4	Extreme erosion (roughness, gaps and haemorrhagic ulcers on stomach wall and separation of epithelia from stomach wall)

4.2.4 Statistical analysis

The following hypotheses were proposed:

H_0: there is no association between gizzard erosion scores and the treatment diets investigated

H_a: there is evidence to suggest that a relationship exists between the severity of gizzard erosion and the treatment diets investigated

Since the data at hand is categorical, a chi-squared test was used to indicate the degree of association between the gizzard score and the treatment diets. Level of association was deemed to be significant at 5% ($p<0.05$), and highly significant on a 1% ($p<0.01$) basis. The chi-squared test was performed using the “table analysis” function, SAS Enterprise 9.1 statistical software.

4.3 Materials and Methods for Gut, Organ and Tibia Bone Analysis

4.3.1 Slaughter process and data collection pre- and post-slaughter

On the day of slaughter, feed was removed from the cages before the lights turned on in the house so that birds could not eat. The usual weighing procedure occurred with all 10 birds per cage being weighed as a group as well as the remaining feed for each cage. Two birds were then selected from each of the 36 cages to be slaughtered for further analysis. These two birds were weighed individually and identified to closely represent the average weight in the pen.

All birds to be used for carcass characteristics and meat analysis were slaughtered first to reduce the risk of contamination. These birds were slaughtered by trained personal in accordance with Department of Agriculture, Forestry & Fisheries [DAFF], 2006. Birds were first stunned with the application of 50-70 volts of electricity for 3-5 seconds to the junction between head and spine, under the beak, before being exsanguinated. Birds were then de-feathered mechanically, washed and the initial pH (pH_i) of the right breast and thigh muscles recorded using a Crison pH25 pH meter (Alella, Barcelona). Both legs were then removed at the tarsal joint, each carcass weighed on a Mettler PC 4400 scale (Mettler-Toledo, Switzerland) to obtain a warm carcass weight and placed in pre-labelled bags for further analysis at a later stage.

The birds intended for organ analysis were then slaughtered in the same way according to Department of Agriculture, Forestry & Fisheries [DAFF] regulations, after the meat birds. The birds were then immediately dissected using scalpels and dissection scissors before the following organs were removed and promptly weighed: heart, spleen, liver, gizzard and the bursa of Fabricius. Small sections of the digestive tract were also removed in order to measure the pH of their contents. These portions included the proventriculus, duodenum, jejunum, ileum and caecum. All organs were removed carefully to avoid any damage and all excess tissue was removed to ensure that accurate weights of each organ were obtained. Birds used for organ analysis were also used to test certain tibia bone characteristics. Before the carcasses were disposed of, the legs/drumsticks were removed and placed in plastic bags according to treatment, before being frozen for analysis of the tibia bones at a later stage.

Once weighed, pH measurements of the gizzards were also recorded using the same Crison pH25 pH meter. They were then cut open longitudinally and washed under clean, running water and placed into bags, pre-labelled according to treatment. These were taken back to the lab and scored according to the four-point system mentioned above, by the same person.

Three measurements (L*, a* and b*) of liver colour were obtained for each liver using a CIE-Lab colour meter (BYK-Gardner GmbH, Gerestried, Germany), immediately after weighing. These colour measurements were repeated four times for each liver and an average was obtained. Care was taken to make sure that liver colour was measured within 15 minutes of the bird being exsanguinated. Measurements (L*, a* and b*) were later used to calculate chroma/saturation and hue values using

Equation 4.1 and Equation 4.2 respectively.

4.3.2 Tibia bone samples

Legs/drumsticks were initially removed from the organ bird carcasses and placed in sealed plastic bags before being frozen at -18°C, with surrounding tissue (muscle and skin) still attached to the bone. This was done by carefully cutting between the femur bone and the periosteum of the tibia, without damaging the tibia in any way. The right legs were then thawed in a fridge at 4°C for approximately 18 hours and carefully deboned. Firstly, the surrounding tissue was cut away after which the periosteum and fibula were removed, taking caution not to damage the bone, thereby compromising its strength. The clean tibia bones were then vacuum sealed, labelled and frozen at -18°C until analysis.

Before analysis, the right tibia bones were thawed in a fridge at 4°C for approximately 12 hours. They were then dabbed dry using tissue paper and weighed individually using a Mettler AE 200 scale (Mettler-Toledo, Switzerland). Each bone was measured for length and mid-point using a digital Vernier calliper, as was the mid-diaphyseal diameter using the same calliper. The mid-point was marked on the diaphysis with a permanent marker to indicate where the acting force should be applied during the strength test. Breaking strength was then determined by a three-point bending test performed on an Instron 3345 material testing machine (model 2519-107), using Fleming *et al.*, 1998 as a guideline. The Instron machine consisted of two fixed supports (14mm wide) and a vertically mobile crosshead, which applied downward force to the mid-diaphyseal point of the bone, and a 5000N load cell capacity. The two supporting ends were set at a distance of 40mm apart, and the crosshead measuring 18mm wide was used to apply downward force at a steady rate of 30mm/min to the mid-point of the bone. The machine recorded the force being applied and the corresponding displacement of the crosshead at intervals of approximately 0.02 seconds, with the maximum force being applied at any point in time indicating the point-of-failure or bending force required to break the bone. The downward movement of the crosshead was initiated manually and stopped shortly after point-of-failure of the bone after which bone fragments were collected

and placed in labelled plastic bags for further analysis. Note that each bone was positioned in the same way between the two supports, with force being applied to the anterior side of the tibia to acquire consistency in the results. Data obtained from the Instron was then used to calculate the breaking strength of each tibia bone using Equation 4.3.

Tibia bones used for breakage analysis were then used to determine moisture/dry matter percentage according to the Official Method 934.01 of the AOAC (2002). After being dried in the drying oven for a minimum of two hours at 100°C, 36 porcelain crucibles were removed and placed in a desiccator for no less than 30 minutes. These were weighed individually and marked clearly. Tibia bones were placed in the crucibles and their weights recorded. Crucibles containing the bones were dried at 100°C for 24 hours to extract all moisture from the bones before being placed in a desiccator for no less than 30 minutes and weighed to determine the dry mass.

Bones were soaked in petroleum ether for 48 hours (Rama Rao & Reddy, 2001) to extract the fat which would have created complications during the ashing process. Once defatted, bones were placed in pre-dried porcelain crucibles of known weight and dried once again at 100°C for 24 hours. Once dried, the crucibles containing the bones were weighed once again to obtain a fat-free dry mass for each bone. Bones were subsequently ashed in a furnace set at 600°C for 24 hours (Zhang & Coon, 1997) and ash weights recorded, after being placed in a desiccator for more than 30 minutes. All weights were recorded using a Mettler AE 200 scale (Mettler-Toledo, Switzerland) accurate to 0.0001g.

Ashed tibia bone samples were ground to a fine powder using a mortar and pestle, before being bottled in air-tight, labelled sample bottles and sent to the Institute of Animal Production, Western Cape Department of Agriculture for mineral analysis. The combustion method (ALASA, 1996) was used to determine mineral composition, whereby each ground sample was mixed with 5ml of a 6M hydrochloric acid solution. Samples were then placed in a 50°C oven for 30 minutes, before 35ml of distilled water was added to each. Each sample was filtered, and distilled water was added to make up 50ml of solution. Mineral composition was determined using an iCAP 6000 Series Inductive Coupled Plasma (ICP) Spectrophotometer (Thermo Electron Corporation, Strada Rivoltana, 20090 Rodana, Milan, Italy) that was fitted with a vertical quartz torch and Cetac ASX-520 auto-sampler. And finally, concentrations of each element were calculated using iTEVA Analyst software.

Equation 4.1 $\text{Chroma} = \sqrt{(a^{*2}+b^{*2})}$

Equation 4.2 $\text{Hue} = \tan^{-1}(b^*/a^*)$

Equation 4.3 $\text{Breaking force} = \frac{\text{Max force (N)}}{\text{Bone Weight (g)}}$

4.3.3 Gastrointestinal pH

Prior to organ removal, small sections of the duodenum (just below the pancreas), jejunum (approximately halfway between Meckel's diverticulum and ileoceacal junction), ileum (5mm above the ileoceacal junction) and caecum were removed. All pH measurements were taken within 15 minutes post-slaughter using a portable Crison pH25 pH meter (calibrated using standard buffers of pH 4.0 and 7.0 at 25°C) (Alella, Barcelona). The pH readings were measured by inserting the electrode of the pH meter into the centre of the sample, and rinsing thoroughly with distilled water between samples. As well as the gut samples, pH measurements of the gizzards were also recorded in the same manner.

4.3.4 Statistical analysis

The following hypotheses were proposed:

H_0: There is no association between bone or organ characteristics and the treatment diets applied.

H_a: There is evidence to suggest that there is a relationship between bone and organ characteristics, and the different treatment diets used.

Before any analysis was performed, assumptions of normality and homoscedasticity were tested using a general linear model (GLM) in Statistica. Subsequently a one-way ANOVA and suitable *post hoc* test (Fisher's LSD or Games-Howell) was performed. The *post hoc* test as stated in the previous chapter was chosen according to whether the assumptions of normality and homoscedasticity were met.

The one-way ANOVA that was performed can be explained by the following model: $Y_{ij} = \mu_i + \alpha_j + \varepsilon_{ij}$, where Y_{ij} *is* the response variable, μ_i the overall mean, α_j the treatment effect and ε_{ij} the unexplained error.

4.4 Results and Discussion

4.4.1 Organ weight

As can be seen in Table 4.4, the heart, liver, gizzard, spleen and bursa weight was not influenced to any significant extent by the inclusion of any of the feed additives and their

differing concentrations ($p>0.05$). Similarly, Table 4.5 indicates that weight of the abovementioned organs relative to live weight was not influenced significantly by any of the treatments ($p>0.05$). Spleen/bursa ratio was also found to show insignificant differences, as can be seen in Table 4.5.

For the most part dietary fenugreek supplementation has been reported to affect neither actual nor relative organ weight in broilers. Studies by Khan *et al.* (2009), Abbas (2010), Duru *et al.* (2013), Elagib & Elamin (2013) and Toaha *et al.* (2016) as well as the current study all concluded that fenugreek as a feed additive had no significant effect on broiler heart, liver or gizzard weight. Other phytogenic supplements such as oregano, cinnamon, pepper, sage, thyme, rosemary (Hernández *et al.*, 2004), black cumin (Al-Beitawi & El-Ghousein, 2008), anise (Yazdi *et al.*, 2014) and ginger (Elagib & Elamin, 2013) have also shown little effect on the abovementioned organ weights. With this being said, there have been exceptions that are worth mentioning.

Mamoun *et al.* (2014) and Khadr & Abdel-Fattah (2015) found that dietary fenugreek reduced gizzard and heart size respectively in relation to live body weight. Ahmed *et al.* (2015) also reported a correlation between relative heart size and the addition of phytogenics to broiler feed, however no fenugreek treatment was applied and variations in heart size were attributed to treatments containing a combination of basil and chamomile. Other relevant visceral organs in all three of these studies were unaffected by phytogenic supplementation, and none of the experiments showed any variation between treatments with regard to actual organ weight. Although again not directly related to fenugreek, liver weight has been known to decrease in Muscovy ducks (Ibraheim *et al.*, 2004) as a result of garlic and onion supplementation. Liver weight was reduced by up to 11% in the instance of this experiment. Such reductions were attributed to the inhibition of fatty acid synthesis in the liver, thereby decreasing the rate of fat accumulation and ultimately lowering the relative weight of the organ (Ibraheim *et al.*, 2004).

In most available literature concerning fenugreek as a natural feed additive, lymphoid organs such as the spleen, bursa or thymus are mentioned briefly. Determining the weight of these organs gives an indication as to the immune status of the birds (Salam & Sunarti, 2013); an important consideration for potential AGP replacements. Birds with a larger bursa of Fabricius are considered to have a higher disease resistance (Jaffe, 1966) which has been attributed to their enhanced immunoglobulin synthesizing capabilities (Yamamoto and Glick, 1982). A larger bursa of Fabricius tends to increase the concentration of blood proteins, particularly globulins, leading to a higher globulins/albumin ratio (Ibraheim *et al.*, 2004). Blood constituents are therefore also useful in determining the state of the immune system in some instances.

Many studies may also employ the use of an antibody titre (Abed & Kadhim, 2014; Wati *et al*., 2015).

The results from all of these methods are however still comparable to a certain extent albeit limited, as they still suggest whether an immune response has been elicited on account of the additive. Results obtained by Durrani *et al.* (2008), Osman *et al.* (2010), Salam & Sunarti (2013), Yazdi *et al.* (2014), Abed & Kadhim (2014), Ahmed *et al.* (2015) and Al-Beitawi & El-Ghousein (2015) all suggest that the dietary inclusion of certain phytogenics could be used to improve immune response in broiler chickens. These additives include chamomile, marjoram, rosemary, garlic, black cumin and aniseed which all led to greater spleen, thymus and/or bursal weights in the abovementioned studies. Similarly, Ibraheim *et al.* (2004) found that phytogenic additives such as fresh onion and garlic also showed a highly significant impact on the immune response of Muscovy broiler ducks. The addition of these supplements resulted in spleen and bursal weights of up to 63- and 74% larger than control treatments respectively. These findings are in agreement with Eid & Iraqi (2014), who found significantly higher antibody titres in post-vaccinated broiler chickens fed on garlic diets.

Studies involving fenugreek specifically however, have not been as consistently successful. Most literature available on broilers seems to indicate that fenugreek is not as practically effective as theory tends to suggest. Fenugreek extract has been known to show a stimulatory effect on the immune system of mice (Bin-Hafeez *et al.*, 2003), although Abbas (2010), Elagib & Elamin (2013), and Weerasingha & Atapattu (2013) are all in agreement with the current study, where no correlation was reported between treatment and immune response in broiler chickens. In fact, Elagib & Elamin (2013) even found that fenugreek supplementation resulted in some of the lowest spleen, bursa and thymus weights compared to antibiotics and other herbs such as cumin, ginger and cinnamon. Statistically these observations were not supported however. The same can be said for layer hens, where results obtained by Awadein *et al.* (2010) and Motamedi & Talkimi (2014) suggest that fenugreek supplementation does not elicit an immune response of any significance, over an extended period of time. Contrary to these findings, Safaei *et al.* (2013) concluded that fenugreek administered at a 0.1% concentration in the drinking water of broilers, significantly increases bursal weight and antibody titre over 42 days. Interestingly, Khadr & Abdel-Fattah (2015) concluded that fenugreek does stimulate the immune system of broilers, based on strong evidence suggested by antibody titre results. With this being said, thymus, spleen and bursal weights were found to be unaffected to any statistical significance. This raises the question as to whether antibody titre measurements would have led to different conclusions being drawn in the abovementioned studies. The contradictory nature of these results makes it difficult to draw

clear conclusions, but the majority of observations lean towards the consensus that fenugreek has little effect on broiler chicken immune status.

Table 4.4 Mean organ weights (±SE) as influenced by the inclusion of Nutrifen® and NutrifenPLUS® feed additives at varying concentrations in broiler diets

Treatment	Heart (g)	Liver (g)	Gizzard (g)	Spleen (g)	Bursa (g)
Neg. CON	9.48 ± 1.43	47.04 ± 3.64	31.50 ± 4.28	2.87 ± 0.91	3.77 ± 0.72
Antibiotic	11.77 ± 1.83	49.66 ± 8.69	32.75 ± 3.53	2.66 ± 0.68	3.60 ± 0.57
0.1%N	10.65 ± 1.56	48.73 ± 6.15	31.81 ± 3.10	3.15 ± 0.28	4.39 ± 0.34
0.2%N	10.28 ± 1.47	48.23 ± 5.85	31.74 ± 3.84	3.19 ± 0.55	4.27 ± 0.73
0.1%N+	10.75 ± 0.94	47.15 ± 3.87	31.48 ± 4.62	3.08 ± 0.78	4.29 ± 0.84
0.2%N+	11.16 ± 2.07	46.32 ± 2.71	31.63 ± 4.50	3.09 ± 0.55	4.39 ± 0.98
p-value	0.236	0.908	0.994	0.727	0.265

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

Table 4.5 Mean relative organ weights (±SE) as influenced by the inclusion of Nutrifen® and NutrifenPLUS® feed additives at varying concentrations in broiler diets

Treatment	Heart %	Liver %	Gizzard %	Spleen %	Bursa %	Spleen:Bursa
Neg. CON	0.50 ± 0.06	2.47 ± 0.21	1.65 ± 0.26	0.15 ± 0.05	0.20 ± 0.03	0.78 ± 0.28
Antibiotic	0.58 ± 0.10	2.45 ± 0.46	1.62 ± 0.16	0.13 ± 0.03	0.18 ± 0.04	0.78 ± 0.29
0.1%N	0.51 ± 0.07	2.35 ± 0.18	1.54 ± 0.10	0.15 ± 0.02	0.21 ± 0.03	0.72 ± 0.08
0.2%N	0.51 ± 0.06	2.40 ± 0.23	1.59 ± 0.23	0.16 ± 0.02	0.21 ± 0.04	0.77 ± 0.19
0.1%N+	0.52 ± 0.05	2.29 ± 0.17	1.52 ± 0.16	0.15 ± 0.03	0.21 ± 0.04	0.73 ± 0.16
0.2%N+	0.55 ± 0.07	2.28 ± 0.15	1.56 ± 0.22	0.15 ± 0.03	0.22 ± 0.05	0.72 ± 0.11
p-value	0.293	0.722	0.85	0.754	0.494	0.978

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

4.4.2 Liver colour

No colour parameters of the liver, namely L* (lightness), a* (redness), b* (yellowness), hue and chroma were affected by the inclusion of Nutrifen® and NutrifenPLUS® in the diet of Cobb500 broiler chickens ($p>0.05$), as indicated in Table 4.6.

Lightness of the liver (L*) can be an indication of an abnormal physiological state, with higher lightness (L*) values being indicative of higher hepatic lipid concentrations and greater overall liver lipid content (Trampel *et al.*, 2005). Although this may be true, mention of liver colour is not common in available literature concerning feed additives and is usually determined in pre-slaughter feed-restriction trials. In the current trial feed restriction was practiced 12 hours pre-slaughter, whereas other available literature shows that feed restriction of between 10 and 12 hours generally yields values higher than those of the current trial for lightness (Trampel *et al.*, 2005; Karacay *et al.*, 2008). This is especially true for 0.2% Nutrifen®, 0.1- and 0.2% NutrifenPLUS®, which showed visibly lower L* values than the negative control, antibiotic and 0.1% Nutrifen® treatments, although not statistically supported. A study by Ocak & Sivri (2008) did however show lower L* values than the current trial, although this feed restriction was performed at different stages throughout the trial, making the results largely incomparable.

Table 4.6 Influence of Nutrifen® and NutrifenPLUS® feed additives on the mean liver colour (±SE) of a Cobb500 broiler

Treatment	L*	a*	b*	Hue	Chroma
Neg. CON	35.77 ± 6.33	12.25 ± 1.70	12.81 ± 2.31	46.00 ± 6.11	17.82 ± 2.20
Antibiotic	34.97 ± 4.32	11.70 ± 1.52	13.23 ± 2.13	48.76 ± 6.83	17.78 ± 1.72
0.1%N	36.99 ± 3.29	11.83 ± 0.94	12.84 ± 1.83	46.84 ± 4.83	17.56 ± 1.39
0.2%N	31.80 ± 2.18	11.91 ± 1.38	11.98 ± 0.89	45.10 ± 3.41	16.99 ± 1.29
0.1%N+	31.65 ± 3.89	12.38 ± 1.66	11.42 ± 1.95	42.85 ± 6.86	17.03 ± 1.70
0.2%N+	31.87 ± 6.04	11.89 ± 1.19	12.37 ± 2.28	45.50 ± 3.95	17.26 ± 2.28
p-value	0.194	0.957	0.644	0.585	0.936

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

4.4.3 Intestinal pH

As indicated by Table 4.7, no significant differences were exhibited between treatments with regard to pH of any of the intestinal tract components or the gizzard ($p>0.05$).

The gastro-intestinal tract (GIT) regulates itself according to physiological requirements of the bird (Mabelebele *et al.*, 2013), which is why functionality of the GIT and overall performance of the broiler is dependent on factors such as the Intestinal pH and overall GIT length (Rahmani *et al.*, 2005). A longer GIT results in greater surface area for absorption of nutrients, which could promote higher growth rates (Mabelebele *et al.*, 2013) however in the current trial, GIT length was not considered. In birds, the pH of the GIT is relatively acidic and is dictated by factors such as the kind of nutrients passing through the gut, makeup and content of microflora and the immune status of the bird (Rahmani *et al.*, 2005). A decrease in microbial content could leave more nutrients available for growth and in doing so, potentially improve weight gain and feed conversion (Allen *et al*., 2013).

Most microorganisms that are beneficial to the bird live at a pH of 5.8-6.2, whereas most pathogens grow at a pH of 7, or slightly higher (Ferd, 1974). For this reason, different areas of the GIT establish specific pH's in order to cultivate different beneficial microbial populations, which stimulate optimal health of the bird (Rahmani *et* al., 2005). Specific microbial populations also relate to digestibility and the value of absorbed nutrients, and the specific function of each component of the GIT (Rahmani *et* al., 2005). pH values of the different GIT components in the current trial were generally within the accepted ranges specified by Van der Klis & Jansman (2002), with the exception of one or two values that were negligibly smaller.

Available literature regarding fenugreek and its effects on intestinal pH however is extremely limited, making it difficult to compare results from the current trial. In piglets, fenugreek supplementation was found to reduce colon and caecal pH (Zentek *et al.*, 2013), which is not in agreement with the current findings. As can be seen in Table 5.4, there were no differences between treatments of the current study, and pH values fell within an acceptable range. This implies that birds were not exposed to harmful pathogens which may have altered GIT conditions. Exposure to stressful conditions or the relevant pathogens may have yielded variation within treatments as with Zentek *et al.* (2013), but far more extensive research is required in this field with regard to fenugreek in order to draw admissible conclusions. Another study by Cherian *et al.* (2013) regarding Sweet Wormwood (*Artemisia annua*) supplementation resulted in lower caecal and ileal pH values. Furthermore, Khalaji *et al.* (2011) found that a combination of black cumin seed and *Artemisia sieberi* leaves reduced jejunal pH significantly. These results are however not supported by any other available

literature and go against results of the current study. Also contrary to the current study, black cumin treatment in this experiment was found to lower gizzard pH. Low gizzard pH can also be indicative to the presence of dietary gizzerosine (Masumura *et al.*, 1985) however, which was not considered.

Table 4.7 Influence of Nutrifen® and NutrifenPLUS® feed additives on the mean intestinal tract and gizzard pH (±SE) of broilers

Treatment	Gizzard pH	Proventriculus pH	Duodenum pH	Jejunum pH	Ileum pH	Caecum pH
Neg. CON	2.27 ± 0.72	2.74 ± 1.13	5.47 ± 0.87	5.92 ± 0.14	6.55 ± 0.20	6.20 ± 0.48
Antibiotic	2.49 ± 1.05	2.97 ± 0.37	5.85 ± 0.43	6.04 ± 0.22	6.09 ± 0.58	6.17 ± 0.71
0.1%N	2.02 ± 0.53	2.81 ± 0.60	5.99 ± 0.40	6.00 ± 0.35	6.26 ± 0.32	6.10 ± 0.71
0.2%N	2.19 ± 0.81	2.37 ± 0.77	5.91 ± 0.22	5.80 ± 0.33	6.53 ± 0.55	6.72 ± 0.43
0.1%N+	2.34 ± 0.51	2.96 ± 1.14	6.07 ± 0.13	5.98 ± 0.08	6.22 ± 0.21	6.25 ± 0.92
0.2%N+	2.63 ± 0.91	3.07 ± 0.71	6.12 ± 0.29	6.04 ± 0.39	6.39 ± 0.67	6.06 ± 0.25
p-value	0.802	0.744	0.198	0.671	0.446	0.486

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

4.4.4 Gizzard erosion

A high p-value (>0.05) as indicated in Table 4.8, suggests that there is no significance between the additives included in the treatment diets and the degree of gizzard erosion at the age of 14 days. Similarly, degree of gizzard erosion cannot be linked to the inclusion of the different feed additives and their varying concentrations at the age of 32 days (p>0.05). This can be seen by the results indicated in Table 4.9.

The aim of the current study was merely to determine if the feed additives Nutrifen® or NutrifenPLUS® may cause, or contribute to the cause of any gizzard erosion in broilers. Any gizzard erosion , whether it was caused by the additives or not, could also have provided a possible explanation as to variation in feed intake or growth rate results, had there been any. For the purpose of this study, two separate experiments were performed. The first occurred over 14 days with exaggerated concentrations of Nutrifen® and NutrifenPLUS®, whereas the other was performed as part of the main trial over 32 days. Although scarce, other available literature (Teuchert, 2014; Akuru, 2016) tends to agree with the current findings of both experiments, suggesting that phytogenics in general do not have any ill effects on the gizzard lining. Between both of the current studies, only one erosion score greater than two was issued, which exhibits a lack of correlation between gizzard damage and treatment. This also indicates that feed was not contaminated with certain mycotoxins that are known to cause lesions. In Table 4.7 Influence of Nutrifen® and NutrifenPLUS® feed additives on the mean intestinal tract and gizzard pH (±SE) of broilers, it can also be seen that gizzard and proventriculus pH was unaffected by treatment, which indicates that phytogenic supplementation had no significant influence on gastric secretion. Another noteworthy study by McReynolds *et al.* (2009) concluded that phytogenic supplementation reduces necrotic enteritis in broiler chickens, which could justify further research.

Table 4.8 Gizzard erosion scores and their association with the inclusion of Nutrifen® and NutrifenPLUS® feed additives at varying concentrations over 14 days

	Score				
Treatment	**0**	**1**	**2**	**3**	**4**
Neg. CON	3	7	0	0	0
Antibiotic	3	7	0	0	0
N 0.2%	6	4	0	0	0
N 0.4%	2	6	2	0	0
N+ 0.2%	6	4	0	0	0
N + 0.4%	6	6	1	0	0
p-value			0.349		

Neg. CON: No non-nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

N 0.2%: Nutrifen 0.2% inclusion

N 0.4%: Nutrifen 0.4% inclusion

N+ 0.2%: NutrifenPLUS 0.2% inclusion

N + 0.4%: NutrifenPLUS 0.4% inclusion

Table 4.9 Gizzard erosion scores and their association with the inclusion of Nutrifen® and NutrifenPLUS® feed additive at varying concentrations over 32 days

	Score				
Treatment	**0**	**1**	**2**	**3**	**4**
Neg. CON	1	2	3	0	0
Antibiotic	2	1	3	0	0
0.1%N	3	1	2	0	0
0.2%N	1	3	2	0	0
0.1%N+	1	1	3	1	0
0.2%N+	2	1	3	0	0
p-value			0.830		

Neg. CON: No non-nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen 0.1% inclusion

0.2%N: Nutrifen 0.2% inclusion

0.1%N+: NutrifenPLUS 0.1% inclusion

0.2%N+: NutrifenPLUS 0.2% inclusion

4.4.5 Tibia bone parameters

No significant differences were observed between treatments with regard to fat-free dry mass, dry matter percentage or fat-free ash percentage ($p>0.05$). The same observations were made for calcium and phosphorus content expressed as a percentage of the fat-free ash content, with both showing non-significance, $p=0.592$ and 0.761 respectively. Significant differences were however observed between the various treatments in the case of the calcium, phosphorus ratio ($p<0.001$) as part of the fat-free ash content. Differences were interpreted statistically as highly significant, due to $p<0.001$ observed in Table 4.10. The table also shows that the negative (no additive) and positive (zinc bacitracin antibiotic) controls and the 0.1 and 0.2% Nutrifen® diets produced a similar ratio, whereas the 0.1% NutrifenPLUS® treatment differed significantly from both control diets. NutrifenPLUS® at a concentration of 0.1% resulted in a similar ratio as both of the Nutrifen® diets; however NutrifenPLUS® at a concentration of 0.2% led to a significantly lower ratio than all other treatment diets applied. No significant differences were observed between any of the treatments regarding the content of various individual bone minerals as illustrated Table 4.11, except for aluminium ($p<0.01$). Furthermore, no significant differences in bone strength were found as a result of treatment (Table 4.12).

Very few studies to date have been performed in order to determine the effects of phytogenic feed additives on the biomechanical properties of bone. Additives tested thus far have varied greatly with regard to species, dosage and combination, and so results on the subject have been largely inconclusive (Olgun, 2016). In the present trial, neither breaking strength (force per gram required to break the bone) nor the actual force applied (Newtons), was significantly influenced by Nutrifen® or NutrifenPLUS® inclusion in the diet of broiler chickens. These observations agree with those of Cardinali *et al.* (2015), where bone strength was unaffected by the inclusion of oregano and rosemary in rabbit diets. Contrary to these findings, Teuchert (2014) suggested that phytogenic feed additives may in fact weaken broiler bone structure. In that case, birds fed an oregano extract based product were found to have a significantly lower tibial breaking strength relative to birds treated with an antibiotic growth promoter (AGP). Although not statistically supported, oregano in the diet even produced weaker tibia bones than the negative control, leading to the conclusion that some phytogenics may in fact have a negative impact on bone structure. On the other hand, Świaotkiewicz *et al.* (2014) observed an increase in breaking strength of the femur and tibia bones in layers as a result of herbal extract (sage, dandelion and nettle) supplementation.

According to Santos *et al*. (2008) and Shaw *et al*. (2011), reductions in tibia ash content can be an indication of lower bone mineralisation levels, or skeletal disorders (Shim *et al.*, 2012) which could result in weaker bone structure. The current trial exhibited no significant differences between treatments with regard to tibia ash content or breaking strength, which suggests sufficient bone mineralisation across all treatment groups. In agreement with the present trial is Folwarczna *et al.* (2014) where dietary fenugreek also had little effect on ash content, Ca and P concentration of rat bones. Bone strength is also affected by the properties of dietary minerals as well as their relative amounts; especially Ca and P (Boskey *et al*., 1999). Seeing as bone strength was not compromised as a result of treatment, it can be concluded that relative mineral composition was also adequate for bone development. With this being said, Nkukwana *et al.* (2014) discovered no correlation between Ca, P or the Ca/P ratio and bone breakage strength having supplemented broiler diets with *Moringa oleifera* leaf meal. In this case, bone strength was unaffected by treatment whereas Ca and P concentrations varied significantly.

Deviations in Ca/P from the normal 2.15:1 weight ratio can be a major cause of tibial dyschondroplasia (TD) (Whitehead, 2007), making it an important parameter to consider when testing a novel feed additive. It can be seen in Table 5.5 that NutrifenPLUS® certainly had the greatest impact on the calcium (Ca), phosphorus (P) bone ash ratio. Although not statistically relevant, Table 5.5 suggests that the ratio was reduced as a result of slightly lower Ca concentrations and relatively stable P concentrations compared to the control group. This is an indication that NutrifenPLUS® had more of an effect on calcium metabolism than it did on phosphorus; therefore the explanation of this phenomenon could possibly be linked to changes in calcium metabolism as a result of the feed additive. A study by Uushona (2015) observed changes in tibia bone Ca content that were attributed to differences in mineral bioavailability, as could be the case with the current trial. Findings by Ziaie *et al.* (2011) and Mamoun *et al.* (2014) support this theory, as both studies reported changes in plasma Ca and P concentrations. A commercial herbal blend led to higher Ca and P levels in the blood and bone, comparable to those observed in antibiotic treatments (Ziaie *et al.*, 2011). Tibial breaking strength was also increased by treatment, contradicting the findings of Nkukwana *et al.* (2014). Mamoun *et al.* (2014) observed increases in P and reductions in Ca plasma concentrations as a result of fenugreek supplementation; however bone characteristics were not determined in this case. Another possible explanation is that NutrifenPLUS® caused a reduction in efficacy of the dietary calcium. This remains a theory however, and more extensive research on the subject is necessary to draw any conclusions.

NutrifenPLUS® consists of 72% Nutrifen® and a combination of other ingredients namely, fennel seed powder, saw palmetto berry powder, kelp powder, vinegar powder and methylsulfonylmethane (MSM). Since Nutrifen® is shown to have an effect on the Ca/P ratio albeit minor; we can therefore attribute some of the variation between treatments to fenugreek. It is clear however, that one of the other ingredients or a synergistic combination within the product had a more significant influence on Ca metabolism of the broiler than did fenugreek alone. Having made this observation, it is also clear that deviation from the ratio specified by Whitehead (2007), was not significant enough to increase the visible prevalence of TD, nor affect the tibial strength. This leads to the conclusion that Nutrifen® and NutrifenPLUS® do not cause any adverse effects on the biomechanical or biochemical integrity of the bone and are both suitable as broiler feed additives in this regard.

Table 4.10 Influence of Nutrifen® and NutrifenPLUS® feed additives on various tibia bone parameters (±SE)

Treatment	Fat-free Dry Mass (g)	Dry Matter %	Fat-free Ash %	Ca % of Bone Ash	P % of Bone Ash	Ca:P of Bone Ash
Neg. CON	4.23 ± 0.63	49.29 ± 1.25	51.81 ± 2.82	35.52 ± 3.73	15.39 ± 0.99	2.30^{a} ± 0.10
Antibiotic	4.84 ± 0.46	48.67 ± 1.78	52.67 ± 3.50	35.44 ± 5.50	15.59 ± 2.64	2.28^{a} ± 0.12
0.1%N	4.75 ± 0.47	48.20 ± 1.57	52.63 ± 3.12	39.75 ± 6.12	17.95 ± 2.17	2.21^{ab} ± 0.17
0.2%N	4.36 ± 0.35	48.13 ± 2.75	51.70 ± 2.58	39.50 ± 7.61	18.11 ± 2.94	2.17^{ab} ± 0.10
0.1%N+	4.67 ± 0.67	48.58 ± 1.33	52.24 ± 1.59	33.86 ± 18.42	15.95 ± 8.15	2.10^{b} ± 0.15
0.2%N+	4.60 ± 0.64	48.21 ± 1.46	51.83 ± 1.19	31.24 ± 6.26	16.24 ± 3.32	1.93^{c} ± 0.09
p-value	0.384	0.864	0.973	0.592	0.761	**< 0.001**

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

Table 4.11 Influence of Nutrifen® and NutrifenPLUS® feed additives on various broiler tibia bone minerals (±SE)

Treatment	K (%)	Mg (%)	Na (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	B (mg/kg)	Al (mg/kg)
Neg. CON	0.98 ± 0.09	1.16 ± 0.21	11278 ± 1224	149.6 ± 20.1	6.46 ± 3.25	396.6 ± 76.7	8.52 ± 0.67	7.55 ± 1.24	1.67 ± 1.45
Antibiotic	0.87 ± 0.07	1.14 ± 0.19	11821 ± 2048	139.7 ± 18.7	4.30 ± 3.78	406.5 ± 28.7	8.83 ± 1.50	7.13 ± 0.98	0.31 ± 0.43
0.1%N	0.89 ± 0.06	1.03 ± 0.08	13025 ± 2055	133.6 ± 16.8	4.73 ± 2.92	392.99 ± 39.6	8.19 ± 1.34	6.98 ± 1.00	0.17 ± 0.23
0.2%N	0.95 ± 0.06	0.91 ± 0.07	13327 ± 2321	141.8 ± 32.7	4.93 ± 3.15	388.4 ± 11.7	8.63 ± 0.94	7.03 ± 0.43	0.17 ± 0.40
0.1%N+	0.97 ± 0.08	0.98 ± 0.51	13631 ± 7066	166.2 ± 64.3	6.60 ± 4.43	381.5 ± 171.4	9.20 ± 2.11	6.92 ± 0.93	0.01 ± 0.00
0.2%N+	0.97 ± 0.10	1.02 ± 0.17	12311 ± 1834	149.2 ± 14.7	5.93 ± 4.68	388.2 ± 35.0	8.27 ± 1.48	6.63 ± 0.67	0.45 ± 0.74
p-value	0.08	0.523	0.816	0.619	0.848	0.997	0.832	0.662	**<0.01**

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

Table 4.12 Influence of Nutrifen® and NutrifenPLUS® feed additives on mean tibia bone breaking strength (±SE) of broilers

Treatment	Force (N)	Breaking Strength (N/g)
Neg. CON	292.94 ± 27.59	31.15 ± 2.90
Antibiotic	346.09 ± 49.08	33.46 ± 5.30
0.1%N	314.18 ± 40.38	29.61 ± 4.11
0.2%N	316.85 ± 27.67	32.95 ± 4.42
0.1%N+	330.81 ± 61.66	31.78 ± 4.24
0.2%N+	332.51 ± 71.44	31.87 ± 4.50
p-value	0.528	0.699

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

4.5 Conclusion

Relative and actual organ weights give an indication as to the well-being of the birds, as well as contributing to dressing percentage values. No treatment differences were found in any of the viscera, including the spleen and bursa. This suggests that the flock was in good health throughout the study and no birds were pathogenically challenged. Low gizzard erosion scores also suggest that treatment did not have any negative impact on gastric secretion or show direct toxicity in the gizzard and that feed was of adequate quality. Lightness as well as overall colour of the liver was unaffected by treatment, suggesting that the birds were in a normal physiological state and fat deposition was comparable to typical commercial standard. Very little literature is available to support or dispute results of the current study with regard to digestive tract pH. No variation in the luminal pH of any of the GIT components was detected, which again is indicative of a healthy flock. Pathogenic exposure may however influence GIT pH values, and further research in this field could be beneficial.

The results of this study indicate that Nutrifen® and NutrifenPLUS® both yielded similar results to those of the control concerning breakage strength, which was within a commercially acceptable range. This also shows that dietary calcium and phosphorus was adequately provided and utilized by the birds. Statistically lower Ca/P ratios observed in NutrifenPLUS® treatments are more likely a result of one or more of the other components of the product either than fenugreek. With this being said, fenugreek does seem to play a role if we consider the numerically lower values produced by Nutrifen® supplementation.

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Chapter 5

The evaluation of Nutrifen® and NutrifenPLUS® as broiler feed additives and their influence on slaughter parameters and meat quality

Abstract

The supplementation of broiler feeds with Nutrifen® and NitrifenPLUS® was investigated with regard to their impact on slaughter parameters and meat quality. Three-hundred and sixty, day-old, mixed gender Cobb500 broiler chicks were fed six treatment diets containing different non-nutritive feed additives until the age of 32 days. Each treatment diet was replicated six times and contained one of the following additives: negative control (no additive), positive control (150g/ton zinc bacitracin antibiotic), 0.1% Nutrifen®, 0.2% Nutrifen®, 0.1% NutrifenPLUS® and 0.2% NutrifenPLUS®. Birds were fed *ad libitum* throughout the trial. No treatment differences were observed when considering live weight, warm carcass and cold carcass weight or dressing percentage. The same lack of significant differences was observed with regard to the weight of the following commercial cuts: breasts, wings, drumsticks and thighs (actual weight and relative to cold carcass weight). Breast portions were also separated into three separate components namely bone, muscle and skin and subcutaneous fat. None of these components showed any weight variation across the six treatments. Muscle pH of the breasts and thighs showed no significant differences with regard to both initial (pH_i) and ultimate (pH_u) pH. Redness (a*) of the breast muscle was found to be affected significantly by treatment. The negative control, antibiotic, 0.2% Nutrifen® and 0.1% NutrifenPLUS® exhibited the highest redness values. The 0.1% Nutrifen® showed similar readings to the antibiotic treatment, but supplementation with 0.2% NutrifenPLUS® resulted in substantially lower readings than all other treatments. Treatment did not influence other colour parameters i.e. L*, b*, hue or chroma. Proximate analysis of the breast muscle showed no treatment differences in moisture, ash or crude protein. Crude fat was however influenced by treatment where supplementation with antibiotic, 0.1- and 0.2% NutrifenPLUS® yielded the highest fat percentages. Nutrifen® supplementation at a concentration of 0.2% produced significantly lower fat percentages than all other treatments.

5.1 Introduction

Broilers raised under current intensive conditions generally express less carcass pigmentation and abnormally dull/pale meat products (Ponsano *et al.*, 2004). This is almost solely as a

consequence of modern broiler diets being based primarily on nutrient dense materials which offer little in terms of colour pigments known as xanthophylls (Ratcliffe *et al*., 1959). Xanthophylls are a type of oxygen containing carotenoid; fat-soluble molecules biosynthesized by a number of higher order plants, and certain yeasts, fungi, algae and bacterial species (Sandmann, 1994). In plants, these are the pigments that are responsible for the colour of fruits and flower petals, but are masked by the green pigment chlorophyll in the leaves (Breithaupt, 2007). Carotenoids however cannot be synthesized by animals *de novo*, meaning that all product pigmentation must be provided through the diet (Goodwin, 1992). Following absorption into the bloodstream dietary carotenoids either accumulate in various tissues, providing colour to meat and egg products or undergo a number of structural modifications that yield secondary metabolites (Breithaupt, 2007).

With colour being one of the most important indications of meat quality as far as consumers are concerned (Velasco & Williams, 2011) it is common for producers to add natural or synthetic colorants to broiler diets in order to enhance the visual appeal of meat products (Castañeda *et al.*, 2005). These colorants generally come in the form of lutein, astaxanthin or canthaxanthin which are considered as the most industrially utilized oxycaratenoids (Breithaupt, 2007), however numerous herbs and spice are too being examined for this very purpose. It is also well known that there is a strong correlation between muscle pH and meat colour *post-mortem* (Warris, 2000); the most notable of which is the inverse relationship that exists between lightness (L*) and the pH of a raw breast fillet (Qiao *et al*., 2001). At the same time, temperature and rate of pH decline as well as pH_u also have an influence on other meat quality characteristics such as water-holding capacity, tenderness and texture (Warris, 2000), although these aspects were not expressed in the current study.

Other natural colourants such as phytogenic additives however may provide alternative benefits to conventional colour enhancers, most notably their antioxidant potencies (Velasco & Williams, 2011). With a recent shift away from the consumption of products containing synthetic antioxidants due to their carcinogenic effects (van Esch, 1986), the introduction of phytogenics may provide a solution to both the colour and anti-oxidative consumer issues. Natural additives are however less effective than synthetic antioxidants and come at a greater cost (Fasseas *et al.*, 2008).

Lipid oxidation during storage is largely influenced by diet (Aouadi *et al.*, 2014) and can lead to muscle quality deterioration, which has a direct effect on flavour, colour and nutritional value of meat (Sharbati *et al.*, 2015). By incorporating natural herbs and/or herb extracts into animal diets, oxidative stability and shelf-life, as well as long term meat colour may be improved (Eleroğlu *et al.*, 2013). This is likely due to the presence of flavonoids and other phenolic

compounds which are known to have certain anti-oxidative and hypocholesterolemic effects on poultry products (Lahucky *et al.*, 2010). In addition to providing oxidative stability, plant extracts may be used to alter fat composition and reduce fat load in poultry (Paravar *et al.*, 2013), which has recently shown a drastic increase as a consequence of high growth rates and the genetic correlation between the two traits (Becker *et al.*, 1979). Apart from product colour and chemical composition, carcass characteristics such as dressing percentage and portion sizes are also important aspects to consider if the birds are to be sold as portion cuts, because the price per kilogram differs depending on the cut (SAPA, 2013).

5.2 Materials and Methods

5.2.1 Carcass characteristics

Birds were raised and fed the same treatment diets as stated in Chapter 3. Briefly, 360 Cobb 500 day-old broiler chicks were used. Six treatment diets consisting of six replications were applied from day one until day 32 and diets were formulated to contain different levels of non-nutritive feed additive as follows: no additive, 150 g/ton Zinc bacitracin antibiotic growth promoter, 0.1% Nutrifen®, 0.2% Nutrifen®, 0.1% NutrifenPLUS® and 0.2% NutrifenPLUS®.

Birds were slaughtered according to standard commercial practice by stunning and subsequent exsanguination as in the previous chapter, after live weight had been recorded. Carcasses were de-feathered and eviscerated (removal of feet, neck and internal organs), before being weighed and the pH of the right breast and thigh muscles being recorded. Both warm carcass weight and the initial breast and thigh pH's (pH_i) were recorded within 15 minutes post mortem. Carcasses were weighed using a Mettler PC 4400 scale (Mettler-Toledo, Switzerland) and pH measurements were taken using a Crison pH25 meter (calibrated using standard pH 4.0 and 7.0 buffers). The pH was measured by making a small incision in the centre of each muscle where the probe was inserted and readings were taken.

Carcasses were kept at 4°C and weighed once again, 24 hours post-mortem to determine cold carcass weight using the same scale as before. The same was done for pH to obtain an ultimate muscle pH reading (pH_u), by inserting the electrode into the same incisions as for the pH_i readings.

Dressing percentage is expressed as the warm carcass weight, after de-feathering and evisceration as a percentage of the live weight of the bird. This was calculated from the recorded weights using Equation 5.1, before the carcasses were separated into commercial portions. Cold carcasses were first cut in half using a portion cutter, before the thighs and drumsticks were separated from the carcass by cutting behind the pubic bone, towards the

acetabulum. Drumsticks and thighs were then separated by cutting through the joint, connecting these two cuts. Wings were subsequently removed by cutting between the scapula and the coracoid, followed by the breasts. Both parts of each commercial cut were weighed together on a Mettler PC 4400 scale (Mettler-Toledo, Switzerland) and expressed as a percentage of the cold carcass weight.

Once weighed, the right breast of each bird was divided into three potions namely, bone, muscle and skin and subcutaneous fat combined. Each of these portions was weighed using a Mettler PC 4400 scale (Mettler-Toledo, Switzerland) and expressed as a percentage of total breast weight.

The muscle portion of the left breast was cut open longitudinally, and placed on a flat surface at 8°C for 30 minutes in order to allow the meat to bloom (Warris, 2000). Once bloomed, colour readings of each breast were taken using a CIE-Lab colour meter (BYK-Gardner GmbH, Gerestried, Germany). Meat colour was expressed using three different values i.e. L* (lightness), a* (redness) and b* (yellowness) (Nollet *et al*., 2007). Colour readings for each breast were measured four times, over the total bloomed area and used to calculate an average in order to get an accurate reflection of the overall breast colour. From the measured L*, a* and b* values, hue and chroma values could be calculated using Equation 5.2 and Equation 5.3. Leftover muscle tissue was vacuum-sealed and labelled accordingly, to be frozen at -18°C awaiting chemical analysis at a later stage.

Equation 5.1 $$DP = \frac{\text{Warm Carcass Weight}}{\text{Live Weight}}$$

Equation 5.2 $$\text{Chroma} = \sqrt{(a^{*2}+b^{*2})}$$

Equation 5.3 $$\text{Hue} = \tan^{-1}(b^{*}/a^{*})$$

5.2.2 Proximate analysis

Meat from the right breast portions was thawed in a fridge at 4°C for 12 hours, after which it was homogenised and re-sealed in the original vacuum packaging. Proximate analysis was performed on this meat, to determine moisture, ash, fat and protein levels.

5.2.2.1 Moisture

Moisture content/ dry matter percentage (DM) was determined using AOAC official method 934.01 as described by the Association of Official Analytical Chemists (2002). In short, 2.5g of homogenised sample was dried at 100-105°C for 24 hours and allowed

to cool in a desiccator for 30 minutes thereafter, before being weighed. Duplicate samples were used to calculate an average for each sample.

5.2.2.2 Ash

Samples used to determine DM percentage were subsequently used to determine ash content, according to the AOAC official method 942.05 as described by the Association of Official Analytical Chemists (2002). Briefly, the moisture-free samples were placed in a furnace at 500°C for 6 hours. Once removed from the furnace samples were allowed to cool in a desiccator for 30 minutes, prior to being weighed on the same scale as was used for moisture determination.

5.2.2.3 Crude fat

Crude fat extraction was determined using the ether extraction method as described by Lee *et al.*, 1996. In summary, 5g of homogenised sample was mixed with a chloroform/methanol solution (1:2) for 1 minute. The resulting solution was filtered through filter paper (Whatman no. 1) into a separation funnel. The residue remaining in the filter paper was placed into an oven at 100°C for 48 hours for later analysis. A 0.5% NaCl (20ml) solution was added to the filtrate before being shaken. The resulting solution was allowed to stand for 60 minutes. Once clear separation of the solution was visible, 5ml of the bottom layer was placed in an Erlenmeyer flask which was allowed to stand on a sand plate for 45 minutes. Once all of the chloroform/methanol solution had evaporated, fat beakers were cooled in a desiccator for 30 minutes and weighed.

5.2.2.4 Crude protein

Crude protein was determined by means of the official AOAC Dumas combustion method 992.15 as described by the Association of Official Analytical Chemists (2002). The moisture-free sample residue obtained from the fat extraction as mentioned, was ground to a fine powder, 0.1000g sample weighed off, and subsequently analysed for quantitative nitrogen content using a LECO FP528. Before use, the LECO was calibrated using EDTA. Following LECO analysis,

Equation 5.4 was used to establish an “as is” nitrogen percentage, which could then be multiplied by a factor of 6.25 to get a crude protein percentage.

Equation 5.4 $$\text{Nitrogen \% (as is)} = \frac{\text{N\%} \times (100 - \text{Moisture\%} - \text{Crude Fat\%})}{100}$$

5.2.3 Statistical analysis

The following hypotheses were proposed:

H_0: There is no association between carcass characteristics and meat quality, and the treatment diets applied.

H_a: There is evidence to suggest that there is a relationship between carcass characteristics and the treatment diets applied.

Before any analysis, tests for normality and homoscedasticity were performed. A one-way ANOVA and appropriate *post hoc* test (Fisher's LSD or Games-Howell) was then performed on each data set, depending on whether the assumptions of normality and homoscedasticity were satisfied or not. A p-value of 0.05 (5%) was used to declare statistical significance and any p-values <0.01 (1%) were considered highly significant. All statistical analysis was performed using Statistica.

The one-way ANOVA that was performed can be explained by the following model: $Y_{ij} = \mu_i + \alpha_j + \varepsilon_{ij}$, where Y_{ij} *is* the response variable, μ_i the overall mean, α_j the treatment effect and ε_{ij} the unexplained error.

5.3 Results and Discussion

5.3.1 Physical carcass characteristics

The inclusion of Nutrifen® and NutrifenPLUS® was observed to have no significant effects on the live weight, warm carcass weight, cold carcass weight or dressing percentage ($p>0.05$) of Cobb500 broiler chickens. Results can be seen in Table 5.1. The same lack of significant differences can be seen in Table 5.2, where thighs, wings, drumsticks and breasts were found to be in similar proportion to cold carcass weight across treatments ($p>0.05$). Breasts were divided into the three components of muscle, bone and skin and subcutaneous fat. These components were weighed individually. Table 5.3 shows differences in weights of each individual component to be non-significant between treatments.

All available literature seems to suggest that phytogenics could have a place in modern feeding systems in terms of improving carcass yield, but effects appear to vary depending on species, genetics and environmental conditions. In general phytogenic supplementation has not been proven to have any negative impact on dressing percentage (DP) in broiler chickens, and literature shows that this may be true for rabbits as well (Cardinali *et al.*, 2015; Hossian *et al.*, 2015). In these cases oregano, rosemary and garlic were tested as rabbit feed additives and all treatments exhibited increases in dressing percentage relative to control diets. Oregano and rosemary showed significant increases, whereas garlic lacked statistical evidence to support any differences. Oregano supplementation in broiler diets however, resulted in no change to dressing percentage (Teuchert, 2014) and garlic was found to be statistically more effective (Sahoo *et al.*, 2013).

With regard to broiler studies, many have produced positive results involving a variety of different herbs and spices, including fenugreek. Sahoo *et al.* (2013), Mamoun *et al.* (2014), Prajapat (2016) and Toaha *et al.* (2016) all found fenugreek to be effective supplements for obtaining higher carcass yields. These results are supported by Durrani *et al.* (2008), Yazdi *et al.* (2014) and Ahmed *et al.* (2015) who found that wild mint, anise and basil supplementation all led to improved dressing percentages in broilers respectively. Keeping this in mind, wild mint was only found to be effective at concentrations of 15g/kg and Sahoo *et al.* (2013) used a combination of garlic, fenugreek and *Commiphora mukul* in their experiment. Positive results could therefore be attributed to the synergistic effects of two or more phytogenic extracts, and not one specifically.

Many other studies have also shown a lack of evidence to support the previously mentioned trends. Soltan *et al*. (2008) and Al-Beitawi & El-Ghousein (2008) found carcass yield to be unaffected by anise and black cumin seeds respectively. Similarly, studies by Khadr & Abdel-Fattah (2007), Abbas (2010), Alloui *et al.* (2012) and Duru *et al.* (2013) are all in agreement with the current trial, concluding that fenugreek has no impact on the dressing percentage of a broiler carcass. Furthermore, Elagib & Elamin (2013) performed a study concerning the effects of cinnamon, ginger and fenugreek against antibiotic treatment on broiler carcass characteristics. Cinnamon and ginger supplementation produced high carcass yields, comparable to those observed in antibiotic treated birds, whereas fenugreek produced significantly lower dressing percentages that were similar to the control group. This emphasizes the need for further research in this field, with regard to plant species and their effectiveness under different environmental conditions.

Cold carcass weight is rarely mentioned in relevant literature and most observations have been made with regard to warm weight. Warm carcass weight in most cases, exhibits

contrasting results to the current study. Although Prajapat (2016) as stated earlier, had fewer than sufficient replications, the results showed similar trends to those of Mamoun *et al.* (2014). In both studies, warm carcass weight was increased by fenugreek supplementation in comparison to the control diets. More importantly however, warm carcass weight was found to be inversely proportional to the concentration of dietary fenugreek i.e. higher fenugreek concentrations resulted in lower carcass weights. Duru *et al.* (2013) reported a similar trend, although supplementation yielded lower overall warm carcass weights than control groups in this particular experiment. In these cases birds were housed in either open-sided houses or in floor cages with bedding. Open-sided houses can lead to temperature variation and bedding can increase exposure to pathogenic organisms. Bedding can also cause dust. These are all factors that could contribute to stress, on which the effectiveness of fenugreek was speculated to be partially dependent on.

Osman *et al.* (2010) also observed higher warm carcass weights for broilers fed marjoram, rosemary and sweet basil, however trends were dissimilar to those of Duru *et al.* (2013), Mamoun *et al.* (2014) and Prajapat (2016), where increases were not as dependent on supplement concentration. These results were also numerical observations and were not statistically supported, apart from marjoram supplementation at a 1.0 g/kg concentration. This follows the theory regarding stress being an integral part of phytogenic supplementation in broiler feeds. Birds in this case were housed in a strictly controlled environment and although some variation was observed across treatments, most was not statistically supported. Furthermore, fenugreek was not included in this study. Similar studies by Cardinali *et al.* (2015) and Hossian *et al.* (2015) also concluded that oregano and garlic supplements were effective for improving carcass weight in meat rabbits. Again, only results for oregano were statistically significant, whereas the results of garlic supplementation were merely numeric observations. In contrast, some available literature does agree with the present findings. Osman *et al.* (n.d.) found the leaves of eucalyptus, pomegranate, tilia or thyme to have little effect on carcass weight in broilers. In agreement with this are Zeweil *et al*. (2015), who observed no correlation between fenugreek or anise supplementation and warm or cold carcass weight in rabbits.

Table 5.1 Influence of Nutrifen® and NutrifenPLUS® on mean live weight and physical carcass characteristics (±SE**)**

Treatment	Live Weight (g)	Warm Weight (g)	Cold Weight (g)	Dressing %
Neg. CON	2057 ± 237.1	1435 ± 99.6	1436 ± 99.5	70.21 ± 5.45
Antibiotic	2062 ± 144.7	1412 ± 106.6	1413 ± 105.8	68.45 ± 1.32
0.1%N	2109 ± 170.3	1432 ± 119.1	1433 ± 118.7	67.91 ± 2.06
0.2%N	2076 ± 177.8	1406 ± 119.5	1406 ± 119.7	67.71 ± 0.97
0.1%N+	1940 ± 45.3	1319 ± 55.8	1318 ± 56.4	67.95 ± 1.65
0.2%N+	2078 ± 213.7	1398 ± 142.4	1399 ± 142.6	67.30 ± 0.88
p-value	0.651	0.499	0.484	0.465

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

In most cases, phytogenic supplementation has shown little effect on commercial carcass cuts as a percentage of the bird's carcass weight. However, literature suggests that fenugreek performs marginally better than other herbs and spices in this regard. Although the current study showed no significant differences, which agrees with the findings of Mamoun *et al.* (2014). Studies by Elagib & Elamin (2013), Khan *et al.* (2013), Sahoo *et al.* (2013) and Toaha *et al.* (2016) all found weight improvements in one or more commercial cuts; most notably the breast portion. All increases were attributed to treatments containing fenugreek, although Sahoo *et al*. (2013) included fenugreek as part of a mixture containing *Commiphora mukul* and garlic. Khan *et al.* (2013) found the relationship between dietary fenugreek concentration and weight increases of the breast, leg and thigh to be linear, whereas other trials did not exhibit such trends. Elagib & Elamin (2013) also tested cumin, ginger and cinnamon as individual supplements in the same experiment. The dietary inclusion of fenugreek and cinnamon resulted in considerably higher breast weights compared to the other two spices, and even outperformed the antibiotic treatment. With this being said, no significant effects were observed with regard to wing or thigh weight. Contradicting all abovementioned results, Duru *et al.* (2013) discovered a negative correlation between breast, wing and leg weights and fenugreek treatment.

Other phytogenic supplements appear largely ineffective at improving commercial cut value, as can be seen in studies concerning pepper (Garcia *et al*., 2007), black cumin (Al-Beitawi & El-Ghousein, 2008), wild mint (Durrani *et al.*, 2008), anise (Simsek *et al*., 2007), oregano (Teuchert, 2014; Garcia *et al*., 2007) and eucalyptus, pomegranate, tilia and thyme (Osman *et al.*, n.d.). The study by Garcia *et al*. (2007) however, does raise a conflict of results obtained by Elagib & Elamin (2013). Cinnamon was also included as an individual additive in this study, and was found to have no significant effect on breast weight, whereas Elagib & Elamin (2013) concluded that cinnamon supplementation was comparable to that of fenugreek in terms of its effects on breast weight.

Literature concerning phytogenic additives and their effects on different components of the breast muscle is very scarce. In fact, only one other source could be found. Teuchert (2014) discusses the impact of dietary oregano on broiler breast composition, and was led to similar conclusions as the present study with regard to fat and skin percentage. Muscle and bone percentages in this study however, were affected by treatment. Muscle percentage was found to be the lowest for birds treated with antibiotics, whereas treatment with oregano led to numerical increases relative to the control. This was especially true for the treatment containing a combination of the oregano product (Ateli plus®) and antibiotics, which showed the greatest breast muscle percentage. The bone percentage of the breast was numerically

reduced by Ateli plus® and was found to be statistically lowest in the Ateli plus®/antibiotic treatment. Antibiotic treatment alone, resulted in the highest proportion of bone; an observation that was statistically significant. Since no other known literature is available and results of these two studies are contradictory, it is impossible to draw conclusions without further research.

Table 5.2 Influence of Nutrifen® and NutrifenPLUS® on commercial carcass cuts and their mean proportion relative to cold carcass weight (±SE)

Treatment	Thighs (%)	Breasts (%)	Drumsticks (%)	Wings (%)
Neg. CON	28.51 ± 1.23	42.61 ± 1.39	13.00 ± 0.42	14.81 ± 0.81
Antibiotic	27.65 ± 1.18	43.08 ± 1.40	13.70 ± 1.04	14.39 ± 1.10
0.1%N	27.86 ± 0.98	42.36 ± 1.06	12.97 ± 1.03	15.40 ± 1.75
0.2%N	28.43 ± 4.03	42.91 ± 4.38	13.52 ± 2.11	14.77 ± 1.66
0.1%N+	28.95 ± 1.86	41.04 ± 1.73	13.58 ± 0.60	15.27 ± 0.88
0.2%N+	30.95 ± 5.88	39.42 ± 6.50	13.36 ± 0.64	15.01 ± 1.06
p-value	0.512	0.414	0.814	0.771

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

Table 5.3 Mean proportions of the components of the commercial breast cut (±SE) as influenced by the dietary inclusion of Nutrifen® and NutrifenPLUS®

Treatment	Bone (%)	Muscle (%)	Skin and Sub. Fat (%)
Neg. CON	26.47 ± 6.84	66.31 ± 7.63	7.22 ± 1.18
Antibiotic	25.26 ± 4.88	67.99 ± 5.20	6.75 ± 1.89
0.1%N	27.95 ± 2.86	65.28 ± 2.61	6.77 ± 1.82
0.2%N	26.02 ± 7.33	66.70 ± 7.64	7.28 ± 0.83
0.1%N+	28.33 ± 6.20	64.31 ± 6.72	7.36 ± 1.35
0.2%N+	28.70 ± 2.63	64.48 ± 2.43	6.82 ± 0.89
p-value	0.849	0.872	0.939

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

5.3.2 Muscle pH and CIE-Lab measurements

Breast and thigh muscle pH's were unaffected by the inclusion of Nutrifen® or NutrifenPLUS® in the diet of Cobb500 broiler chickens. This was found to be true for both initial and ultimate pH of the aforementioned muscles ($p>0.05$). Refer to Table 5.4 for results regarding the pH of the muscles. The inclusion of Nutrifen® and NutrifenPLUS® did not affect colour parameters of an uncooked broiler breast muscle to any significant extent except for the redness, as represented by the "a*"- reading ($p<0.05$). Results can be found in Table 5.5, where the negative (no additive) and positive (zinc bac antibiotic) controls, the 0.1- and 0.2% NutrifenPLUS® inclusion and the 0.2% Nutrifen® inclusion all produced similar readings for redness of the breast meat. The inclusion of Nutrifen® at a concentration of 0.1% resulted in similar redness values to treatments including zinc bacitracin antibiotic and 0.2% NutrifenPLUS®. With this in mind, 0.2% NutrifenPLUS® showed similarities only to 0.1% Nutrifen® inclusion levels with regard to redness and had a significantly lower a*-value than the other treatment diets.

It is well-known that a strong correlation exists between muscle pH and meat colour (Mancini & Hunt, 2005). Major changes occurring in a muscle post-mortem include acidification and the resolution of rigor, where acidification affects the water-holding capacity and the colour of the meat (Warris, 2000). Meat colour has been reported to be the most important aspect of meat quality to the consumer, as it is associated with freshness (Velasco & Williams, 2011). Post-mortem colour changes can be attributed to the oxidation of red oxymyoglobin to metmyoglobin (MMG) which turns the meat an unattractive brown colour, thereby discouraging consumers (Pirmohamammadi *et al.*, 2016). The ability of certain phytogenic feed additives to reduce or slow down the rate of pH decline post-mortem, may improve meat colour by reducing lightness (L*), thus maintaining a less pale and more attractive colour. Such observations were made by Pirmohamammadi *et al.* (2016), where thyme and mentha supplementation to heat-stressed broilers significantly reduced L* readings of the thigh muscle without affecting redness (a*) values. Yellowness (b*) values were reduced by supplementation, but to a non-significant extent. Although not statistically supported, the lower L* values also correlated with numerically higher pH values. This relationship between L* and pH was however statistically proven by Rahman *et al.* (2016) where L* was reduced corresponding to an increase in pH, as a result of cumin supplementation. In contrast to these results, *Satureja Khuzestanic* which belongs to the same family as rosemary and thyme, only yielded a non-significant reduction in breast muscle pH_i (Paravar *et al.*, 2013).

Oregano supplementation was found to have a similar influence to cumin and thyme, on the L* readings of broiler breast and thigh meat (Teuchert, 2014). Eleroğlu *et al.* (2013) however

found variation only in yellowness (b*) when oregano was included in the diet, as did Young *et al*. (2003). With this being said, the results of Teuchert (2014) and Young *et al*. (2003) contradict those of Eleroğlu *et al.* (2013). Teuchert (2014) and Young *et al*. (2003) concluded that oregano supplementation led to higher b* values for breast and thigh meat, whereas Eleroğlu *et al.* (2013) observed reductions in b* values in the thigh muscles of birds fed oregano. All of these findings were statistically supported, making it difficult to draw conclusions based on the contrasting results. The influence of black cumin supplementation on meat colour has also proven somewhat contraversial. Significant variation was observed by Rahman *et al.*, 2016 concerning pH, L*, a* and b* values, but colour parameters were unaffected by the dietary inclusion of both cumin and *Echinacea purpurea* (purple coneflower) in a similar study by Nasir & Grashorn (2010).

Meat colour is generally defined by a point within a three-dimensional sphere or “colour space” (Warris, 2000). The first dimension is given by lightness and reflectance, which indicates how light or dark the meat is. This plane is accounted for by the L* value mentioned above. The second function is hue, which describes what is commonly referred to as colour ie. red, green etc. The third characteristic is chroma or saturation, which indicates the purity or intensity/lack of dullness of a colour. Hue and chroma values were not recorded in most available literature, but in agreement with the present study, Eleroğlu *et al.* (2013) found the dietary inclusion of oregano and lemon balm leaves to have no significant influence on the hue of broiler meat. Chroma values of the thigh were however reduced by both of these supplements. Another study that did not involve phytogenic feed additives (Ponsano *et al.*, 2004), obtained similar breast meat chroma values as the current study for all treatments. The hue values of the present study were considerably higher than those of Ponsano *et al.* (2004), but this could be due to the basal diet being maize based rather than sorghum based. No other literature relating to the hue or chroma of poultry meat could be found.

Table 5.4 Influence of Nutrifen® and NutrifenPLUS® on the mean initial (pHi) and ultimate (pHu) pH of the breast and thigh muscles (±SE)

Treatment	Breast pH_i	Breast pH_u	Thigh pH_i	Thigh pH_u
Neg. CON	5.93 ± 0.45	5.78 ± 0.19	6.12 ± 0.15	6.35 ± 0.10
Antibiotic	5.98 ± 0.13	5.93 ± 0.21	6.23 ± 0.19	6.35 ± 0.20
0.1%N	5.95 ± 0.13	5.90 ± 0.10	6.15 ± 0.18	6.49 ± 0.13
0.2%N	5.82 ± 0.18	5.89 ± 0.11	6.04 ± 0.18	6.35 ± 0.10
0.1%N+	5.87 ± 0.07	5.90 ± 0.13	6.17 ± 0.25	6.18 ± 0.30
0.2%N+	5.90 ± 0.17	5.91 ± 0.21	6.16 ± 0.14	6.35 ± 0.19
p-value	0.845	0.673	0.630	0.144

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

Table 5.5 Influence of Nutrifen® and NutrifenPLUS® on mean CIE-Lab colour readings (±SE) of the broiler breast muscle

Treatment	L*	a*	b*	Hue	Chroma
Neg. CON	49.95 ± 3.20	3.30[a] ± 1.06	11.98 ± 0.96	74.50 ± 5.71	12.54 ± 0.79
Antibiotic	49.05 ± 0.94	3.27[ab] ± 0.77	11.66 ± 1.17	74.42 ± 4.11	12.20 ± 1.20
0.1%N	50.76 ± 1.39	2.39[cb] ± 0.38	12.50 ± 1.15	78.75 ± 2.49	12.76 ± 1.14
0.2%N	49.20 ± 2.74	3.51[a] ± 0.48	12.47 ± 1.80	73.85 ± 4.71	13.05 ± 1.57
0.1%N+	49.48 ± 3.16	3.39[a] ± 0.54	13.06 ± 1.05	75.40 ± 2.42	13.53 ± 1.07
0.2%N+	51.55 ± 1.25	2.29[c] ± 1.02	12.81 ± 1.30	79.53 ± 5.08	13.10 ± 1.19
p-value	0.395	**<0.05**	0.436	0.115	0.465

[a,b]Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

5.3.3 Proximate analysis of the breast muscle

Moisture and crude protein content of the broiler breast muscle was not affected by Nutrifen® and NutrifenPLUS® dietary inclusion, however significant results were acquired in the cases of fat and ash expressed as a percentage of the dry matter content ($p<0.05$).

The highest fat concentrations resulted from 0.1- and 0.2% Nutrifen® and 0.1% NutrifenPLUS® supplementation, along with the negative control (no additive), as indicated in Table 5.6. The inclusion of zinc bacitracin antibiotic produced similar crude fat concentrations to the negative control and the 0.2% NutrifenPLUS®; however the 0.2% NutrifenPLUS® treatment produced significantly lower crude fat concentrations than those resulting from the Nutrifen supplementation at either concentration.

Ash levels were found to be greatest in the antibiotic treatment; however these levels did not differ significantly from both 0.1- and 0.2% NutrifenPLUS® treatments. The negative control, 0.1% Nutrifen®, 0.1- and 0.2% NutrifenPLUS® all shared similar ash concentrations and only the negative control, 0.1% Nutrifen® and 0.2% NutrifenPLUS® showed similar ash characteristics to the 0.2% Nutrifen®. NutrifenPLUS® at a 0.2% concentration was proven to have the lowest ash percentage on a dry matter basis. Results can be found in Table 5.6.

Literature concerning the effects of fenugreek specifically, on broiler meat quality is rather limited. A study by Sahoo *et al*. (2013) involving a treatment combining fenugreek with *Commiphora mukul* and garlic, resulted in an increase in the breast fat percentage however no significance could be reported in the current trial. Both trials are in agreement with regard to meat protein percentage however, where no variation among treatments was observed. Other trials by Rahman *et al.* (2016) and Toaha *et al.* (2016) involving a black cumin and 2% fenugreek treatment respectively, found that dietary inclusion reduced abdominal fat content and breast fat percentage in broilers significantly. Abdominal fat content was however found to be greater in treated groups when broilers were fed a marjoram supplements at a concentration of 1 g/kg (Mona Osman *et al.*, 2010). Abdominal fat is linked to total body fat in avian species, making it a reliable parameter for predicting the total body fat content of a broiler (Becker *et al.*, 1979). Toaha *et al.* (2016) also concluded that antibiotic treatment increased abdominal fat content significantly which directly contradicts the findings of Sahoo *et al*. (2013) and goes against indications of the current study.

Osman *et al.* (n.d.) found no correlation between the fat and protein content of broiler meat, and phytogenic supplementation. This study did however; find that the breast muscles of broiler chickens fed thyme, exhibited statistically higher values for ash and moisture content, which agrees with the findings of Herkel' *et al.* (2016) who fed a mixture of oregano, anise and

citrus essential oils to turkeys. Similarly, oregano supplementation in rabbit diets also resulted in a higher moisture content of the *Longissimus dorsi* (LD) muscle, but had no influence on other meat quality parameters (Cardinali *et al.*, 2015). The same study concluded that rosemary had a positive effect on protein content of the LD as did Hossian *et al.* (2015) with regard to garlic supplementation. The effects of garlic were only numerical, but this was also found to be statistically true for poultry receiving phytogenic supplements in some cases (Herkel' *et al.*, 2016; Rahman *et al.*, 2016). A numerical reduction in LD fat percentage was also observed by Hossian *et al.* (2015), which again disagrees with the findings of Sahoo *et al*. (2013).

Table 5.6 Influence of Nutrifen® and NutrifenPLUS® on mean nutritional composition (±SE) of the broiler breast muscle

Treatment	Moisture %	Crude Protein %DM	Crude Fat %DM	Ash %DM
Neg. CON	75.21 ± 0.91	22.01 ± 1.06	10.15^{abc} ± 0.91	4.70^{bc} ± 0.16
Antibiotic	75.99 ± 0.48	21.46 ± 0.65	9.38^{bc} ± 0.97	5.05^{a} ± 0.28
0.1%N	75.95 ± 0.73	21.21 ± 0.51	10.98^{a} ± 0.87	4.70^{bc} ± 0.20
0.2%N	75.43 ± 0.71	21.45 ± 0.78	11.12^{a} ± 0.88	4.64^{c} ± 0.19
0.1%N+	76.25 ± 0.79	20.99 ± 1.13	10.52^{ab} ± 1.61	4.91^{ab} ± 0.20
0.2%N+	75.81 ± 0.80	21.78 ± 0.84	9.08^{c} ± 1.68	4.82^{abc} ± 0.24
p-value	0.193	0.371	**<0.05**	**<0.05**

^{a,b}Means with different superscripts differ significantly from each other (P<0.05)

Neg. CON: No non- nutritive additive

Antibiotic: 150g/ton zinc bac antibiotic inclusion

0.1%N: Nutrifen® 0.1% inclusion

0.2%N: Nutrifen® 0.2% inclusion

0.1%N+: NutrifenPLUS® 0.1% inclusion

0.2%N+: NutrifenPLUS® 0.2% inclusion

5.4 Conclusion

Dressing percentage and the relative weights of commercial carcass cuts are among the most important aspects of a broiler carcass' physical traits and none of these characteristics, as well as warm and cold carcass weight were affected by phytogenic or antibiotic treatment. Breast composition that may have a certain influence on consumer preference was also found to be unaffected by treatment. Breast and thigh muscle pH were found to be of no significance between treatments.

Redness (a*) of the breast meat was statistically influenced by phytogenic supplementation, but more importantly, lightness (L*) and/or chroma values were not affected adversely. Light/pale meat (lower L* values) generally appears less attractive to the consumer and would compromise its value significantly. Similarly, higher chroma/saturation values indicate greater colour intensity, making the meat more visually appealing. With consumers growing more health conscious in recent times, it is also important to limit intramuscular fat deposition as far as possible. In this study, phytogenic treatment showed some statistical differences in fat content of the breast meat, although all were comparable to the negative control. Differences in meat ash percentage can be attributed to treatment, where the supplements could be responsible for altering the host microbiota and thus, the bioavailability of certain minerals.

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Chapter 6

General conclusion and recommendations

Although not shown in the current study, the general consensus drawn from available literature is that fenugreek increases live weight and body weight gain during the latter stages of broiler production. Concentrations of the additive, conditions under which it is most influential, and its mechanisms of action are still largely unknown, although exposure to stress factors seems to play a role in its effectiveness. Further research into the effects of fenugreek under stressful conditions (i.e. heat/temperature variation) or following pathogenic exposure would possibly yield more conclusive results in future. Results regarding feed intake are far more difficult to interpret, as a much wider scope of variation across studies was observed. Concentration of the phytogenic supplement in the feed seems to be of more importance when it comes to intake than body weight gains. This may be an indirect effect of its contribution to the palatability of a feed mixture however this is unlikely in the case of poultry. The synergistic effects of two or more phytogenic substances may be of value in such cases, where the addition of an individual herb/spice may compromise the palatability of a feed. With this noted, changes in feed intake may also stem from changes in digestibility, or nutrient availability that results from histological changes caused by the additive. These are mere speculations however that exist among numerous others at this point. More extensive research into specific plant compounds that may have the potential to influence feed intake, and their mechanisms of action could breakdown some of the complexity surrounding the matter.

Most phytogenic supplements seem to have an economically significant influence on the feed conversion ratios (FCR) of certain poultry species, as well as other commercially farmed animals. Most instances produce positive changes in FCR, although the exact mechanisms of action are still misunderstood. This study however could not demonstrate the economical potential value in FCR thus further research regarding different plant species and the impacts that they may have on the FCR of different animals would be beneficial to the agricultural sector. Based on the lack of treatment effects in the current study, further research may also yield better results if birds were subject to some kind of external stress. European production efficiency factor (EPEF) appears to be of more importance when mortality rates are high within the flock. In cases where mortality is low and conditions are suitably controlled, EPEF values seem relatively constant between treatments. Unless phytogenic supplementation has a drastic influence on FCR or live weight and birds are subject to the same conditions, little variation in EPEF should be observed. Protein efficiency ratio (PER) seems to be one of the most conclusive areas of study with regard to phytogenic supplementation in broiler feeds.

Most available literature suggests; be it statistical or numerical, that the use of phytogenic additives leads to better dietary protein utilization by the bird. This was even supported by the current study, although no statistical evidence was found to substantiate this. Furthermore, results of some studies indicate that fenugreek may have a role to play in altering ADG, but results are generally scattered and inconclusive in this regard. The results of this study however as well as certain others, suggest that fenugreek has very little influence on average daily gain (ADG) and further research concerning other aspects of production would be more valuable.

Fenugreek treatment was found to have a significant influence on breast fat percentage in the current study, as well as numerous other studies not concerning poultry. With very little literature available on this subject however, it is difficult to provide further recommendation, although other herbs and spices with similar hypocholesterolemic effects have been known to reduce carcass fat and alter composition somewhat. Further research in this field may be of benefit, but other areas of production are likely to yield more conclusive results.

Similar can be said for calcium and phosphorus bone ash ratio, where statistically significant differences were observed in the present study. Changes however, did not translate into any differences in bone breakage strength or any other bone parameters for that matter. With very few studies having performed bone breakage analysis on phytogenically supplemented birds' further research may be of value, although more emphasis should be placed on production parameters.

The redness (a*) of raw breast fillet was affected by treatment in the current study as well as in other research based on phytogenic additives. Although literature on the subject was scarce, there has also been evidence to suggest that lightness values may be influenced by the dietary inclusion of certain herbs and spices. With the importance of colour to the consumer and the recent shift away from synthetic antioxidants, this field of research begs more attention. Very little significance was observed with regard to liver colour, immune status as indicated by lymph organ weights, intestinal and muscle pH, carcass characteristics such as dressing percentage and portion size, or any other parameters measured in the current study. Furthermore, no significant differences in gizzard erosion were observed between treatments, indicating that the feed provided was not contaminated with any significant levels of toxic substances and that the additives Nutrifen® and NutrifenPlus® are not causative agents of gizzard erosion.