Bromelain as a feed additive to promote broiler performance, nutrient digestibility and health

By

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DECLARATION

I, Emmanuela Sarrimanolis, declare that this dissertation, which I hereby submit for the degree MSc. Agric Animal Science: Animal Nutrition at the University of Pretoria is my own work and has not been previously submitted by me for a degree at this or any other tertiary institution.

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ABSTRACT

The broiler industry faces a growing pressure to reduce antibiotic growth promotors (AGP) from broiler feed due to public concern and the threat of antibiotic resistance. This has caused an upsurge in studies relating to natural alternatives to antibiotic growth promoters. Some such alternatives are exogenous proteases, which improves the digestibility of dietary protein. Bromelain, a natural protease found in pineapple, may be beneficial in livestock, however, there exists little literature on the effect of bromelain on broiler production specifically. This study aimed at determining the effects of supplementation of bromelain on broiler health and performance.

Two trials were conducted: a performance trial and a digestibility trial. The performance trial was carried out to determine whether bromelain supplementation would improve broiler gut health and therefore broiler growth and performance. The aim of the digestibility trial was to determine whether bromelain supplementation would have any effect on the crude protein and dry matter digestibility of feed.

Two-thousand four hundred male Ross 308 chicks were used in the performance trial and were reared in standard commercial conditions. All birds received a standard maize-soya based diet and were separated into 12 treatments of a combination of three levels of bromelain (0, 0.125, 0.75 g/kg), two levels of crude protein (standard or high) and either in the presence or absence of an AGP (zinc bacitracin) and there were 8 replicates per treatment. The trial lasted 34 days and birds were weighed weekly to determine body weight, body weight gain, feed intake and feed conversion ratio. On day 34, duodenum, jejunum and ileal samples of 16 birds from each treatment were analysed for villi height, crypt depth and villus height-to-crypt depth ratio. Bromelain had a significant effect on intestinal crypt depth, where a high level of bromelain lead to a lower crypt depth. Birds that received a standard crude protein diet without an AGP performed the best, and birds that received an AGP showed shorter villi and crypt depths. Bromelain did not have a significant effect on body weight, feed intake or FCR.

For the digestibility trial, Ross 308 chicks were reared in floor pens receiving the same maize-soya based diet. On day 14, 180 birds with a body weight closest to the average were transferred to 30 metabolic cages with six birds per cage. From day 15 to 21, the birds received either a negative control diet, a diet supplemented with bromelain, or a diet supplemented with a commercially available protease that served as a positive control. On day 21, all birds were euthanised and ileal digesta was removed and analysed. Neither bromelain, nor the commercial protease product improved crude protein digestibility compared to the negative control. However, both bromelain and

the commercial protease significantly increased dry matter digestibility, compared to the control.

This study found that the performance and gut morphology of the birds that received supplemental bromelain was not significantly better than birds that did not receive supplemental bromelain, even though dry matter digestibility was improved. Thus, there might be room for bromelain in poultry production as it was seen to improve digestibility and gut health in this trial. Its benefits could perhaps be more attainable in different conditions, for example if a larger gut challenge was presented.

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Conflicts of interest:
None

LIST OF ABBREVIATIONS

3D	Three dimensional
AA	Amino acids
AGP	Antibiotic growth promoter
ANOVA	Analysis of variance
AOAC	The Association of Official Analytical Chemists
°C	Degrees Celsius
cm	Centimetre
CP	Crude protein
d	days
DM	Dry matter
DNA	Deoxyribonucleic acid
EU	European Union
FCR	Feed conversion ratio
FTUs	Phytase units
g	Grams
g/kg	Grams per kilogram
GIT	Gastrointestinal tract
H_0	Null hypothesis
H_1	Alternative hypothesis
HCL	Hydrochloride
igG	Immunoglobulin G
kg	Kilogram
LD50	Median lethal dose
MDCP	Mono-dicalcium phosphate
ME	Metabolisable energy
Min	Mineral
MJ	megaJoule
mL	Millilitre
mm	milimetre
NC	Negative control
NH_3	Ammonia
NMR	Nuclear magnetic resonance
O/C	Oil cake
Q-PCR	Quantitative polymerase chain reaction

RNA	Ribonucleic acid
SEM	Standard error of means
TiO_2	Titanium dioxide
USA	United states of America
Vit	Vitamin

CHAPTER 1: INTRODUCTION

The broiler industry is under pressure to produce a cost effective yet large carcass to ensure profitability and saleability. In 2017, 42% of South Africans preferred poultry (Shahbandeh, 2020), and broiler meat consumption increased to 40 kg per person per annum (Oirere, 2019). Considering the short lifespan of a broiler (approximately five weeks), feeding strategies such as the inclusion of antibiotic growth promoters (AGPs) are employed to meet the intense market demands. These AGPs yield higher feed efficiency, proving to be exceptionally profitable. However, AGP use is now either frowned upon or prohibited due to the risk for development of antibiotic resistance as well as consumer preference, leaving a gap in the market for a sustainable, natural alternative.

Pineapple (*Ananas comosus*) is a member of the *Bromeliaceae* family and contains the enzyme bromelain in its fruit and stems. Bromelain contains proteases, phosphatases, glucosidases, peroxidases and cellulases. It is highly absorbable and when ingested, does not produce notable side effects (Pavan *et al.,* 2012). Bromelain has a lethal dose (LD50) of greater than 10 g/kg in mice, badgers, and rabbits (Mohamed Tap *et al.,* 2018). However, bromelain can become unpalatable if given in too high concentrations.

Bromelain is known for its medicinal properties and has been studied and used in human medicine for centuries, however only a few studies have been carried out in pigs and poultry. In humans, bromelain was effective in reducing inflammation, bacterial load, boosts the immune system and is anti-carcinogenic (Pavan *et al.,* 2012). In other human studies, bromelain has been seen to reduce the risk of oedema (Bayat *et al.,* 2019). This could be especially relevant in the broiler industry where ascites is a common problem. It was found that weanling pigs supplemented with bromelain in their diets had improved average daily gains and average daily feed intakes. Furthermore, they also displayed decreased faecal *E. coli* counts and faecal NH_3 gas emission and increased apparent total tract digestibility of dry matter and nitrogen (Zhao *et al.,* 2015). When multiparous sows were fed bromelain supplemented diets, the sows had linearly higher apparent total tract digestibility of nitrogen, lower blood urea nitrogen and higher lymphocyte counts. Piglets suckling bromelain-supplemented sows had increased average daily gain and weaning weight, higher IgG counts and lower blood urea nitrogen (Begum *et al.,* 2015). One of the few studies using bromelain in chickens was conducted by Yenice *et al.* (2019), who discovered that feeding diets containing different concentrations of bromelain to laying hens increased serum protein concentrations in egg yolk and decreased egg yolk triacylglycerol fraction and serum cholesterol concentration.

The chicken gut is a complicated environment which should be in a constant state of dynamic equilibrium. The presence or absence of certain microorganisms in the gut influence the delicate balance of the microbiome. When the balance is disrupted and dysbiosis develops, consequences like disease or reduced performance may occur in the chicken. For example, pathogenic bacteria can damage the intestinal tract, which can cause reduced feed conversion efficiency and therefore slower growth (Yegani & Korver, 2008).

Amino acids are macronutrients that form peptides and proteins and are essential nutrients for muscle growth. Apart from muscle growth, amino acids also perform other important functions in the body, which include multiple important syntheses and functions to maintain gut health. In the broiler industry, high protein concentrations in the feed are often used in an attempt to maximise growth rate. Depending on the dietary protein level and source, this may lead to excessive amounts of undigested protein reaching the hind gut, which may act as substrate for the protein fermenting microorganisms (Rajaguru *et al.,* 1966). These microbes then produce unfavourable by-products such as phenols, thiols, amines, ammonia, and indoles and increases the pH of the hind gut, which encourages the proliferation of pathogenic bacteria such as *Clostridium perfringens*. These anaerobic, gram-positive bacteria are naturally found in low levels in healthy chicken intestines but causes necrotic enteritis when it converts into a toxin producing form (Fu *et al.,* 2022). It is the most common bacterial disease in poultry and causes acute enterotoxaemia and normally the symptoms are depression followed by increased flock mortality (Duff, 2019). High protein concentrations or imbalanced amino acid concentrations in the diet may alter the gut environment in such a way that the protein becomes even less digestible. This creates a build-up of protein in the gut and allows for amino acid fermenting bacteria to flourish. Diets high in animal proteins, like fishmeal, predispose chickens to necrotic enteritis, which may be ascribed to the higher zinc, glycine and methionine concentrations in animal proteins that encourage *C. perfringens* proliferation (M'Sadeq *et al.,* 2015).

Another reason why excess protein and amino acids in the gut should be prevented is because of its negative effect on the environment and bird welfare. Excess protein leads to excess nitrogen excretion in the form of uric acid, which is an environmental pollutant (Cowieson, 2018). Excess excretion of uric acid can affect performance as the chicken uses energy to convert protein to uric acid where it would otherwise be used for feed efficiency and growth. High-CP diets were included in the performance trial of this study to test bromelain's antimicrobial characteristics, and whether it is capable of minimising the effects of excess protein.

The gastrointestinal tract (GIT) of the chicken is not properly developed at hatch, leading to ineffective digestion. Dietary supplementation of exogenous protease enzymes like bromelain are likely to promote protein digestibility in the feed, which can improve growth rate, reduce the required concentration of total crude protein and amino acids in the feed, and also reduce the amount of undigested protein ending up in the hindgut as fermentation substrate. Angel *et al.* (2011) found that growth and performance of broilers increased when fed exogenous proteases. Supplemental proteases increased feed efficiency, jejunal goblet cell numbers and epithelial thickness, and increased gut tensile strength and villus height, according to Cowieson *et al.* (2017).

This experiment is highly relevant and important to the broiler industry. If bromelain can improve broiler growth and performance, health and feed digestibility, then the broiler industry will benefit enormously. There is also little to no research on the effect of bromelain in broilers, specifically Ross 308 chickens, with regards to growth, performance, health or any other important aspects in broiler production.

1.1 Problem Statement

The broiler industry faces an incredible demand with 42% of South African consumers preferring poultry meat (Shahbandeh, 2020). Together with the shift away from AGP use and the short broiler life span of approximately five weeks, there is a need for alternative natural growth promoters that can improve the growth efficiency of the broiler.

Crude protein/amino acids are essential nutrients as building blocks in muscle growth and are in high demand by the fast-growing broiler. Protein sources are limited and expensive, however any improvement in protein or amino acid digestibility will improve feed efficiency and profitability. Cost effective exogenous protease products that can be added to the feed to improve digestibility are therefore in high demand.

Maintaining the delicate balance in the GIT is a prerequisite for efficient production in broilers. The main function of AGPs in the diets of broilers is to limit pathogenic bacteria in the GIT. With the removal of AGPs, development of other natural alternative products with antimicrobial activity is necessary in the broiler industry. Furthermore, overfeeding protein to broilers with the desired outcome of maximised growth is common but may negatively affect gut health and therefore production: it may reduce income by increasing carcass condemnations or premature mortalities such as breast blisters (Cowieson, 2018) or necrotic enteritis. There is thus a need for a product that will limit the unfavourable bacteria in the gut of the chicken and improve overall health.

There is limited to no research on the effect of bromelain in chickens, specifically the Ross 308 strain. Bromelain remains an unknown agent in the broiler industry until now.

1.2 Aims and objectives

Pilot trial

The aim of the pilot trial was to ensure that the anticipated dietary inclusion levels of bromelain that would promote appetite and feed intake in Ross broiler chickens in the subsequent performance and digestibility trials.
The objective of the pilot trial was to include various inclusion levels of bromelain in different treatments and measure the effects on broiler performance.

Performance trial

The aims of the performance trial were:

1) To determine whether bromelain supplementation would increase broiler growth and performance.
2) To determine whether bromelain would improve/have an effect on chicken gut morphology.
3) To determine whether bromelain would be effective in reducing the negative effect of excess levels of protein in the gut.

The objectives of the performance trial were:

- To feed broilers diets containing different levels of bromelain and crude protein (with and without AGP) and measure the following:
- Broiler performance, by weighing the broilers weekly, determine feed intake by weighing the feed weekly and calculate the FCR using the body weight and feed intake.
- Gut morphology, by measuring the villus height-to-crypt depth ratio in the duodenum, jejunum and ileum of broilers.

Digestibility trial

The aim of the digestibility trial was to determine the effect of bromelain supplementation on crude protein and dry matter digestibility of feed in broilers.

The objective of the digestibility trial was to analyse the concentration of indigestible matter in feed and ileal digesta and to subsequently calculate the dry matter digestibility and crude protein digestibility of the feed.

1.3 Hypotheses

H_0: Supplementation of bromelain will have no significant effect on broiler growth and performance.
H_1: Supplementation of bromelain will have a significant effect on broiler growth and performance.

H_0: Supplementation of bromelain will have no significant effect on the nutrient digestibility of the broiler.
H_1: Supplementation of bromelain will have a significant effect on the nutrient digestibility of the broiler.

H_0: Supplementation of bromelain will not significantly ameliorate the negative effects of excess dietary protein on the performance and health of broilers.
H_1: Supplementation of bromelain will significantly ameliorate the negative effects of excess dietary protein on the performance and health of broilers.

H_0: Supplementation of bromelain will not be as effective as an antibiotic growth promoter to enhance broiler production.
H_1: Supplementation of bromelain will be an effective alternative to an antibiotic growth promoter

H_0: Supplementation of a high level of crude protein will not provide a more challenging gut environment than a standard level of crude protein.
H_1: Supplementation of a high level of crude protein will provide a more challenging gut environment than a standard level of crude protein.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Under current difficult economic circumstances, the use of feed additives in broiler enterprises may be an effective way to increase production and profitability. One such group of feed additives is antibiotic growth promoters (AGPs), which may have many benefits. Antibiotic growth promoters are popular in the South African broiler industry because of the higher stocking densities. A higher stocking density provides a bacterial and microbial challenge, which can be remedied using AGPs (Kleyn, 2014).

An AGP alternative is any exogenous feed additive that mimics the mode of action of an AGP. Some are able to positively manipulate gut morphology, which in turn influences feed digestibility and therefore production (and profit), making them a sought-after feed additive. These include but are not limited to enzymes, probiotics, prebiotics, organic or inorganic acids, and essential oils (Huyghebaert *et al.,* 2011). In particular, this literature review investigates the protease enzyme bromelain and its effects on broiler health, performance, feed digestibility and growth.

2.2 Chicken digestive system

2.2.1 Anatomy and function

The chicken gut is a complex and intricate system wherein food is broken down and nutrients are made available to the body via the blood. When food is swallowed, it reaches the crop first, where it is temporarily stored. During this time, it is partly fermented by bacteria. It then moves to the proventriculus, otherwise known as the glandular stomach, where it is mixed with hydrochloric acid and mucus secreted by the oxynticipeptic cells and mucus secreted by the columnar epithelial cells. The proventriculus has a pH of 2,5–3,5 (Dharne, 2008, Macwhirter, 2009, Bailey, 2019). The peptides and broken-down food then move to the ventriculus, or gizzard, which is separated from the proventriculus by a muscular sphincter. The ventriculus contains two strong muscle bundles arranged circularly and concentrically (Gabella, 1985). These protected muscles, along with some salivary amylase and the abovementioned digestive juices from the proventriculus, grind the food into even smaller pieces. It then travels to the small intestine, comprised of the upper section named the duodenum, and the lower sections, the jejunum and ileum. The jejunum and ileum are separated by the Meckel's diverticulum, a bulge which is the remnant of the yolk sac and yolk stalk (Jacob, 2019). The liver and pancreas secrete bile and bicarbonate respectively, which is taken to the duodenum via the gall bladder. The duodenum requires the bicarbonate in order to neutralise the

hydrochloric acid from the proventriculus and ventriculus, as the duodenum's pH is 5–6 (Dharne, 2008). The bile digests lipids and aids in the absorption of fat-soluble vitamins. The end of digestion occurs in the duodenum and nutrient absorption takes place in the ileum and jejunum, which have a high surface area due to the numerous villi and microvilli. The undigested feed is then either passed through the cloaca and mixed with uric acid, to be excreted from the vent as solid, uric acid capped faecal droppings, or is taken up by the paired caeca which further ferment the material to form short chain fatty acids, organic acids or vitamins for the bird's absorption and use. The vent then excretes the unfermented caecal material as dark brown, liquid caecal droppings (Bailey, 2019).

2.2.2 Intestinal microbiota, gut health and integrity

Intestinal microbiota are resident bacteria, protozoans and fungi inside the chicken gut that maintain bird nutrition and metabolism and provide resistance to disease and illness thereby preserving general bird immunity. They can be found either in the gut lumen, within the mucus layer or attached to the digestive mucosa. Bacteria are in the highest concentration in the crop and the caeca; mostly facultative anaerobic gram-positive bacteria are present in the crop to the ileum, whereas strict anaerobes are present in the caeca and rectum (Adil & Magray, 2012). Large quantities of *lactobacilli* and fewer *streptococci* and *coliforms* are present in the crop, where they grow before being exposed to the low pH of the proventriculus and ventriculus. These bacteria remain dominant due to their ability to attach themselves to the epithelial cells of the crop. Further aiding their dominance are the squamous epithelial cells, which have receptors specifically for *lactobacilli*. When the food passes to the proventriculus, large numbers of *lactobacilli* remain adhered to the crop wall, awaiting incoming food to inoculate (Fuller, 2001).

There are little to no bacteria colonies in the stomach due to its acidic environment. *Lactobacilli* are again the dominant bacteria in the small intestine, but in the caeca the colonies change. The caeca have different nutritional conditions that allow for longer residence time, providing an optimal environment for strict anaerobes. The rectum intermittently receives caecal contents, which allow for the same environment and therefore the same bacteria to flourish inside it (Fuller, 2001).
The chicken seldom hatches with pre-existing microbiota and must acquire an optimal gut environment after hatch. Sometimes micro-organisms can pass through the pores of the eggshell and inhabit the chicken gut before hatch, but the principle of acquiring a sound microbiota environment after hatch remains the same in these chicks. Day-old chicks raised in a free-range setting will consume part of its mother's faeces to obtain some of the intestinal bacteria excreted by the mother. By day 3 of life, significant numbers of bacteria such as *Enterobacteriaceae* can be found in the gastrointestinal tract of the chicken and by day 7, the gut is successfully colonised by

Firmicutes. The microbiota will begin to resemble that of an established chicken, dominated by specific bacterial species belonging to the *Enterobacteriaceae* and *Firmicutes* family once contact with the environment and bacteria in the food is established (Diaz Carrasco *et al.,* 2019). However, the microbiota will grow and diversify throughout the chicken's life; *lactobacilli* are only dominant in the small intestine after 40 days of age, and the caecal microbiota only becomes established after 6 weeks of life (Adil & Magray, 2012). This is depicted in Figure 1.

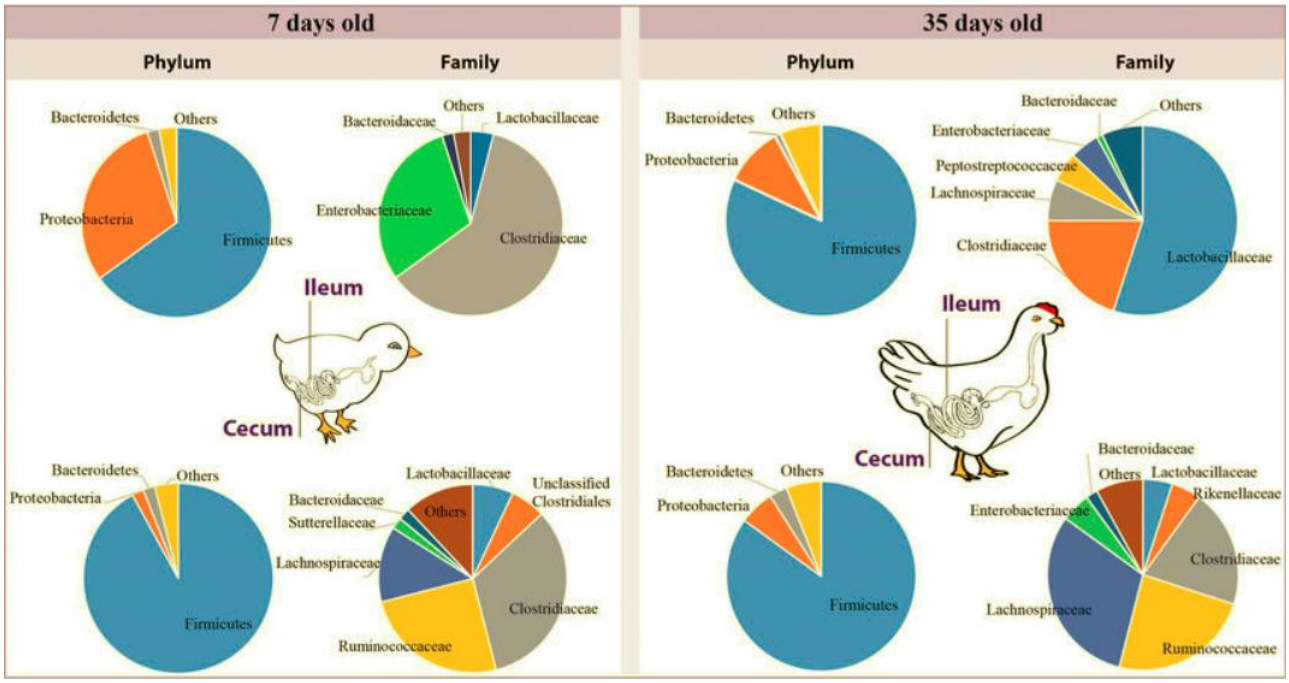

Figure 1 Ileal and caecal microbial population in the chicken on day 1 and day 35 (Pourabedin & Zhao, 2015)

This sterilised environment disallows favourable bacteria from colonising the gut and therefore the chick must be supplemented with these bacteria immediately. This is done by applying a form of prebiotics, probiotics and other microorganisms directly into or onto the egg before hatch, allowing a colonisation of favourable bacteria after hatch (Diaz Carrasco *et al., 2*019). Other ways to improve the chance of a healthy gut are to minimise stress factors such as handling, transport and overstocking; removing all toxic elements from the feed; enforcing biosecurity so as not to contaminate and disease the birds; preventing beak deformities which would otherwise inhibit proper feed intake and providing beneficial feed additives such as probiotics and prebiotics to the feed (Dharne, 2008).

Measuring and analysing biomarkers is a good way to understand gut health. Non-invasive markers such as faecal microbiota can be used to establish the internal bacterial population. For example, a

negative correlation between *Enterobacteriaceae* and performance (as expressed through gut health) has been seen (Aruwa *et al.,* 2021). Identifying the quantification of *Enterobacteriaceae* using Q-PCR can be used to assess disequilibrium in the chicken gut (Aruwa *et al.,* 2021). Invasive markers such as blood and liver markers can also be a good measure of gut health and integrity. If the tight junctions of the intestinal epithelium are even slightly damaged, bacteria can move through the bloodstream and then to the liver, causing inflammation. Therefore, an inflamed liver can be an indicator of poor gut health and more specifically, intestinal permeability (Ducatelle *et al.,* 2018). Furthermore, when there is intestinal permeability, intestinal epithelial cell-specific proteins are released into the bloodstream and can be used as biomarkers. One such protein is the enzyme diaminoxidase.

Another notable metabolite of intestinal bacteria is D-lactate. D-lactate can be further metabolised by bacteria, however in chickens with increased intestinal permeability, D-lactate can be found in the blood serum in large amounts. This makes it a serum biomarker for intestinal integrity (Ducatelle *et al.,* 2018). Lastly, very common biomarkers for gut health are measurements of the villus height, crypt depth and the villus: crypt depth ratio (Ducatelle *et al.,* 2018). Intestinal villi are projections that extend into the intestinal lumen, providing increased surface area for nutrient absorption. Intestinal crypts are glands found anchored to both the villi and the epithelial lining of the intestine. They consist of Paneth cells, which secrete antimicrobial peptides and proteins (Bowen, 2019). In an optimally functioning chicken intestine, its layer of epithelial cells is constantly replaced by stem cells found at the base of the crypt. The new cells move to the tip of the villus where they await cell death and then are removed from the villus tips. Differentiation of the newly formed cells occurs during their migration, making them an integral part in nutrient absorption. Enteric bacteria such as *coccidia* can cause epithelial cell death. The loss of epithelial cells will result in a decreased villus length and increased crypt depth (as illustrated in Figure 2) and therefore deterioration of the gut health following decreased absorption. Villus height, crypt depth and villus: crypt depth ratio are normally measured from the duodenum, jejunum and ileum (Ducatelle *et al.,* 2018).

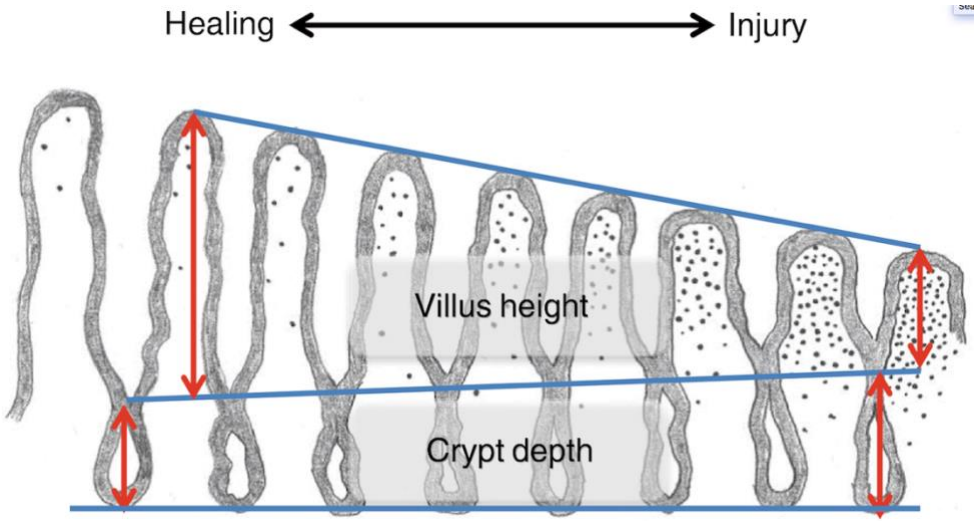

Figure 2 Diagram of villi height and crypt depth during health and injury (Daveson *et al.,* 2020)

2.2.3 Gut health and broiler performance

Gut health has never been a major factor in improving broiler performance. It was believed that gut integrity was important to maintain the overall health of the chicken, but no emphasis was placed on its optimisation in terms of research and monetization. It is only in recent years that importance has been placed on gut health to maximise broiler performance, as it was discovered that the two have a linear positive relationship and the former can be manipulated and optimised to increase profit. When the eminence of gut health was realised, the broiler industry began to thrive on the effects of antibiotics (Wielsma, 2017).

In the ideal chicken gut the resident, favourable bacteria produce antimicrobial metabolites that create a hostile micro-environment for unwanted bacteria and eliminate any chance of their colonisation. This practice is known as competitive exclusion and ensures intestinal homeostasis at all times. It is highly beneficial to the chicken as it decreases the amount of energy the chicken would have spent combatting these pathogens and reallocates it towards production. Therefore, this translates into an improvement in bird performance (Diaz Carrasco *et al.,* 2019). Certain antibiotics used in the broiler industry work to the same end, for example tetracycline which reversibly binds to the 30S ribosomal subunit receptors of the bacteria, thereby inhibiting protein synthesis in the unfavourable bacteria. The antibiotics given to poultry decrease the incidence of dysbiosis and in turn maintain gut integrity and increase FCR (Kapoor *et al.,* 2017).

2.3 Protein digestibility

2.3.1 Protein structure and function

Protein molecules are intricate structures that are essential for the chemical processes that maintain life. They are responsible for majority of cellular processes and for DNA replication and transcription. They also control cell division and cell metabolism as well as producing other proteins (Alberts *et al.,* 2002). Approximately 20 amino acids are used to form between 10000 and 6 billion different proteins by arranging themselves in varying sequences and numbers of amino acids (Ponomarenko *et al.,* 2016). This creates a large molecule that is specific to the species and organ in which it is destined to perform its function. For example, ovocalyxin-36 is a protein specific to the chicken eggshell membrane and is not found in humans, though it consists of amino acids that humans have (Cordeiro *et al.,* 2013). It is not fully possible to determine the structure and function of a protein solely by analysing its amino acid sequence, however correlations have been found to link the function of a protein with the properties of its amino acids (Haurowitz & Koshland, 2019). Amino acids are groups of organic molecules containing a central carbon atom, a basic amino group and a unique side chain belonging only to that amino acid (Reddy, 2019). There are two groups of amino acids, namely the essential and non-essential amino acids. Essential amino acids are those that cannot be synthesised in the body and must be obtained from plants in the diet as plants can synthesize all amino acids (Lopez & Mohiudden, 2022). Non-essential amino acids are those that occur naturally in the body and do not need to be obtained from the diet (Lopez & Mohiudden, 2022).

The whole structure of a three-dimensional protein can be defined using four complex levels of structure: primary, secondary, tertiary and quaternary. The term protein sequence is often interchangeable with protein structure, as it is defined as the linear amino acid sequence of a protein's polypeptide chain. A protein's secondary structure is the backbone of the protein's spatial conformation without including the side chains. Secondary structures include α-helices and β-pleated sheets, loops and turns, which can vary greatly in length (Sun *et al.,* 2004). α-helices are formed when a nitrogen-hydrogen group in the backbone forms a hydrogen bond with the carbon=oxygen (C=O) group of the amino acid four residues earlier in the helix. β-pleated sheets are formed when N-H groups in the backbone of one strand form hydrogen bonds with C=O groups in the backbone of a fully extended strand next to it. Proteins can have multiple functional groups, for example alcohols, carboxylic acids and thiols, that cause the protein to form a specific shape and function. Turns and loops are what connect α-helices and β-pleated sheets. The tertiary structure of a protein refers to the protein's three-dimensional shape and arrangement. The precise conformation and coordination of the secondary structures and functional groups influence the tertiary structure

(Smith, 2018). Only proteins consisting of multiple polypeptide chains have a quaternary structure. The arrangement and orientation of subunits defines a protein's quaternary structure. The three-dimensional structure that results from the folding of the protein to accommodate its amino acid sequence directly determines the protein's function. Some proteins can also form macromolecules with other molecules in the body, which allow for a completely different three-dimensional structure and function compared to the protein alone. Further aiding a protein's function is its ability to be flexible. Some proteins are rigid and function as structural units, whereas others are more fluid and can act as supporting structures such as hinges and springs (Smith, 2018). Figure 3 provides an example of the three-dimensional structure of a protein.

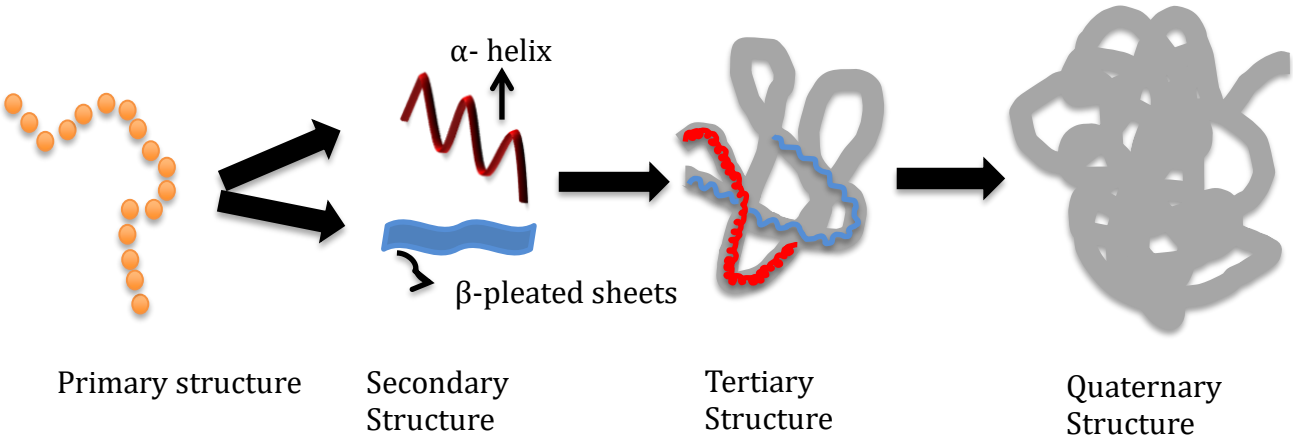

Figure 3 Illustration of the structure of a three-dimensional protein (Binet, 2013)

For one to fully comprehend the structure of a protein, x-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy is used. It is not yet possible to determine the three-dimensional structure of a protein using only its amino acid sequence, therefore the above methods are needed for a clearer understanding. When an x-ray is taken of a protein, most of the x-ray will pass through it due to its short wavelength. However, the atoms in the protein will scatter a small fraction of the x-ray, and if the sample is a well-ordered crystal, those waves will reinforce each other at different points in the crystal, called diffraction points. It is then possible to determine the three-dimensional structure of the protein using these points. It is however necessary to use a sample of very pure protein in order to generate suitable crystals. NMR spectroscopy is unlike x-ray crystallography in that it does not rely on crystal structures within the protein to determine its 3D structure, but rather makes use of the electrical charge and spin that every nucleus has. When an external magnetic field is applied to these nuclei, they emit a radiofrequency radiation, which is displayed as a spectrum and can be measured. As only a small sample is required for an electric field to take effect, NMR spectroscopy only determines the 3D structure of small proteins (Alberts *et al.,* 2018).

Protein is an essential nutrient due to its life-giving functions. Proteins can function as structural components, enzymes, hormones, defence units, transport mechanisms and regulators of acid-base balance within the chicken (Callahan *et al.,* 2020). Examples of protein structural components are actin, myosin, keratin, collagen and elastin. These proteins can be present in cell cytoskeletons to give cells shape and movement, provide elasticity and strength to connective tissue, and provide support to large organs, bones, feathers, muscles etc. (Dean, 2021). Pepsin, amylase, trypsin and lipase are examples of protein enzymes in the chicken. These digestive enzymes act as catalysts to substrates in the gut, rendering them monomers. Examples of protein hormones are insulin and glucagon. Hormones are chemical messengers made by specialist cells (usually in endocrine glands) that transport messages to parts of the body otherwise unattainable. This works to provide an internal communication system between cells located in distant areas of the body. Insulin is secreted by the beta cells in the pancreatic islets of Langerhans and maintains normal blood sugar levels in the body by facilitating the uptake of glucose into the cells. When available glucose is present in the body, insulin is released in corresponding amounts (Wilcox, 2005). Conversely, glucagon, which is secreted by alpha cells in the pancreatic islets of Langerhans, stimulates glucose and fatty acid release from the cells of the liver when blood glucose levels are low (Rix *et al.,* 2019).

Proteins can also help transport substances via the blood or lymph around the body or help substances cross semi-permeable or permeable membranes. Examples of these transport proteins are haemoglobin or carrier proteins. Haemoglobin facilitates oxygen transport on the red blood cells around the body. Carrier proteins are usually embedded in the cell membrane and are involved in facilitated diffusion and active transport of ions and small and big molecules in and out of the cell (Alberts *et al., 2*002). Haemoglobin is also an example of a protein that maintains the acid-base balance within cells and the body. When carbon dioxide is converted to bicarbonate, haemoglobin buffers hydrogen ions liberated during the process which would have otherwise dissociated oxygen, therefore maintaining the pH balance (Biga *et al.,* 2019). Finally, antibodies are examples of defence proteins. Antibodies such as immunoglobulin identify antigens on foreign bodies and then seek to neutralise them. Foreign bodies range from pathogenic viruses and bacteria to exogenous drugs to particles, for example a thorn (Forthal, 2014). Table 1 shows the different protein types and their functions.

Table 1 Protein types with an example and their function (Chadha, 2022)

Protein type	Example	Function
Structural	Actin	Provide shape, movement, strength and structure
Hormonal	Insulin	Enable chemical messages to and from distant cells
Enzymatic	Pepsin	Digest macromolecules into smaller monomers
Acid-base balance	Haemoglobin	Maintains pH balance throughout the body
Transport	Haemoglobin	Transports molecules via the blood and lymph
Defence	Immunoglobulin	Identify and neutralise foreign particles in the body

2.3.2 Protein digestion and absorption

Protein digestion refers to the amount of amino acids yielded from the protein after digestion. The digestion of protein in a chicken starts in the stomach and ends in the small intestine. The chicken has enzymes in its mouth to support the initial breakdown of food, but only starch degrading enzymes such as amylase. Therefore, protein remains essentially whole as it passes to the crop. It then passes to the proventriculus, where the chief cells secrete pepsinogen. Pepsinogen mixes with the hydrochloric acid also produced in the proventriculus and becomes activated. The product of this activation is a protease called pepsin. Pepsin moves to the gizzard with the protein and starts to work when the gizzard walls begin to grind the food and digestive enzymes together. Once the food is sufficiently pulverized, it moves to the small intestine where most of protein digestion occurs (Jacob, 2019). The duodenum receives mainly proteolytic enzymes from the liver and pancreas, and this is the site of most protein digestion. The proteolytic enzymes from the pancreas include trypsin, chymotrypsin, aminopeptidase, tripeptidase, dipeptidase, carboxypeptidase, ribonuclease, elastase and collaginase. The proteolytic enzymes from the intestinal juices are erepsin, polynucleotidase, nucleosidase and nucleotidase (Molnar & Gair, 2019). The function of each of these proteases can be found in Table 2.

Table 2 Function and site of pancreatic proteolytic enzymes (Rogers, 2020)

Site of protease production	Protease name	Protease function
Pancreas	Trypsin	Converts proteins to peptides
	Chymotrypsin	Cleaves aromatic amino acids in proteins thereby creating peptides and amino acids from proteins
	Aminopeptidase	Converts proteins to polypeptides
	Tripeptidase	Converts tripeptides to amino acids
	Dipeptidase	Converts dipeptides to amino acids
	Carboxypeptidase	Converts polypeptides to amino acids
	Ribonuclease	Converts nucleic acids to nucleotides
	Elastase	Converts elastin proteins to peptones
	Collaginase	Converts collagen proteins to peptones
Intestine	Erepsin	Converts polypeptides to amino acids
	Polynucleotidase	Converts nucleic acids to nucleotides
	Nucleosidase	Converts nucleosides to purines and pyrimidines.
	Nucleotidase	Converts nucleotides to nucleosides and purines and pyrimidines.

Once these enzymes have broken down the proteins into polypeptides, tri- and dipeptides and amino acids, the duodenal muscles push the available protein fragments to absorption sites in the lower small intestine. These fragments are moved from the ileal and proximal jejunal lumens into the intestinal cells via the interstitial brush border and then into the blood surrounding the intestines using facilitative diffusion (Zimmerman & Snow, 2012). After entering the blood, the amino acids enter a process called enterohepatic circulation, and are carried to the liver via the hepatic portal vein where they are sorted into readily available amino acids and peptides needing further degradation. Ninety percent of amino acids remain whole and are used to form new proteins and approximately 10% are broken down further. All amino acids contain nitrogen, and so this process releases nitrogen-containing ammonia, which is transformed into uric acid by the liver due its toxicity. The uric acid is carried to the kidneys via the blood and is excreted via the cloaca. Some of the amino acids that are not broken down remain in the liver for future use. Others are transported to the rest of the body and taken up by the cells to be used there. The liver regulates the amino acid content in the body, and so when muscles or other organs need protein, DNA, RNA, energy or other molecules, amino acids can be summoned from the 'pool' of amino acids broken down from ingested protein; a process called protein turnover (Byerley, 2020). The process of protein turnover is illustrated in Figure 4.

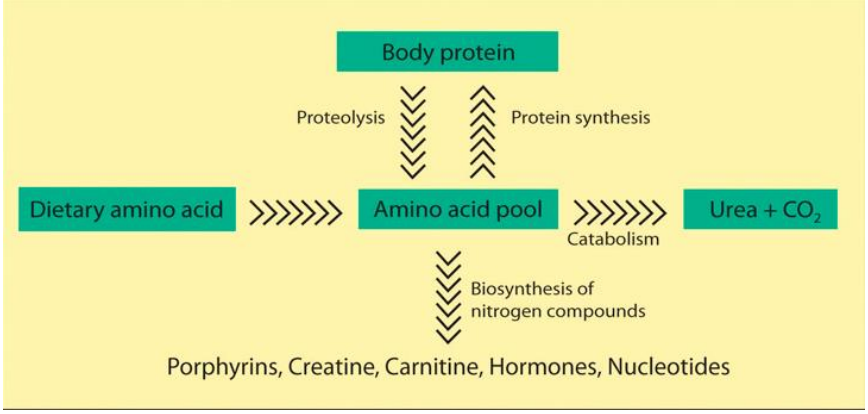

Figure 4 Illustration of protein turnover (Zimmerman & Snow, 2012)

2.3.3 Quality versus amount of protein

A protein is of good quality if it has a high biological value. A protein's biological value refers to the amount and availability of essential amino acids in the protein, in other words how well the chicken can utilise the protein ingested. The biological value of protein is influenced by the rate at which the protein enters the hepatic portal vein and consequently its end location. When this occurs slowly, for example when only high-quality protein is ingested, more nitrogen can be retained. A protein with a low biological value is one that does not provide enough essential and non-essential amino acids to be totally useful to the body (Moore & Soeters, 2015). For example, a good quality protein for a

broiler would be blood meal, as it has a similar amino acid composition to a broiler and contains a high amount of the first limiting amino acids: methionine, lysine, tryptophan and threonine. A low-quality protein might be soybean meal due to its amino acid composition lacking enough methionine and lysine. One study by Wethli *et al.* (1975) found that chickens raised on a cereal-based diet (lacking in essential amino acids) did not reach maximum live-weight gain and efficiency of food utilisation at any level of protein fed. When the same chickens were fed a cereal-based diet and supplemented lysine and methionine, they showed the normal growth pattern and efficiency of feed utilisation as a diet containing cereals and herring meal. It might be wrongly concluded that in order to counteract a certain lower quality protein, a larger quantity of protein can be fed. Broilers require on average, highly depending on their breed, sex and environment, 22–23% crude protein (CP) in the starter diet, 20% CP in the grower diet and 18–19% CP in the finisher diet (Esmail, 2016).

As stated before, when protein is ingested, it is broken down into amino acids. These amino acids are either stored or broken down further. When the body has reached its requirement for stored amino acids, any excess amino acids are broken down to prevent over-storage. Amino acids that are de-aminated yield ammonia as a by-product, and this ammonia is transported to the kidneys via the blood. Once there, it is excreted. However, an excess amount of ammonia in the kidneys is toxic to the bird and must be immediately transformed into uric acid. To form extra uric acid requires a large amount of energy that could rather be used for growth and production. This unnecessary energy use also results in decreased feed efficiency, causing feed and production costs to increase. An excess amount of uric acid in the kidneys requires an excess amount of water to be efficiently excreted, causing the excretion to become wet (Zimmerman & Snow, 2012). This can result in wet litter, which can lead to an increase in pathogenic bacteria and bird discomfort in the form of breast blisters, bruising, scabbing, hock burn, and foot lesions, and therefore decreased growth.

Excess protein is also transported to the hindgut, where not only healthy gut microbiota will process it, but also unwanted pathogenic bacteria such as *colibacteria* and *salmonella*. This can result in diarrhea, which causes increased morbidities, mortalities and medicine costs while also decreasing efficient feed utilisation and therefore growth and production (Mavromichalis, 2016). Another study by Dittoe *et al.* (2022) explains that protein entering the caeca from the intestinal tract can be degraded by bacteria in the caeca into potentially toxic metabolites including ammonia, amines, and phenols. This putrefaction can be detrimental to the chicken's health in large amounts, which is explained in a study by Apajalahti & Vienola (2016), where chickens that were challenged with *Eimeria maxima* (a protozoa that causes coccidiosis and therefore damage to the small intestine) showed an increase in biogenic amine level in the caecum, due to protein not being correctly broken down. Therefore, a high protein diet can create a challenged gut environment (Yadav & Jha, 2019).

Conversely, feeding less protein to the broiler does not necessarily lead to a decreased growth rate; feed intake or mortality and can have a beneficial effect on litter quality. In a study by Van Harn *et al.* (2019) it was found that feeding broilers 1% and 2% less protein than recommended yielded similar, if not the same, body weight gain, feed intake and mortality rate as feeding the broiler the recommended protein percentage. Broilers fed 2% and 3% less protein (containing adequate amino acid profiles) had lower FCRs than the control. This can be explained by the decrease in nitrogen excretion presented by the birds, thereby improving the quality of the litter and foot lesions: a broiler experiencing discomfort via bad quality litter will present a higher FCR. Only broilers fed the 3% lower protein diet displayed a lower breast meat yield, however they exhibited a decrease in FCR. It is therefore concluded that feeding broilers an excess of protein or any amount of low-quality protein will result in decreased production whereas feeding broilers slightly less protein than recommended can slightly improve production.

2.4 Antibiotic growth promoters

2.4.1 Modes of action of antibiotic growth promoters

Antibiotic growth promoters are antibiotics given in sub therapeutic amounts to promote growth and production efficiency in the livestock industry. Antibiotics, or antibacterials, are a class of antimicrobial medication used to kill or inhibit the growth of bacterial cells. Antibiotics are used to exterminate pathogenic bacteria that cause infection; however, antibiotics cannot distinguish between pathogenic and healthy bacteria, and will eradicate whichever they come into contact (Dibner & Richards, 2005). Both gram-positive and gram-negative bacteria have structured cell walls that provide shape and protection to the bacterial cell. Gram-positive bacteria are surrounded by an inflexible and unyielding cell wall that is difficult to permeate. Gram-negative bacteria have a thinner cell wall that is surrounded by an outer cell membrane. This membrane encloses porins that allow for the movement of certain molecules in an out of the cell (Kapoor *et al.,* 2017). Antibiotics have many different modes of action. Some work by interfering with bacterial cell wall synthesis, which therefore exposes the bacterial cell to the elements; some inhibit bacterial DNA and/or RNA synthesis; some inhibit protein synthesis and some disrupt bacterial metabolic pathways such as folic acid synthesis and some disrupt the bacterial membrane (Moore, 2019). Table 3 provides examples of antibiotics that work in each of these manners.

Table 3 Examples of antibiotics and their different modes of action (Chander et al., 2007)

Mode of action	Antibiotic example
Cell wall synthesis interference	B-Lactams, Glycopeptides
Inhibition of protein synthesis (binding to 50S ribosomal subunit)	Macrolides, Chloramphenicols
Inhibition of protein synthesis (binding to 30S ribosomal subunit)	Aminoglycosides, Tetracyclines
Inhibition of DNA synthesis	Fluoroquinolones
Inhibition of RNA synthesis	Rifampin
Inhibition of metabolic pathways	Sulfonamides
Disruption of bacterial membrane	Polymixins

2.4.2 The role of antibiotic growth promoters in the broiler industry

Antibiotic growth promoters (AGPs) were first used in the 1940's as a means to increase production on livestock farms. They are used in sub-therapeutic amounts to improve animal health and therefore performance because they have been shown to increase growth rates by up to 16% and feed efficiency by up to 7%. There are many speculations as to why AGPs are so successful in improving growth; most studies focus on the antibacterial properties of AGPs and how their ability to inhibit/kill pathogenic bacteria allows for the animal to remain healthy during the production process. Ways in which antibiotic growth promoters do this are by reducing the microbial load in the gastrointestinal tract (GIT); reducing the amount of pathogenic bacteria within the GIT; decreasing the formation of toxic bacterial metabolites and thinning the gut epithelium, therefore increasing nutrient absorption (Broom *et al.,* 2017). New studies show that AGPs can also prevent the negative effects of inflammatory intestinal cells. Although the reason AGPs are so successful is still being debated, it has been accepted that the level of microbial challenge in an animal is directly related to the response seen by the AGP. Depending on the AGP used, a reduction in certain bacteria can be seen. Most AGPs will for example reduce the *Lactobacillus* and *Streptococcus* populations, allowing for certain other bacteria, for example *Clostridia,* to flourish. This decrease in certain populations allows for a decline in a variety of bacteria and overall community diversity (Broom *et al.,* 2017). Due to the low concentration of AGP given, some professionals doubt the AGPs ability to cause such a drastic change in microbial environment. They believe that an amount above the minimum inhibitory

concentration is necessary to see results described above. Taylor *et al.* (1981), who found that even sub-therapeutic amounts of AGP could alter bacterial properties enough that the bacteria became increasingly susceptible to defence mechanisms carried out by the animal itself, negates this. He continued to say that any current screening technology would not identify this phenomenon; therefore, AGPs are indirectly allowing the host to perform optimally. These results are supported by multiple other studies such as those by Hacker *et al.* (1993*)* and Nanduri *et al.* (2006).

The South African commercial broiler industry is known for having a generally high stocking density in the houses. This is a clever tactic to increase turnover and therefore profit but can expose the broilers to an increased incidence of pathogens and a weaker immune system due to the high stress levels in a densely packed environment. Antibiotic growth promoters have been a source of respite for South African farmers and have been used extensively on broiler farms. Common AGPs given to broilers are bacitracin, chlortetracycline, virginiamycin, tylosin, avoparcin, neomysin, oxytetracycline and others. These AGPs are normally given orally within the feed or supplied through the water source (Apata, 2009). AGPs are most effective at younger ages, due to the chicken's gastrointestinal tract being underdeveloped and the chicken's microbial population having not been established yet. This allows the AGP to formulate a population of certain beneficial microbes as discussed above. Antibiotic growth promoters given from birth to market age can also decrease mucosa thickness, lamina propria amounts, duodenum and ileum weight and increasing absorption surface area (Miles *et al.,* 2006). According to Zhou *et al. (*2004), AGPs can increase intestinal villus height by 12% in the duodenum and 14% in the ileum and jejunum. It is evident that AGPs provide welcome therapeutic effects in the chicken GIT, allowing for increased feed efficiency (more so than growth).

2.4.3 Antibiotic resistance in broilers and humans

When an AGP is given from birth, it can immediately dictate the populations of bacteria that will grow by inhibiting the proliferation of others. It is common for an AGP to eliminate those bacterial strains that are naturally more susceptible to them, leaving the stronger, more resistant strains behind. The population that is left behind will flourish, multiplying until they are the dominant micro-organism in the GIT (Apata, 2009).. When the bacteria multiply, and it is time to transmit genetically stronger genes to their progeny, the resistant bacteria will almost always pass on their resistant gene. They can also pass the gene on to other bacterial species through mutation. This is done by the bacteria to survive against antimicrobial agents.

There are two groups of antibiotic resistance. The first is when bacteria can resist the effect of a specific antibiotic due to its ability to enzymatically deactivate the antibiotic. The second group is one where the bacteria can survive in the presence of the antibiotic without interacting directly. This does not involve enzymatic deactivation. An example of the first group is *Staphylococcus* producing penicillinase. Penicillinase is able to destroy the molecular structure of penicillin (Apata, 2009).

Speculation about the safety of using AGPs in livestock arose not long after AGPs became popular in the industry. The main concern was not for the animal itself, but for the human consuming it. Van den Bogaard & Stobberingh (2000) have proved that it is possible for the resistant bacteria from broiler meat to transfer to humans through the consumption and/or handling of meat contaminated by the pathogens. In the past it was more common for antibiotic residues to be present in the meat, a sure way for the resistant antibiotic to enter the human body, however nowadays this is scarce as antibiotic withdrawal regulations have been widely implemented. If the antibiotic does enter the human intestine, it can begin to colonise the gut and transfer the antibiotic resistant genes to the already present micro-population. When the human is next given the same antibiotic, it becomes almost impossible for the medicine to inhibit/kill the resistant bacteria. In the Western Cape, a study showed that spent hens were 100% resistant to oxacillin, vancomycin and methicillin antibiotics, posing a threat to the humans who eat them (Selaledi *et al.,* 2020). The modes of transmission of antibiotic resistance from poultry to humans is illustrated in Figure 5.

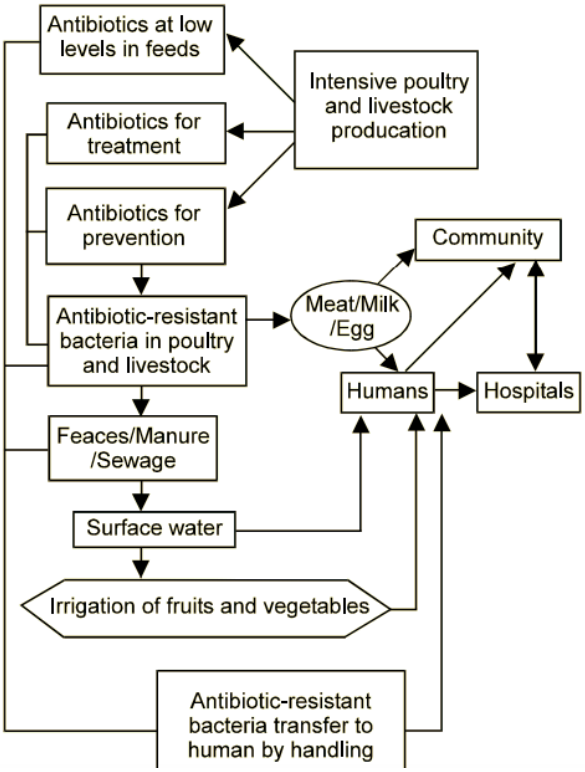

Figure 5 Modes of transmission of antibiotic resistance from poultry to humans (Wegener, 2012)

Consequently, the use of several antibiotics has been banned in the livestock industry across the globe (Kirchhelle, 2018). Experts began warning the public about the potential risk caused by antibiotic use in livestock in the 1940s. These initial warnings were ignored and only in 1967 were some regulations put in place to control AGP use in Germany. In 1969, Britain began reviewing the use of AGPs in livestock, but did not warn against them until pressure from Sweden in 1995. Sweden had totally banned AGP use in 1986, and Denmark and Norway followed suit shortly after. By 2006, all AGP use was banned in the EU. Some higher income countries like Japan have implemented reforms such as banning specific AGPs like avoparcin and orienticin. If not outright banned, new and on-going regulations and restrictions have decreased the amount of AGPs used in the livestock industry (Kirchhelle, 2018). A survey by Alltech of 59 countries where AGP restrictions exist were taken into consideration, which included 28 from the EU and the top seven countries in terms of livestock production. The USA, Canada, Mexico, Japan, Hong Kong, China and India have restricted the use of antibiotics in animal feed. Although the USA has not banned antibiotic use in the livestock industry, as of 2017 antibiotics can no longer be used for growth promoting purposes (Salim *et al.,* 2018). South Africa is a country that does not have many limitations of using AGPs in the poultry industry. South Africa faces the challenge of a low profit margin with little to no government subsidies. The South African poultry industry also must contend with cheap imports from other countries. Banning antibiotic growth promoters in South Africa would lead to a short-term negative impact on

the food security sector. It is therefore not possible at present to discontinue AGP use, however it is probable that some regulations will be put in place in the future, potentially leading to an outright ban (Selaledi *et al.,* 2020).

2.5 Exogenous enzymes as alternatives to antibiotic growth promoters

Feed additives are commonly included in broiler diets to enhance performance. There exists a wide range of feed additives that promote ingestion, absorption and assimilation of feed and therefore nutrients. They can be found in the form of molecules, organisms and/or compounds. Antibiotic growth promoters have seen a decline in use in recent years and so instead, the broiler industry has endeavoured to discover natural feed additive alternatives that might assist broiler production as efficiently as AGPs. This enterprise has proven successful, with products such as probiotics, prebiotics, antioxidants, enzymes, essential oils and mould inhibitors that exhibit positive performance results when included in broiler diets (Cooper & Sunderland, 2000).

Enzymes are proteins that act as catalysts to biochemical reactions. They bind to their respective substrates and lower the activation energy of a reaction. They are therefore used to improve nutrient digestion and absorption, by hastening the reaction time of biochemical reactions within the gut (Cooper & Sunderland, 2000). All animals produce enzymes *in vivo*, however exogenous enzyme supplementation has been used, particularly in poultry, as an aid to digestion. This is because monogastric animals do not produce the enzymes required for optimal nutrient digestion and with supplementation of exogenous enzymes it has been noted that broilers yield higher performance parameters and therefore profit. Exogenous enzymes have shown to increase feed conversion efficiency as well as release trapped nutrients within insoluble diet ingredients and release oligosaccharides that support the growth of beneficial bacteria while decreasing pathogenic bacteria populations (Alagawany *et al.,* 2018). They can either be amylolytic, proteolytic, ß-glucanase, xylanase, or ß-mannanase. These classes of enzymes have been seen to increase the utilisation of dietary starch, protein, ß-glucans, arabinoxylans, and mannan, respectively. Proteolytic enzymes are used most often for their protein degradation properties, releasing amino acids within the small intestine. If a diet has a low digestibility, these enzymes can be effective (Roque *et al.,* 2017).

It is also important to note that because dietary enzymes optimise nutrient utilisation, less nutrients are excreted by the broiler into the environment. This results in less nutrients wasted and has a decreased effect on the environment (Chang'a *et al.,* 2019). This also leads to a lower feed cost as the broiler can receive a lower nutrient level and still yield optimal production results. Other benefits of using exogenous enzymes in broiler production include: providing young birds with

underdeveloped digestive tracts a sufficient enzyme supply; increasing overall health and performance of broilers by increasing fibre digestibility and therefore reducing the incidence of digestive distress; decrease water in the excreta which can reduce incidence of pasty vents; reducing the amount of anti-nutritional factors in the diet and increasing the accuracy of feed formulation which can lead to decreased feed cost (Alagawany *et al.,* 2018). More often than not, supplementing a broiler diet with only one exogenous enzyme does not yield the above-mentioned results, and so a mixture of enzymes is given, sometimes referred to as an enzyme cocktail. However, it can also be the case that an enzyme cocktail may not yield the sought after results due to the complicated nature of enzymes. The method of integration into the feed; the interaction effects of the enzymes and the other feed ingredients; the dose of enzymes; the age and species of the animal being supplemented and many more factors will have an effect on whether the enzymes given will increase broiler performance and consequently profit (Munir & Maqsood, 2013).

Proteases

Proteins are an essential component of the broiler diet as they are a crucial element in broiler survival. Proteins consumed by the bird are broken down to supply the body with smaller chain peptides and amino acids. Proteases are a class of enzyme that are found naturally in the broiler (either produced by the bird itself or the bird's intestinal microbes) and they assist in digesting and absorbing nutrients within the diet by hydrolysing proteins and peptides into the above-mentioned simplest forms: amino acids and peptides (Pan & Yu, 2013). These molecules are then easily absorbed by the small intestine (the site of major protein digestion by proteases) to be used in multiple pathways throughout the body. A protease must be activated before it can begin to cleave peptide bonds. This is done through an activation cascade wherein a series of stepwise reactions occur resulting in an amplified response (Walk *et al.,* 2019). The addition of a protease in broiler diets can also alleviate the environmental effects of excess nitrogen produced by the bird due to the improvement in ileal amino acid digestibility caused by the presence of a protease, and therefore a lower excretion of undigested nutrients into the environment.

There is little research on the exact mode of action of proteases, however the results on protease supplementation are vast. By supplementing proteases within the broiler diet, the maintenance requirements of the broiler are reduced, allowing for reduced endogenous enzyme production and the superfluous energy can be used for performance (Barekatain *et al.,* 2013). One study by Mohammadigheisar & Kim (2018) showed that supplementing diets containing a standard level of CP with protease attenuated the negative effects on body weight gain and FCR caused. Dry matter (DM) digestibility and digestibility of total essential and total non-essential amino acids was reduced

as a result of the standard CP diets; however, the addition of a protease countered these effects. A study by Park *et al.* (2020) yielded similar results of increased body weight, average daily gain and nutrient digestibility and lower FCRs. Xu *et al.* (2017) reported that the use of a protease increased villus height: crypt depth ratio, indicating enhanced absorption of nutrients in the intestine. It has also been seen that proteases have a larger effect when combined with diets of a poorer quality, for example in the study by Mohammadigheisar & Kim (2018) where a standard CP diet was used.

According to the literature available and the claim that protein digestion is an inefficient process due to the commonly large portion of undigested protein, it can be concluded that diets supplemented with proteases yield positive performance results, and literature provides no significant adverse effects in using proteases (Pan & Yu, 2013). When supplementing a protease, it is important to carefully select a protease that will produce the desired effect, as some proteases can be unstable and their composition and efficiency can be adversely affected by heat or the pelleting process that is almost always used when producing broiler feed. To overcome this, proteases are often supplemented with various other exogenous enzymes, however the interacting effects are poorly understood. Proteases are also added to the feed in powder form prior to pelleting (Yildiz*.,* 2021). There is little information on the extent to which proteases are included in broiler diets in South Africa, however some protease enzymes used in the broiler industry are listed in Table 4 below.

Table 4 Protease enzymes used in the broiler industry as well as their classification, production organisms and their function (Munir & Maqsood, 2013)

Name	Type	Organism	Function
Bromelain	Protease	Pineapple *(Ananas comosus)* stem and fruit	Hydrolyses proteins
Ficain	Protease	*Ficus glabrata*	Hydrolyses proteins
Keratinase	Protease	*Bacillus licheniformis*	
Papain	Protease	*Papaya (Carica papaya)*	Hydrolyses proteins
Pepsin	Protease	Animal stomachs	Hydrolyses proteins
Protease	Protease	*Aspergillus niger, Aspergillus sp., Bacillus spp.*	Hydrolyses proteins
Trypsin	Protease	Animal pancreas	Hydrolyses proteins

Bromelain

Pineapple (*Ananas comosus*) is a member of the *Bromeliaceae* family and contains the proteolytic enzyme bromelain. It is believed that the plant uses bromelain to extract nitrogen and phosphorous from some microbes, however the absolute physiological function of bromelain in the plant is undecided. Both modern and ancient human medicine has utilised bromelain as a phytomedical compound, the first record of bromelain being extracted for human consumption dates back to 1875. According to Pavan *et al.* (2012), bromelain has been used to treat angina pectoris, bronchitis, sinusitis, surgical trauma, thrombophlebitis, debridement of wounds, and enhanced absorption of drugs, particularly antibiotics and more in humans. It is also used in the meat processing industry as a meat tenderising agent (Ketnawa & Rawdkuen, 2011).

Bromelain can be found in most parts of the pineapple but the bromelain found in the fruit and stems has been commercially extracted for the livestock industry. The pineapple fruit accounts for 23% of the plant and is the main source of income in the pineapple production industry. The remaining 77% of the plant is considered surplus and is under-utilised and mostly discarded, despite it containing substantial amounts of bromelain (Martins *et al.,* 2014). Because the stems fall into this waste category, the resulting bromelain is cost effective.

Bromelain comprises of multiple thiol endopeptidases and other compounds like phosphatase, glucosidase, peroxidase, cellulase and several protease inhibitors. It is known that bromelain evinces various fibrinolytic, antithrombotic and anti-inflammatory activities and it is also highly absorbable and when ingested, does not produce notable side effects (Pavan *et al.,* 2012). Bromelain has a lethal dose (LD50) of greater than 10 g/kg in mice, badgers, and rabbits (Mohamed Tap *et al.,* 2018). However, bromelain can become unpalatable if given in too high concentrations.

Studies on the effects of bromelain are mainly concerning humans, however some have been carried out in pigs and poultry. It was reported that weanling pigs supplemented with 0, 0.5, 1.0 and 2.0 g bromelain/kg in their diets had improved average daily gains and average daily feed intakes (Zhao *et al.,* 2015). Furthermore, they also displayed decreased faecal *E. coli* counts and faecal NH_3 gas emission and increased apparent total tract digestibility of DM and nitrogen (Zhao *et al.,* 2015). When multiparous sows were fed bromelain supplemented diets, specifically 0, 0.5, 1, and 2 g bromelain/kg feed, the sows had linearly higher apparent total tract digestibility of nitrogen, lower blood urea nitrogen and higher lymphocyte counts. Piglets suckling bromelain-supplemented sows had increased average daily gain and weaning weight, higher IgG counts and lower blood urea nitrogen (Begum *et al.,* 2015). One of the few studies using bromelain in chickens was conducted by Yenice *et al.* (2019), who discovered that feeding diets containing different concentrations of bromelain (0, 0.15, 0.30 and 0.45 g bromelain/kg feed) to laying hens increased serum protein ($P< 0.04$) concentrations and egg yolk ($P<0.0001$) and decreased egg yolk triacylglycerol fraction ($P<0.0001$) and serum cholesterol concentration ($P<0.0003$). Human related studies in a medicinal context show bromelain to be effective in reducing inflammation, bacterial load, boosts the immune system and is anti-carcinogenic (Manzoor *et al.,* 2016). In other studies, bromelain has been seen to reduce the risk of oedema (Bayat *et al.,* 2019). This may be especially relevant in the broiler industry where ascites is common. One study by Yu *et al.* (2010) showed that when 180 Arbour Acre strain commercial male broilers were fed on diets with varied sources and qualities of protein, the supplementation of bromelain did not yield any positive results on protein digestion. A study by Akit *et al.* (2019) yielded positive results when 180 broilers were randomly allocated one of five treatments and fed a commercial basal diet. Starter birds fed 0.05 and 0.1%

bromelain had higher (P<0.05) fat and protein digestibility than the control, and finisher birds fed 0.05, 0.2 and 0.5% bromelain had higher (P<0.05) fat digestibility than the control. Bromelain showed increased protein digestibility and therefore decreased faecal nitrogen content (P=0.096). However, the improved protein digestion was not used for lean gain as bromelain did not improve the body weight gain and FCR (P<0.05). Bromelain did improve intestinal villus height and reduced digesta viscosity in the starter birds (P<0.05). The results can be seen in more detail in Table 5, Table 6 and Table 7. Another study examined the possible protective effect of bromelain on renal and hepatic toxicity induced by 100 mg/kg ciprofloxacin (a broad-spectrum antibiotic) in broiler chicks. Female Ross 308 broiler chicks were divided into four treatments: the control treatment of 1 mL/kg saline in drinking water, the second treatment of 100 mg/kg ciprofloxacin in drinking water and the third and fourth treatments were treated with basal diet and supplemented with ciprofloxacin and 20 mg/kg bromelain and ciprofloxacin and 40mg/kg bromelain, respectively. Bromelain was seen to significantly increase body weights and haematological parameters which lead to a rise in antibodies against the virus (Albawi, 2019). The results proved that bromelain's antioxidant effects can protect against ciprofloxacin induced hepatic and renal toxicity.

Table 5 Effect of dietary bromelain on growth performance of broiler chickens (Akit *et al.,* 2019)

Parameters	Treatment[1]					
	Control	BR 0.05	BR 0.1	BR 0.2	BR 0.5	SEM[2]
Body weight gain (kg)						
At day 21	0.83[a]	0.82[a]	0.81[a]	0.82[a]	0.74[b]	0.009
At day 42	1.44	1.49	1.43	1.54	1.37	0.019
Overall period	2.27[a]	2.30[a]	2.23[ab]	2.36[a]	2.12[b]	0.044
Feed intake (kg)						
At day 21	1.07	1.10	1.07	1.11	1.03	0.009
At day 42	2.87	2.93	2.88	2.92	2.64	0.037
Overall period	3.94	4.03	3.96	4.03	3.68	0.024
Feed conversion ratio						
At day 21	1.29[c]	1.35[ab]	1.33[bc]	1.35[ab]	1.39[a]	0.011
At day 42	1.99	1.98	2.02	1.90	1.93	0.023
Overall period	1.74	1.75	1.77	1.71	1.74	0.012

[1]Control basal diet + 0 bromelain, BR 0.05 basal diet +0.05% bromelain, BR 0.1 basal diet + 0.1% bromelain, BR 0.2 basal diet + 0.2% bromelain, BR 0.5 basal diet + 0.5% bromelain.

[2]SEM Standard Error of the Mean.

[a-c] Mean values with different letters in the same row are significantly different (P<0.05).

Table 6 Effect of dietary bromelain on nutrient digestibility of broilers (Akit *et al.,* 2019)

Parameter	Treatment[1]					SEM[2]
	Control	BR 0.05	BR 0.1	BR 0.2	BR 0.5	
At day 21						
Dry matter	91.3^{c}	91.1^{c}	94.2^{a}	90.2^{c}	92.9^{b}	0.63
Ether extract	65.3^{e}	77.4^{b}	80.5^{a}	71.9^{c}	68.6^{d}	1.44
Crude protein	74.3^{bc}	76.8^{b}	80.9^{a}	73.2^{c}	65.4^{d}	1.75
Crude fibre	84.9	89.3	94.2	85.8	92.0	1.42
Ash	63.4^{a}	61.6^{a}	64.7^{a}	53.7^{b}	44.4^{c}	2.22
At day 42						
Dry matter	91.2	91.8	92.0	92.0	92.7	0.53
Ether extract	68.8^{b}	75.1^{a}	68.6^{b}	75.9^{a}	75.9^{a}	1.71
Crude protein	68.1^{b}	67.6^{b}	63.6^{c}	68.7^{ab}	71.4^{a}	1.59
Crude fibre	84.8	86.0	87.3	88.4	81.8	1.12
Ash	48.1	54.2	42.0	44.4	36.6	4.67

[1]Control: basal diet +0 bromelain, BR 0.05: basal diet +0.05% bromelain, BR 0.1: basal diet + 0.1% bromelain, BR 0.2: basal diet + 0.2% bromelain, BR 0.5: basal diet + 0.5% bromelain.

[2]SEM Standard Error of the Mean.

[a-e] Mean values with different letters in the same row are significantly different (P<0.05).

Table 7 Effect of dietary bromelain on villus height, crypt depth and villus height-to-crypt depth ratio (VH:CD) of broilers (Akit *et al.,* 2019)

Parameters		Treatment[1]					SEM[2]
		Control	BR 0.05	BR 0.1	BR 0.2	BR 0.5	
At day 21							
Duodenum	Villus height	011^{d}	1537^{a}	1354^{c}	1410^{b}	1395^{b}	13.5
	Crypt depth	168^{e}	223^{a}	208^{d}	210^{c}	215^{b}	1.3
	VH:CD	6.0^{d}	6.9^{a}	6.5^{c}	6.7^{b}	6.5^{c}	0.08
Jejunum	Villus height	813^{b}	812^{bc}	798^{c}	830^{a}	826^{ab}	7.7
	Crypt depth	149^{d}	176^{b}	168^{c}	182^{a}	177^{b}	2.0
	VH:CD	5.5^{a}	4.6^{c}	4.8^{b}	4.6^{c}	4.7^{bc}	0.07
Ileum	Villus height	739^{d}	695^{e}	836^{c}	978^{a}	878^{b}	2.9
	Crypt depth	182^{d}	195^{c}	193^{c}	203^{b}	207^{a}	1.9
	VH:CD	4.1^{d}	3.6^{e}	4.3^{b}	4.8^{a}	4.2^{c}	0.04
At day 42							
Duodenum	Villus height	1270^{d}	1368^{c}	1441^{b}	1725^{a}	1741^{a}	10.6
	Crypt depth	286^{a}	222^{d}	223^{d}	251^{c}	276^{b}	3.0
	VH:CD	4.5^{c}	6.2^{b}	6.5^{ab}	6.7^{a}	6.3^{ab}	0.20
Jejunum	Villus height	942^{e}	959^{d}	1041^{c}	1076^{b}	1094^{a}	5.2
	Crypt depth	264^{a}	187^{c}	196^{b}	196^{b}	198^{b}	2.1
	VH:CD	3.6^{d}	5.1^{c}	5.3^{b}	5.5^{a}	5.5^{a}	0.06
Ileum	Villus height	1319^{a}	917^{d}	950^{c}	951^{c}	1061^{b}	7.5
	Crypt depth	205^{b}	186^{d}	184^{e}	194^{c}	217^{a}	1.0
	VH:CD	6.4^{a}	4.9^{c}	5.2^{b}	4.9^{c}	4.9^{c}	0.06

[1]Control basal diet +0 bromelain, BR 0.05 basal diet + 0.05% bromelain, BR 0.1 basal diet + 0.1% bromelain, BR 0.2 basal diet + 0.2% bromelain, BR 0.5 basal diet + 0.5% bromelain.

[2]SEM Standard Error of the Mean.

[a-e] Mean values with different letters in the same row are significantly different (P<0.05).

2.6 Conclusion

The South African broiler industry faces ongoing and increasing demand, and one of the repercussions of this is that commercial broiler houses have increased their stocking rates. An increased stocking rate creates an environment in which the animals might experience an element of stress which can increase microbial yield and induce health issues such as an imbalanced gut biome. Results like this can heavily impact the performance of the birds and therefore the farmer's profit. To counter this repercussion the broiler industry has been using antibiotic growth promoters as a means to maintain broiler health and increase performance. However, the recent dislike or in some cases prohibition of antibiotic growth promoters has led the industry to increase its use of exogenous enzymes as natural alternatives to these antibiotics. Exogenous protease enzymes are used for their proteolytic activity, increasing protein digestibility and energy for production. There are other benefits to protease use such as decreasing nitrogen and phosphorous excretion into the environment. Studies show that although there is undeniable benefit in using proteases, the method of inclusion, protease used and other important decisions highly influence the protease's beneficial activity. There exists little literature on the effect of bromelain on broilers and specifically on high protein diet More research is therefore needed to determine if bromelain is an effective replacement of antibiotics or not.

CHAPTER 3: MATERIALS AND METHODS

A total of three trials were conducted: a pilot trial to determine the levels of dietary bromelain for use in the performance trail; a performance trial to determine the effect of bromelain on broiler production when supplemented with two different levels of protein (standard and high), with and without an AGP; and a digestibility trial to determine the effects of supplemental bromelain on dietary protein digestibility. The bromelain was supplied by Enzyme Technologies (PTY) Ltd (South Africa).

All trials were approved by the Animal Ethics Committee of the University of Pretoria (pilot, performance and digestibility trial reference numbers were NAS358/2020, NAS109/2021 and NAS060/2022, respectively) and the Department of Agriculture, Forestry and Fisheries under Section 20 of the Animal Disease Act of 1984. The trials were conducted in the environmentally controlled broiler houses of the University of Pretoria's experimental farm (Innovation Africa @ UP Research Park, Hillcrest, Pretoria).

3.1 Pilot trial

Facilities and bird housing

A total of 200 one-day-old chicks were used in the pilot trial. All chickens were purchased from Eagle's Pride Hatchery. The chicks were not sexed for the pilot trial so each pen contained an unknown number of males and females. An environmentally controlled broiler house at the Innovation Africa @UP Research Park, University of Pretoria, was used to conduct the pilot trial. It was equipped with 10 identical pens, five on each side of the house, with concrete flooring.

A randomised block design was used to account for environmental variables. In the pilot trial, two replicates of each treatment (20 chicks) were allocated randomly to one of the 10 pens on each side of the house. In each pen, one infrared heating lamp was positioned in a way that the chicks could regress to a cooler area if they became too hot. The heat lamps were all the same height and remained on for the first three days, after which they were switched off. Ventilation was provided naturally; airflow was controlled by one fan. Clean drinking water was provided daily in two bell drinkers per pen. Every bell drinker was placed at the same height and lifted weekly to account for bird growth.

Cleaned and disinfected pine shavings were used to absorb waste and assist with heat insulation. Prior to placing the day-old chicks, the broiler house was washed, disinfected with VET GL 20 and

SAN QUAT disinfectants (Immuno-Vet, South Africa) and preheated for two days. To uphold strict biosecurity, F10 disinfectant was used as a footbath on entry to the house and labourers were provided with boots that remained in the houses at all times. The broiler house had ambient and litter (floor) temperatures of 35°C and 32°C, respectively.

One water fountain, one chick sheet (approximately 60 cm of parchment paper suitable for animal use), one round chick pan, and one large feeder were placed in each pen. The water fountains were cleaned and replaced with fresh water once a day for one week. New feed was added to the chick sheet, chick tray, and large feeder as necessary. The feed and water sources were kept separate throughout the trial so as to ensure no contamination or feed wastage due to water leakage. After one week, the chick sheet and chick tray were removed, along with the water fountain, leaving the large feeder and bell drinker. Twice-daily chicken care was conducted at 8:30 and 16:30, during which the pens were extensively examined as follows: excreta and/or pine shavings were removed from the feeders; the water nipples were checked for leaks and/or blockages; the feeders were filled where necessary to provide unlimited access to feed; the chickens were monitored for any behavioural abnormalities, including panting, digging into litter, or huddling; eating habits were monitored; any sick/tired appearances were observed; the temperature of three pens in the house were taken along with the set temperature and house temperature; and finally, any dead animals were weighed, recorded, and an autopsy was conducted to determine likely cause of death. The frequency of these checks enabled the thorough and careful care of the chickens.

The chicks in the pilot trial received 1 h of darkness and 23 h of light for the first 7 d. Thereafter, they received 6 h of darkness and 18 h of light for the remainder of the period, as they weighed <200 g at 14 d.

Experimental design and dietary treatments

The pilot trial was designed as a complete randomised study with five treatments and two cage replicates per treatment, including 20 mixed-sex Ross 308 broilers per pen (10 pens and 200 chicks in total). The treatments differed in terms of levels of bromelain to ascertain the optimum levels that could be used in the performance trial (Table 8).

All diets were maize-soya based, containing fine phytase (1000 FTUs) and a coccidiostat (Salinomycin, 500 g/ton feed) to mimic typical South African commercial broiler diets. A total of three feed phases were implemented throughout the duration of the trial, namely, a ‘starter’ diet from 1 to 14 d old, a ‘grower’ diet from 14 to 28 d old, and a ‘finisher’ diet from 28 to 35 d old. All feed was

provided in mash form, as the amount of feed that was mixed was too low to be pelleted. All feed bags were labelled to ensure the correct feed was given to each treatment group during respective phases.

Table 8 Dietary treatments used in the pilot trial

	Treatment
1	Positive Control (NC) (basal diet, 0 g/kg bromelain)
2	PC + 0.125 g bromelain/kg feed (0.012%)
3	PC + 0.25 g/kg bromelain (0.025%)
4	PC + 0.50 g/kg bromelain (0.050%)
5	PC + 0.75 g/kg bromelain (0.075%)

Table 9 Nutrient content of the basal diet for the pilot trial

Nutrient	**Units**	**Starter**	**Grower**	**Finisher**
VOLUME		100.00	100.00	100.00
ME Poultry	MJ/kg	12.5	13.0	13.2
Crude Protein	g/kg	242	201	201
Lysine (total)	g/kg	13.9	13.0	12.0
TSAA (total)	g/kg	10.7	8.71	8.89
Arginine (available)	g/kg	13.6	10.9	11.2
Isoleucine (available)	g/kg	8.75	6.96	7.02
Lysine (available)	g/kg	12.7	12.0	11.0
Methionine (available)	g/kg	6.58	5.22	5.40
Threonine (available)	g/kg	8.47	6.83	7.06
Tryptophan (available)	g/kg	2.31	1.82	1.86
TSAA (available)	g/kg	9.82	7.99	8.15
Valine (available)	g/kg	9.94	8.00	8.20
Phosphorus (available)	g/kg	4.79	4.03	3.53
Total Phosphorus	g/kg	6.64	5.52	4.96
Calcium	g/kg	8.96	8.06	6.91

Chloride	g/kg	2.13	2.42	2.18
Potassium	g/kg	9.14	7.63	7.92
Sodium	g/kg	1.74	1.74	1.74
Fat	g/kg	31.8	39.3	48.9
Linoleic acid	g/kg	15.0	19.3	24.5
Crude fibre	g/kg	34.7	33.1	34.7

ME: Metabolisable energy

TSAA: total sulphur amino acids

Table 10 Feed ingredient content (%) of the basal diet in the pilot trial

Ingredient	Starter	Grower	Finisher
Yellow Maize 8.7	565	665	648
Prime Gluten 65%	30.0	15.0	10.0
Extruded Full Fat Soya 35%	25.0	61.0	122
Soya O/C 46.5%	304	191.0	158
Sunflower O/C 35.5%	34.0	30.0	30.0
Limestone 32%	12.0	12.0	10.0
Monocalcium Phosphate	12.5	9.00	6.00
Salt	2.00	2.00	2.00
Sodium Bicarbonate	2.00	2.00	2.00
DL Methionine	3.10	2.30	2.50
L Threonine	0.90	0.70	0.90
L Valine	0.40	0.20	0.40
Lysine HCl	3.10	5.00	3.40

Parameters measured

At placement and at 7, 14, 21, 28, and 35 d of age, all birds from each pen were weighed together and the average body weight per bird was calculated. Residual feed was weighed on the same days as bird body weight, which was used to calculate weekly as well as cumulative feed intake. The weekly and cumulative FCRs were corrected for mortality by adding the weight of the mortalities to the final weight of the associated pen. Number, age, and weight of birds that died during the trial were recorded to calculate mortality rate.

Feed analysis

Feed analysis was not conducted for the treatments in the pilot trial as it was unnecessary to do so, because the aim of the pilot trial was to check palatability of the diets.

Sampling and processing

Birds were weighed weekly and their weights recorded. The feed was also weighed weekly which allowed the FCR calculation. These results indicated which levels of bromelain would be most suitable for the performance trial.

Statistical analysis

As there were only two replicates and one degree of freedom in the pilot trial's experimental design, statistical analysis was of limited value, and therefore was not conducted.

3.2 Performance trial

Facilities and bird housing

A total of 2400 day-old, mixed-sex chicks were used in the performance trial. All chicks were purchased from Eagle's Pride Hatchery and came from a breeder flock aged 55 weeks. The 2400 chickens were randomly divided into the 96 pens, with 25 chicks per pen to induce the slight stress that is present in all commercial broiler farms in South Africa. Due to the Covid-19 pandemic and outbreaks of avian influenza during the start of the trial, the hatchery did not allow sexing of any chickens on their premises, and thus, both male and female chickens were delivered to the farm. However, on placement day of the performance trial, all chickens were sexed, and the result was six pens of females and two pens of males per treatment.

An environmentally controlled broiler house at the Innovation Africa @ UP Research Park, University of Pretoria, was used to conduct the performance trial. The house consisted of two adjacent sides with 48 pens in each, summing to a total of 96 pens. Each side of the house was fitted with a SKOV computer system, which regulated the environmental conditions in the house by controlling the electric heaters and mist sprayers and adjusting the fan speed and opening of air inlets. Housing and care of the birds were done in such a way as to represent commercial conditions.

For bird welfare, clean pine shavings were used on the floor of the pens to absorb waste and assist in heat insulation. Chicks were vaccinated against Newcastle Disease and Gumboro Disease at the hatchery via spraying and on day 13 via the drinking water. On day 19, all birds were again vaccinated against Newcastle disease via drinking water. A commercial lighting and temperature programme was followed throughout the study, according to recommendations by Aviagen (Ross 308, Production Manual).

Prior to placing the day-old chicks, the house was washed, disinfected with VET GL 20 and SAN QUAT disinfectants (Immuno-Vet), and preheated for two days. To uphold strict biosecurity, F10 disinfectant was used as a footbath on entry to the house and labourers were provided with boots that remained inside the house at all times.

Feed and water were provided *ad libitum* throughout the trial. To ensure easy access to feed and water during the brooding stage (week 1), a water fountain, chick paper, and tray feeder were added to the pens, in addition to the water nipples and tube feeder. Pens were extensively examined twice daily as follows: faeces and/or pine shavings were removed from the feeding and watering equipment; water nipples were checked for leaks and/or blockages; feeders were filled where necessary; chickens were monitored for any abnormalities, such as panting, digging into litter, or huddling; eating habits and sick/tired appearance were monitored; the temperature of six pens in the house were taken, along with the set temperature and house temperature; and finally, any deceased animals were weighed, recorded, and an autopsy was conducted to determine cause of death. The frequency of these checks enabled the thorough and careful care of the chickens.

The birds received 1 h of darkness and 23 h of light for the first 3 d, followed by 3 h of darkness and 21 h of light for the next 12 d. For the remaining period, they received 6 h of darkness and 18 h of light per day.

Experimental design and dietary treatments

A complete randomised block design was used to account for environmental variables. A total of 12 dietary treatments were included in this trial, with a 2×3×2 factorial arrangement of treatments and eight replicates per treatment. The dietary variables in the study included CP level, bromelain inclusion level, and presence of an AGP, as well as interaction effects, namely, CP × AGP, CP × bromelain, AGP × bromelain, and CP × AGP × bromelain. The 12 treatments are described in Table 11.

The pilot trial showed no adverse effects of bromelain on feed intake and it was decided to include the lowest (0.125 g/kg) and highest (0.75 g/kg) levels as treatments in the performance trial. The lowest level was chosen as this represents a level that would be economically viable should the industry ever adopt bromelain as a feasible feed additive. The higher level was chosen to investigate efficacy at higher levels of inclusion.

Table 11 Dietary treatments included in the performance trial

	Treatment
1	0 g/kg bromelain, standard CP, no AGP
2	0 g/kg bromelain, standard CP, AGP included
3	0 g/kg bromelain, high CP, no AGP
4	0 g/kg bromelain, high CP, AGP included
5	0.125 g/kg bromelain, standard CP, no AGP
6	0.125 g/kg bromelain, standard CP, AGP included
7	0.125 g/kg bromelain, high CP, no AGP
8	0.125 g/kg bromelain, high CP, AGP included
9	0.75 g/kg bromelain, standard CP, no AGP
10	0.75 g/kg bromelain, standard CP, AGP included
11	0.75 g/kg bromelain, high CP, no AGP
12	0.75 g/kg bromelain, high CP, AGP included

CP, crude protein; AGP, antibiotic growth promoter (zinc bacitracin)

The standard-CP diets were formulated to contain approximately 4% less CP and amino acids, while the high-CP diets were formulated to contain 3% higher CP and amino acids than that of the Ross 308 recommendation.

The diets contained CP and AA levels which were either marginally lower than levels recommended by Avigen or slightly higher. The marginally lower levels of CP and AA were used for formulation to provide a contrast which did not have excess levels of CP or AAs. High-CP diets were included in the trial to test bromelain's antimicrobial characteristics, as it is known that a high load of undigested CP in the hindgut may negatively affect the gut microbiome. The treatment diets were iso-energetic and only differed in CP content, bromelain inclusion level, and presence of zinc bacitracin as an AGP. Ross 308-recommended CP and amino acid levels (2019) were used as a baseline.
The chicks received a maize-soya-based diet *ad libitum* throughout the trial. All diets contained fine phytase (1000 FTUs) and a coccidiostat (Salinomycin, 500 g/ton feed) as typically found in South African commercial diets.

A total of three feed phases were fed throughout the duration of the trial. The chicks received a starter diet from day-old until 14 d-of- age. They then received a grower diet from 14 to 28 d-of-age and a finisher diet from 28 until 35 d-of-age. The starter feed was fed as a crumble and the grower and finisher feed in pellet form. All bags of feed were clearly labelled with an identifier matching its respective pen. This ensured that the correct feed was fed to each treatment during the different phases. The nutrient density and feed composition of the two basal diets during the three phases with standard CP and high CP content are shown in Table 12, Table 13 and Table 14.

Table 12 Nutrient content of the basal diet with standard crude protein in the performance trial

Nutrient	Units	Starter	Grower	Finisher
AME Poultry	MJ/kg	12.55	12.97	13.39
Crude protein	g/kg	220	210	187
Lysine (total)	g/kg	13.6	12.4	11.0
Methionine (total)	g/kg	6.43	5.88	5.45
TSAA (total)	g/kg	10.0	9.25	8.50
Valine (total)	g/kg	10.4	10.2	9.04
Histidine (available)	g/kg	4.74	4.61	4.08
Arginine (available)	g/kg	13.4	13.2	11.6
Threonine (available)	g/kg	8.26	7.82	7.41
Isoleucine (available)	g/kg	8.72	8.46	7.41
Lysine (available)	g/kg	12.3	11.0	9.89
Methionine (available)	g/kg	6.14	5.58	5.17
TSAA (available)	g/kg	9.12	8.35	7.68
Tryptophan (available)	g/kg	2.22	2.23	1.94
Crude fibre	g/kg	39.6	38.5	37.2
Fat (ether extract)	g/kg	39.8	54.0	60.0
Linoleic acid	g/kg	19.3	26.8	30.3
Calcium	g/kg	9.60	8.70	7.90
Total phosphorus	g/kg	6.32	5.69	5.10
Available phosphorus	g/kg	4.80	4.35	3.95
Chloride	g/kg	2.30	2.30	2.30
Potassium	g/kg	9.76	9.24	8.07
Sodium	g/kg	1.60	1.60	1.60

TSAA, Total sulphur amino acids; AME, apparent metabolizable energy.

Table 13 The nutrient content of the basal diet with high crude protein

Nutrient	Units	Starter	Grower	Finisher
ME Poultry	MJ/kg	12.55	12.97	13.39
Crude protein	g/kg	236	221	200
Lysine (total)	g/kg	15.2	13.9	12.6
Methionine (total)	g/kg	7.09	6.44	5.96
TSAA (total)	g/kg	10.8	9.98	9.19
Valine (total)	g/kg	12.1	12.0	10.5
Histidine (available)	g/kg	6.04	6.00	5.61
Arginine (available)	g/kg	14.6	13.4	11.8
Threonine (available)	g/kg	8.86	7.93	7.57
Isoleucine (available)	g/kg	9.07	8.24	7.31
Lysine (available)	g/kg	13.2	11.8	10.6
Methionine (available)	g/kg	6.79	6.16	5.71
TSAA (available)	g/kg	9.78	8.96	8.24
Tryptophan (available)	g/kg	2.47	2.27	2.01
Crude fibre	g/kg	41.1	40.1	36.4
Fat	g/kg	47.1	54.9	60.0
Linoleic Acid	g/kg	23.0	27.4	30.3
Calcium	g/kg	9.60	8.70	7.90
Total phosphorus	g/kg	6.32	5.69	5.05
Available phosphorus	g/kg	4.80	4.35	3.95
Chloride	g/kg	2.30	2.30	2.30
Potassium	g/kg	9.76	8.81	7.87
Sodium	g/kg	1.60	1.60	1.60

TSAA, Total sulphur amino acids; AME, apparent metabolizable energy.

Table 14 Feed ingredient content (%) of the standard and high crude protein basal diets

Ingredient	Standard/High CP	Starter	Grower	Finisher
Yellow Maize 7.5%	standard	56.0	56.9	63.1
	high	51.3	55.8	61.8
Soya O/C 44%	standard	26.8	22.1	14.0
	high	27.6	19.1	12.9
Full fat soya	standard	5.52	13.8	16.6
	high	10.5	14.5	16.9
Sunflower O/C 38 %	standard	5.00	3.00	3.00
	high	5.00	5.00	3.00
Gluten 60 prime	standard	2.50	-	-
	high	-	-	-
Spray dried blood	standard	-	-	-
	high	1.50	2.00	2.10
Limestone	standard	1.42	1.33	1.26
	high	1.40	1.32	1.25
Monocalcium phosphate	standard	1.01	0.73	0.56
	high	0.96	0.74	0.56
DL Methionine SA	standard	0.28	0.26	0.24
	high	0.35	0.31	0.29
Lysine HCl SA	standard	0.33	0.14	0.18
	high	0.25	0.20	0.19
L Threonine SA	standard	0.10	0.08	0.13
	high	0.11	0.07	0.10
Salt	standard	0.22	0.27	0.26
	high	0.23	0.24	0.25
Sodium bicarbonate	standard	0.19	0.12	0.14
	high	0.16	0.15	0.14
Mycotoxin binder[1]	standard	0.15	0.15	0.15
	high	0.15	0.15	0.15
Coccidiostat[2]	standard	0.05	0.05	0.05
	high	0.05	0.05	0.05
Premix (broiler)	standard	0.30	0.25	0.20
	high	0.30	0.25	0.20

Phytase (broiler[3])	standard	0.01	0.01	0.01
	high	0.01	0.01	0.01

[1] *Mycosorb*

[2] *Salinomycin*

[3] *AxtraPhy*

Parameters measured

The same methods used in the pilot trial to determine the average bird body weight, weekly and cumulative feed intake, and FCR were used in the performance trial; however, the final measurement was taken at 34 d. The mortalities were also determined in a manner similar to the pilot trial.

Feed analysis

Six feed samples used in the performance trial were analysed for crude fat content, crude fibre content, CP content, ash, and DM content. The six feed samples used were treatment 1 (0 g/kg bromelain, standard CP, no AGP) for the starter, grower, and finisher diets and treatment 7 (0.125 g/kg bromelain, high CP, no AGP) for the starter, grower, and finisher diets. The samples were analysed at Nutrilab, Department of Animal Science, University of Pretoria (Hatfield, Pretoria) according to standard methods described by the Association of Official Analytical Chemists (AOAC, 2000).

Dry matter was determined by placing 2.0 g of feed sample into dry crucibles, which had been previously dried in an oven for 1 h at 105 °C. Once containing the 2.0 g of feed, the crucibles were placed into an oven and left to dry for 24 h at 105 °C. After oven drying, the samples were cooled in a desiccator and weighed. This method is according to the AOAC (2000), Official Method of Analysis 934.01, and the DM percentage was calculated using the following formula:

$$\%DM = \frac{mass\ of\ crucible\ and\ sample\ after\ drying}{mass\ of\ crucible\ and\ sample\ before\ drying} \times 100$$

The ash content of the feed samples was obtained according to AOAC's Official Method of Analysis (AOAC, 2000, Official Method of Analysis 942.05). Once the weights of the oven-dried samples were calculated, the crucibles containing the dried samples were incinerated in a muffle 32 furnace for 2 h at 250 °C and then a further 4 h at 600 °C. The crucibles and ash were then left in the furnace for another 2 h to cool, before being placed in the desiccator for 1 h to cool further. Once completely

cooled, the crucibles and ash samples were weighed. Tongs were used to handle the crucibles at all times so as not to alter the moisture content of the crucibles. The ash percentage was determined using the following formula:

$$\%Ash = \frac{mass\ of\ crucible\ and\ ash\ sample}{mass\ of\ crucible\ and\ sample\ after\ drying} \times 100$$

The Dumas method, which followed the AOAC's Official Method of Analysis (AOAC, 2000, Official Method of Analysis 968.06), was used to determine the CP content of the feed. The percentage of CP was then calculated by multiplying the nitrogen content of the feed by a conversion factor of 6.25, and the moisture percentage was calculated by subtracting the DM percentage from 100.
The crude fibre content of the samples was determined using the AOAC's Official Method of Analysis (AOAC., 2000, Official Method of Analysis 962.09). Glass crucibles containing 0.95 g of the feed sample, 150 mL of hot sulphuric acid, and three drops of n-octanol were boiled for 30 min in a hot extraction unit and then rinsed three times with 30 mL hot distilled water each time. Thereafter, three more drops of n-octanol and 150 mL of a sodium hydroxide solution were added to each crucible. The crucibles were placed back into the hot extraction unit and boiled for another 30 min before rinsing with boiling distilled water. They were then placed in a drying oven and thereafter cooled in a desiccator overnight. The crucibles were weighed the next day. Ash samples were measured by placing the crucibles into a furnace oven for 3 h at 600 °C and then slowly cooled to 250 °C before being placed into a desiccator for a further 2 h to cool completely. Once cooled, the samples were weighed. The crude fibre percentage was determined by the following formula:

$$\%CF = \frac{mass\ of\ crucible\ and\ sample\ after\ boiling - mass\ of\ crucible\ and\ ash\ sample}{mass\ of\ crucible\ and\ sample\ before\ boilling} \times 100$$

Crude fat was determined using the Foss Soxtec method, according to the AOAC's Official Method of Analysis (AOAC, 2000, Official Method of Analysis 920.39). Approximately 2 g of feed sample was placed into filter paper, which was folded and sealed to prevent any spilling. The samples were then placed into marked extraction thimbles, and these were placed into an extraction tube. Forty mL of hot petroleum ether was added and allowed to boil and condense for 2.5 h. The remaining petroleum ether was collected for 30 min and the beakers were placed into the drying oven for a minimum of 2 h before being weighed. The percentage of crude fat in the feed sample was determined using the following formula:

$$\%Crude\ fat = \frac{mass\ of\ crude\ fat}{mass\ of\ sample} \times 100$$

Sampling and processing

Sampling and processing of the 192 intestinal villi took place at the Department of Pathology, Faculty of Veterinary Sciences, University of Pretoria (Onderstepoort, Pretoria) and the Laboratory for Microscopy & Microanalysis at the University of Pretoria (Hatfield, Pretoria). Two days before sampling, 16 birds from each treatment were randomly chosen and marked with a cable tie on their leg. One day before sampling, 192 collection jars were filled halfway with formaldehyde and stored in the abattoir on the University of Pretoria Experimental Farm. On sampling day, all 192 marked birds were brought to the abattoir and euthanised by cervical dislocation in groups according to treatment, and their intestines were immediately exposed. The small intestine was divided into three distinct intestinal sections of approximately 3 cm long: the duodenum, the jejunum, and the ileum. A small piece from the middle of each duodenal, jejunal, and ileal sample was cut out cleanly using a scalpel, and the digesta was removed from each piece using a syringe and distilled water. This was done carefully so as not to damage the villi. Each sample was thoroughly rinsed in phosphate-buffered saline to remove the digesta and then placed in a clearly labelled container and covered with formaldehyde for preservation The intestinal sections were placed into jars of formaldehyde and further processed at the department of Pathology, Faculty of Veterinary Sciences, University of Pretoria (Onderstepoort, Pretoria). Once there, each sample was cut into an even smaller piece using a sharp scalpel, placed onto a metal slide, and submerged in formaldehyde. The samples were then transferred to plastic slides and solidified using wax. Once solid, each sample was sliced thinly and fixed onto a microscope slide. All microscope slides were viewed at the Laboratory for Microscopy & Microanalysis, University of Pretoria (Hatfield, Pretoria) under a light microscope (Zeiss AXIO Imager.M2). Pictures of the amplified intestines were taken and the scale of each picture was noted. Approximately 10 villi and 10 crypt depths were measured using images from each treatment. The software used to measure these images was ImageJ. The mean value of the 10 measurements were calculated and recorded for each sample.

Statistical analysis

The performance and digestibility trials used the same method of statistical analysis.

SAS (Statistical Analysis System) software was used to statistically analyse data such as treatment means. Significant treatment means will be separated using Tukey HSD test at ($P<0.05$). Data was analysed by ANOVA using a general linear model (GLM) as described by the following equation:

$$Y = \mu + Ti + Lj + TLij + eij$$

Where:

Y = variable studied during the period

μ =overall mean of the population
Ti = effect of the ith treatment
Li = effect of the jth source
TLij = effect of the kth level
eij = error associated with each Y

Means and standard errors were calculated and the significance of difference ($P < 0.05$) between means determined by Fisher's Test (Samuels, 1989).

3.3 Digestibility trial

Facilities and bird housing

The digestibility trial was conducted at the Innovation @ UP Research Park, Hillcrest, Pretoria. A total of 300-day-old male Ross 308 broiler chickens were purchased from Eagle's Pride Hatchery and individually sexed using the feather sexing method. The birds had an average body weight of 44.35 g at one-day-old. The broiler house on the experimental farm was preheated to 33 °C for optimum bird comfort on arrival and used for the first 15 d of the trial. All birds were reared from day 0 to day 14 on concrete floors covered with pine shavings. Feed and water were available *ad libitum* throughout the first two weeks of the trial. There were two tube feeders and two bell drinkers per cage, and the 300 birds were randomly placed in 4 pens, with 75 birds per pen. They received the same lighting programme as the performance trial, and the same biosecurity and chick care protocols were followed throughout the trial.

On day 14, all birds were weighed individually and an average bird weight of 540 g was recorded. One hundred eighty birds with body weights closest to the average were then transferred to 30 metabolic cages with six birds per cage. The cages were housed in the metabolic house on University of Pretoria's Experimental Farm, and the house was fitted with a SKOV computer system and extraction fan. Each cage was fitted with a feeding tray and a water line with three water nipples per cage. Birds received feed *ad libitum* throughout the trial.

Experimental design and dietary treatments

On day 14, all birds were individually weighed. On day 15, 180 broilers with body weights closest to the population average (540 g) were moved to 30 metabolic cages with six broilers per cage. These 180 birds were randomly assigned to one of three treatments, with 10 replicates per treatment. They were distributed throughout the cages in such a way as to prevent significant differences in body weight between cages. Cage allocation was done using a randomised block design to avoid environmental factors that could influence the results. Once in the metabolic cages, there was an adaptation period of 6 days (days 15–20) while receiving the treatment diets *ad libitum*. The basal diet for all three treatments contained a maize-soya-based diet containing fine phytase (Axtraphy, 1000 FTUs) and a coccidiostat (Salinomycin, 500 g/ton feed) as typically found in South African commercial chicken diets. All treatment diets also contained titanium dioxide at 5 g/kg feed as an indigestible marker to assist in determining ileal digestibility. Treatment one (the negative control) consisted of the basal diet and 0 g/kg bromelain. Treatment two contained the negative control and 0.75 g/kg bromelain. Treatment three (the positive control) consisted of 0.25 g/kg AxtraPro (Du Pont), a commercially available protease. The three treatments can be found in table 15. The feed ingredients and nutrient content of the basal diets can be found in tables 16 and 17.

Table 15 Dietary treatments included in the digestibility trial

	Treatment
1	Negative Control (NC) (standard diet, 0 g/kg bromelain)
2	NC + 0.75 g bromelain/kg feed (0.075%)
3	Positive Control (0.25 g/kg AxtraPro, Du Pont)

All treatments contained a standard amount of crude protein (isonitrogenous).

Table 16 Feed ingredients of the basal grower diet

Ingredient	Feed ingredients (%)
Yellow Maize 8.5%	63.6
Extruded FF Soya	5.00
Soya O/C 44%	22.7
Sunflower O/C 38 %	4.02
L- Arginine	0.01
L Isoleucine	0.02
L Threonine SA	0.13
L- Valine	0.05
L-Methionine CJ	0.27
Lysine HCL SA	0.29
Sunflower Oil	0.42
Limestone Slow Solubility	1.21
Monodicalcium phosphate	0.35
Salt	0.25
Sodium bicarbonate	0.15
Mineral Premix	0.13
Vitamin Premix	0.13
Choline Chloride (60%)	0.20
Pellibond	1.00

Table 17 Nutrient content of the basal diet

Nutrient	Units	Content
ME Broiler	MJ/kg	12.2
Crude protein	g/kg	20.0
Calcium	g/kg	0.75
Total Phosphorus	g/kg	0.39
Phytate Phosphorous	g/kg	0.26

ME, Metabolisable energy

Parameters measured

On day 21, the birds were euthanised using cervical dislocation. After confirming death, the small intestine was immediately exposed and ileal samples were collected from the lower (distal) half of the ileum (the ileum being defined as the portion of the small intestine from Meckel's diverticulum to 40 mm proximal to the ileo-caecal junction). Using distilled water, digesta was then gently flushed from the ileum. Digesta from all birds per cage were pooled together into one clearly labelled container. Frozen digesta samples were freeze-dried for one week and then ground and stored in airtight containers before analysis for DM, total crude protein, and titanium dioxide. The analysis took place at the Chem Nutri laboratory (Olifantsfontein, Centurion).

Feed analysis

The three treatment feed samples used in the digestibility trial were analysed for CP, titanium dioxide, and DM in the Chem Nutri laboratory.

The Dumas method, which followed the AOAC's Official Method of Analysis (AOAC, 2000, Official Method of Analysis 968.06), was used to determine the CP content of the feed. The percentage of CP was then calculated by multiplying the nitrogen content of the feed by a conversion factor of 6.25, and the moisture percentage was calculated by subtracting the DM percentage from 100.

Dry matter was determined by placing 2.0 g of feed sample into dry crucibles, which had been previously dried in an oven for 1 h at 105 °C. Once containing the 2.0 g of feed, the crucibles were placed into an oven and left to dry for 24 h at 105 °C. After oven drying, the samples were cooled in a desiccator and weighed. This method is according to the AOAC (2000), Official Method of Analysis 934.01, and the DM percentage was calculated using the following formula:

$$\%DM = \frac{mass\ of\ crucible\ and\ sample\ after\ drying}{mass\ of\ crucible\ and\ sample\ before\ drying} \times 100$$

Sampling and processing

Once euthanised, the ileum of 10 birds from each treatment was exposed, sectioned, and immediately moved to a cold granite slab surrounded by ice. The digesta from each ileum was removed into a clearly marked container using a 20 mL syringe with 2 mL distilled water and 18 mL air. All ileal and faecal samples—in their clearly marked containers—were immediately stored at

Nutrilab, (Department of Animal Science, University of Pretoria, Hatfield) in a freezer at –36 °C until frozen solid. Once frozen, they were removed and placed into clearly labelled aluminium containers, and dried in a freeze dryer (Vacutec, South Africa) at –50 °C for one week. Upon removal, the samples had a clumped powder consistency. The samples were then ground and stored in airtight containers before analysis for DM, total crude protein, and titanium dioxide. The analysis took place at the Chem Nutri laboratory (Olifantsfontein, Centurion) and followed the AOAC (2000) Official Methods of Analysis.

Using titanium dioxide as an indigestible marker, DM and CP digestibility were then calculated using the following equation:

$$\frac{\left[\left(\frac{Nutrient}{TiO_2}\right)_d - \left(\frac{Nutrient}{TiO_2}\right)_i\right]}{\left(\frac{Nutrient}{TiO_2}\right)_d} x100$$

This equation holds where (nutrient/ TiO_2)$_d$ is the ratio of the analysed nutrient to TiO_2 in the diet and where (nutrient/ TiO_2)$_i$ is the ratio of the analysed nutrient to TiO_2 in the ileal digesta.

Statistical analysis

The performance and digestibility trials used the same method of statistical analysis.

SAS (Statistical Analysis System) software was used to statistically analyse data such as treatment means. Significant treatment means will be separated using Tukey HSD test at ($P<0.05$). Data was analysed by ANOVA using a general linear model (GLM) as described by the following equation:

$Y = \mu + Ti + Lj + TLij + eij$

Where:

Y = variable studied during the period

μ =overall mean of the population

Ti = effect of the ith treatment

Li = effect of the jth source

TLij = effect of the kth level

eij = error associated with each Y

Means and standard errors were calculated and the significance of difference ($P < 0.05$) between means determined by Fisher's Test (Samuels, 1989).

CHAPTER 4: RESULTS

4.1 Pilot trial

The data did not show any significant differences in intake between the treatments containing high and low bromelain.

4.2 Performance trial

Weekly performance

The main and interaction effects of CP, bromelain supplementation, and AGP on weekly body weight, weight gain, feed intake, and FCRs, along with the sources of variation for each category is shown in Tables 18–29.

On days 7 and 14, the birds given no AGP had a significantly higher (P<0.05) body weight compared to those that were given an AGP. On days 21 and 28, the high CP group had a significantly higher (P<0.05) body weight compared to the standard CP group. Bromelain had no significant effect on the body weight of broilers (table 18).

On days 7, 14, and 21, birds that received AGP had significantly lower (P<0.05) body weights with the interaction effects of 0.125 g/kg bromelain and a standard amount of CP. On days 28 and 34, birds consuming feed of the same parameters also showed significantly lower (P<0.05) body weights. The level of bromelain did not have a significant effect (P<0.05) on body weight (table 19).

Crude protein had a significant effect (P<0.05) on body weight on day 21 and 28, while AGP significantly affected (P<0.05) body weight on days 7 and 14. No significant interaction effects were noted for weekly body weight (table 20).

In the first week, birds that received an AGP showed significantly less (P<0.05) body weight gain than those that did not receive an AGP (P<0.05). In the third and fourth week, birds that received a standard level of CP showed significantly less (P<0.05) body weight gain than those that received a high level of CP (table 21).

On days 0-7, 7-14 and 14-21, birds that received a combination of 0.125 g/kg bromelain, a standard level of CP and the inclusion of an AGP had significantly lower (P<0.05) weight gain. The level of bromelain included did not have a significant effect on body weight gain across all days of age (table 22).

Crude protein had a significant effect (P<0.05) on weight gain in days 14-34. Antibiotic growth promoter also significantly affected weight gain in week 1. No significant interaction effects were seen (table 23).

In the first and third weeks, the birds that were given high-CP feed had significantly higher (P<0.05) feed intakes than the birds given a standard-CP feed. Birds given 0.75 g/kg bromelain had significantly higher (P<0.05) feed intakes in week 1. Birds given an AGP had a significantly decreased (P<0.05) FI in days 28-34 (table 24).

In days 7-14, birds that received a combination of the effects of 0.75 g/kg bromelain, a high amount of CP and the inclusion of an AGP had significantly higher (P<0.05) feed intake than the birds that received 0 g/kg bromelain with all combinations of CP and AGP. The level of CP did not have a significant effect (P<0.05) on feed intake from days 0 to 34, and in days 21–28 no significant difference (P<0.05) was seen across all combinations of bromelain, CP, and AGP (table 25).

It is evident that CP had a significant effect (P<0.05) on feed intake in week 1 and 3, and AGP had a significant effect (P<0.05) on feed intake in week 5. Bromelain significantly affected feed intake in week 1. Crude protein, AGP and bromelain showed a significant interaction effect during the first week (table 26).

In weeks 1 and 2 across all treatments, the FCR of birds not given an AGP were significantly lower (P<0.05) than those of birds given an AGP. However, the opposite was seen in week 4. Furthermore, in the first two weeks, birds given bromelain at 0.125 g/kg had significantly higher (P<0.05) FCR. In the fourth week, birds given a standard level of CP had significantly higher (P<0.05) FCR than those given a high level of CP (table 27).

In days 0-7, birds that received a combination of 0 g/kg bromelain, a standard amount of CP and the inclusion of AGP had significantly lower (P<0.05) FCR compared to the 0 g/kg bromelain category. In days 7-14, birds given 0.125 g/kg bromelain, a standard amount of CP and the inclusion of AGP had significantly higher (P<0.05) FCR. The level of CP, bromelain and AGP given caused no significant differences (P<0.05) to the birds' FCR in days 14-21. In the last 28–34 d, there was a notable increase in FCR (table 28).

In week 1, AGP and bromelain had a significant effect on FCR as main effects. In week 2, AGP also had a significant effect on FCR. Crude protein significantly affected FCR in week 4. In week 1, a

significant interaction effect of AGP and bromelain was seen, and in week 5, a significant effect was also seen from the interaction between CP and bromelain (table 29).

Table 18 The main effect of crude protein (CP) concentration, bromelain supplementation, and antibiotic growth promoter (AGP) on the weekly mean body weight (g) of broilers (± standard error of the mean)

	Day 0	Day 7	Day 14	Day 21	Day 28	Day 34
CP						
Standard	41.21 (±0.14)	169.2 (±1.34)	459.6 (±3.50)	941.1 (±5.60) [b]	1693 (±8.35) [b]	2360 (±12.44)
High	41.35 (±0.14)	169.4 (±1.34)	465.8 (±3.50)	969.3 (±5.60) [a]	1748 (±8.35) [a]	2386 (±12.43)
AGP						
Without	41.45 (±0.14)	171.8 (±1.34) [a]	469.0 (±3.49) [a]	960.2 (±5.59)	1727 (±8.33)	2387 (±12.40)
With	41.08 (±0.14)	166.9 (±1.33) [b]	456.4 (±3.48) [b]	950.2 (±5.58)	1713 (±8.32)	2359 (±12.39)
Bromelain (g/kg)						
0	41.46 (±0.18)	171.5 (±1.63)	466.3 (±4.27)	960.5 (±6.84)	1724 (±10.19)	2386 (±15.18)
0.125	41.25 (±0.18)	167.5 (±1.64)	456.6 (±4.29)	949.2 (±6.87)	1712 (±10.24)	2351 (±15.25)
0.75	41.04 (±0.18)	168.9 (±1.64)	465.2 (±4.27)	955.8 (±6.84)	1725 (±10.20)	2382 (±15.19)

[a,b] Mean values within a column without a common superscript letter differ significantly ($P<0.05$)

Table 19 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on the weekly mean body weight (g) of broilers (± standard error of the mean)

Treatment		Day 0	Day 7	Day 14	Day 21	Day 28	Day 34
0 g/kg bromelain							
Standard CP	- AGP	42.00 (±0.35) ^{a}	169.7 (±3.29) ^{ad}	459.9 (±8.58) ^{ab}	941.1 (±13.74) ^{ab}	1707 (±20.49) ^{abc}	2392 (±30.50) ^{ab}
	+ AGP	41.00(±0.35) ^{bc}	172.6 (±3.29) ^{ad}	467.6 (±8.58) ^{a}	957.5 (±13.74) ^{a}	1691 (±20.48) ^{ace}	2349 (±30.50) ^{ab}
High CP	- AGP	41.23 (±0.35) ^{ac}	173.7 (±3.29) ^{ae}	477.1 (±8.58) ^{a}	972.7 (±13.74) ^{a}	1751 (±20.48) ^{bd}	2407 (±30.50) ^{a}
	+ AGP	41.33 (±0.35) ^{ac}	169.9 (±3.28) ^{abc}	460.7 (±8.58) ^{ab}	970.9 (±13.74) ^{a}	1744 (±20.48) ^{bde}	2395 (±30.50) ^{ab}
0.125 g/kg bromelain							
Standard CP	- AGP	41.62 (±0.35) ^{ab}	171.0 (±3.28) ^{ad}	461.3 (±8.58) ^{ab}	954.8 (±13.74) ^{a}	1700 (±20.48) ^{acd}	2358 (±30.50) ^{ab}
	+ AGP	40.69 (±0.35) ^{c}	161.4 (±3.28) ^{bc}	439.3 (±8.58) ^{b}	903.0 (±13.74) ^{b}	1655 (±20.48) ^{c}	2319 (±30.50) ^{bc}
High CP	- AGP	41.28 (±0.35) ^{ac}	172.2 (±3.28) ^{ae}	468.8 (±8.58) ^{a}	968.6 (±13.74) ^{a}	1750 (±20.48) ^{bd}	2401 (±30.50) ^{ab}
	+ AGP	41.58 (±0.35) ^{ac}	165.4 (±3.29) ^{cde}	457.2 (±8.59) ^{ab}	970.3 (±13.76) ^{a}	1742 (±20.51) ^{bde}	2325 (±30.54) ^{ab}
0.75 g/kg bromelain							
Standard CP	- AGP	41.11 (±0.35) ^{abc}	175.2 (±3.29) ^{a}	475.5 (±8.59) ^{a}	956.0 (±13.76) ^{a}	1720 (±20.51) ^{ab}	2363 (±30.54) ^{ab}
	+ AGP	40.64 (±0.35) ^{c}	165.6 (±3.29) ^{bde}	453.9 (±8.59) ^{ab}	934.1 (±13.76) ^{ab}	1682 (±20.51) ^{ac}	2378 (±30.50) ^{ac}
High CP	- AGP	41.10 (±0.35) ^{ac}	168.8 (±3.28) ^{abc}	471.3 (±8.58) ^{a}	967.8 (±13.74) ^{a}	1733 (±20.48) ^{ab}	2402 (±30.50) ^{ab}
	+ AGP	41.30 (±0.35) ^{ac}	166.3 (±3.29) ^{abc}	459.9 (±8.59) ^{ab}	965.3 (±13.76) ^{a}	1763 (±20.51) ^{b}	2385 (±30.54) ^{ab}

$^{a–e}$ Mean values within a column without a common superscript letter differ significantly (P<0.05)

Table 20 Sources of variation (P-values) in weekly body weight of broilers that received feed with different levels of crude protein (CP) and bromelain, with and without antibiotic growth promoter (AGP) (± standard error of the mean)

	***P*-values**					
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 34**
Main effects						
CP	0.519	0.937	0.214	0.001	<0.0001	0.150
AGP	0.090	0.012	0.013	0.211	0.237	0.109
Bromelain	0.321	0.231	0.227	0.505	0.625	0.213
Interaction effects						
CP*AGP	0.012	0.783	0.907	0.261	0.119	0.732
CP*Bromelain	0.369	0.499	0.619	0.548	0.698	0.985
AGP*Bromelain	0.801	0.237	0.512	0.257	0.746	0.421
CP*AGP*Bromelain	0.817	0.318	0.269	0.189	0.599	0.680

Table 21 The main effect of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on the weekly mean weight gain (g) of broilers (± standard error of the mean)

Treatment	Days 0–7	Days 7–14	Days 14–21	Days 21–28	Days 28–34
CP					
Standard	128.1 (±1.35)	291.7 (±2.82)	481.5 (±3.92) [a]	751.9 (±5.69) [a]	667.0 (±9.75)
High	128.1 (±1.35)	296.3 (±2.82)	503.4 (±3.92) [b]	778.2 (±5.69) [b]	638.2 (±9.75)
AGP					
Without	130.4 (±1.35) [a]	297.0 (±2.81)	491.1 (±3.91)	767.1 (±5.67)	659.8 (±9.72)
With	125.8 (±1.35) [b]	291.0 (±2.81)	493.8 (±3.90)	763.0 (±5.67)	645.4 (±9.72)
Bromelain (g/kg)					
0	130.1 (±1.65)	294.3 (±3.44)	494.2 (±4.78)	762.9 (±6.94)	662.4 (±11.90)
0.125	126.3 (±1.66)	289.2 (±3.46)	492.5 (±4.80)	763.0 (±6.98)	638.6 (±11.96)
0.75	127.9 (±1.65)	298.6 (±3.45)	490.7 (±4.78)	769.3 (±6.95)	656.8 (±11.91)

[a,b] Mean values within a column without a common superscript differ significantly ($P<0.05$)

Table 22 The interaction effects of crude protein (CP) concentration, bromelain supplementation and inclusion of an antibiotic growth promoter (AGP) on the mean weekly weight gain (g) of broilers (± standard error of the mean)

Treatment		Days 0–7	Days 7–14	Days 14–21	Days 21–28	Days 28–34
0 g/kg bromelain						
Standard CP	- AGP	127.6 (±3.31) ^{abc}	291.1 (±6.92) ^{ab}	481.1 (±9.61) ^{ac}	766.1 (±13.96) ^{abc}	684.9 (±23.92) ^{ad}
	+ AGP	131.6 (±3.31) ^{ac}	293.3 (±9.92) ^{ab}	489.9 (±9.61) ^{abc}	733.9 (±13.96) ^{c}	658.1 (±23.92) ^{ac}
High CP	- AGP	132.5 (±3.31) ^{ac}	303.6 (±6.92) ^{a}	495.6 (±9.61) ^{ab}	778.5 (±13.96) ^{ab}	655.4 (±23.92) ^{ac}
	+ AGP	128.7 (±3.31) ^{abc}	289.3 (±6.92) ^{ab}	510.2 (±9.61) ^{b}	773.2 (±13.96) ^{ab}	651.1 (±23.92) ^{ac}
0.125 g/kg bromelain						
Standard CP	- AGP	129.4 (±3.31) ^{abc}	289.0 (±6.92) ^{ab}	493.4 (±9.61) ^{ab}	745.4 (±13.97) ^{ac}	658.2 (±23.92) ^{ad}
	+ AGP	120.9 (±3.31) ^{b}	278.2 (±6.92) ^{b}	463.7 (±9.61) ^{c}	752.5 (±13.96) ^{ac}	663.3 (±23.92) ^{ac}
High CP	- AGP	131.0 (±3.31) ^{ac}	296.9 (±6.92) ^{ab}	499.8 (±9.61) ^{ab}	781.8 (±13.96) ^{ab}	650.4 (±23.91) ^{ac}
	+ AGP	123.9 (±3.32) ^{bc}	292.5 (±6.93) ^{ab}	513.1 (±9.62) ^{b}	772.4 (±13.97) ^{ab}	582.4 (±23.95) ^{b}
0.75 g/kg bromelain						
Standard CP	- AGP	134.1 (±3.32) ^{a}	300.4 (±6.93) ^{a}	480.5 (±9.62) ^{ac}	765.0 (±13.97) ^{abc}	641.9 (±23.95) ^{abc}
	+AGP	125.0 (±3.31) ^{abc}	298.4 (±6.93) ^{a}	480.2 (±9.62) ^{ac}	748.6 (±13.97) ^{ac}	695.4 (±23.95) ^{cd}
High CP	- AGP	127.6 (±3.31) ^{abc}	301.2 (±6.92) ^{a}	496.5 (±9.61) ^{ab}	765.9 (±13.96) ^{ab}	667.9 (±23.92) ^{ac}
	+ AGP	125.0 (±3.32) ^{abc}	294.4 (±6.93) ^{ab}	505.4 (±9.62) ^{ab}	797.7 (±13.97) ^{b}	621.9 (±23.95) ^{ab}

$^{a-d}$ Values within a column without a common superscript letter differ significantly (P<0.05)

Table 23 Sources of variation (P-values) in weekly weight gain of broilers that received feed with different levels of crude protein (CP) and bromelain, with and without the inclusion of an antibiotic growth promoter (AGP) (± standard error of the mean)

	***P*-values**				
	Days 0–7	**Days 7–14**	**Days 14–21**	**Days 21–28**	**Days 28–34**
Main effects					
CP	0.992	0.255	0.0002	0.002	0.041
AGP	0.020	0.136	0.637	0.613	0.298
Bromelain	0.273	0.162	0.869	0.760	0.346
Interaction effects					
CP*AGP	0.998	0.543	0.089	0.234	0.078
CP*Bromelain	0.475	0.434	0.734	0.986	0.720
AGP*Bromelain	0.227	0.946	0.345	0.403	0.582
CP*AGP*Bromelain	0.312	0.505	0.320	0.252	0.171

Table 24 The main effect of crude protein (CP) concentration, bromelain supplementation and inclusion of an antibiotic growth promoter (AGP) on the weekly mean feed intake (g) of broilers (± standard error of the mean)

Treatment	Days 0–7	Days 7–14	Days 14–21	Days 21–28	Days 28–34
Crude Protein					
Standard	177.6 (±1.94) [a]	357.0 (±3.72)	733.5 (±8.32) [a]	984.4 (±7.04)	1445 (±13.49)
High	185.0 (±1.94) [b]	365.5 (±3.72)	765.7 (±8.32) [b]	975.4 (±7.04)	1426 (±13.48)
AGP					
Without	179.1 (±1.94)	361.4 (±3.71)	749.0 (±8.30)	988.7 (±7.02)	1457 (±13.45) [a]
With	183.5 (±1.94)	361.1 (±3.70)	750.1 (±8.29)	971.1 (±7.02)	1414 (±13.44) [b]
Bromelain (g/kg)					
0	181.3 (±2.37) [b]	354.4 (±4.54)	742.7 (±10.16)	976.3 (±8.59)	1434 (±16.46)
0.125	185.5 (±2.38) [b]	365.4 (±4.56)	748.2 (±10.21)	980.5 (±8.63)	1442 (±16.54)
0.75	177.1 (±2.37) [a]	363.9 (±4.54)	757.7 (±10.16)	982.9 (±8.60)	1430 (±16.47)

[a-b] Mean values within a column without a common superscript differ significantly (P<0.05)

Table 25 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on the weekly mean feed intake (g) of broilers (± standard error of the mean)

Treatment		Days 0–7	Days 7–14	14–21	21–28	28–34
0 g/kg bromelain						
Standard CP	- AGP	174.8 (±4.76) ^{bc}	356.1 (±9.12) ^{bc}	748.7 (±20.41) ^{abcd}	1003 (±17.27)	1483 (±33.08) ^{ac}
	+ AGP	174.5 (±4.76) ^{c}	344.7 (±9.12) ^{b}	732.6 (±20.42) ^{abc}	957.2 (±17.27)	1430 (±33.08) ^{abc}
High CP	- AGP	189.7 (±4.76) ^{ad}	358.2 (±9.12) ^{bc}	728.1 (±20.42) ^{bd}	987.7 (±17.27)	1474 (±33.08) ^{ad}
	+ AGP	186.2 (±4.76) ^{abd}	358.5 (±9.12) ^{bc}	761.6 (±20.41) ^{abcd}	956.9 (±17.27)	1349 (±33.08) ^{be}
0.125 g/kg bromelain						
Standard CP	- AGP	176.6 (±4.76) ^{abcd}	361.0 (±9.12) ^{abc}	749.4 (±20.41) ^{abcd}	988.5 (±17.27)	1428 (±33.08) ^{abc}
	+ AGP	188.9 (±4.76) ^{a}	368.3 (±9.12) ^{abc}	714.9 (±20.41) ^{bc}	967.2 (±17.27)	1407 (±33.08) ^{abc}
High CP	- AGP	188.2 (±4.76) ^{abd}	372.4 (±9.12) ^{ac}	747.9 (±20.41) ^{abcd}	990.8 (±17.28)	1496 (±33.07) ^{cd}
	+ AGP	188.5 (±4.76) ^{a}	359.9 (±9.13) ^{bcd}	780.5 (±20.44) ^{abcd}	975.3 (±17.29)	1435 (±33.12) ^{ace}
0.75 g/kg bromelain						
Standard CP	- AGP	176.6 (±4.76) ^{abc}	361.9 (±9.13) ^{abc}	730.4 (±20.44) ^{bd}	1002 (±17.29)	1456 (±33.12) ^{ad}
	+ AGP	174.1 (±4.76) ^{c}	350.2 (±9.13) ^{bc}	724.8 (±20.44) ^{bd}	987.3 (±17.29)	1460 (±33.12) ^{ad}
High CP	- AGP	168.5 (±4.76) ^{c}	358.9 (±9.12) ^{abc}	789.4 (±20.41) ^{ac}	959.2 (±17.27)	1402 (±33.08) ^{ab}
	+ AGP	189.0 (±4.76) ^{a}	384.7 (±11.13) ^{ad}	786.4 (±20.44) ^{d}	982.5 (±17.29)	1399 (±33.12) ^{ab}

$^{a-d}$ Mean values within a column without a common superscript differ significantly (P<0.05)

Table 26 Sources of variation (P-values) in weekly feed intake (g) of broilers based on different levels of crude protein (CP) and bromelain, with or without an antibiotic growth promoter (AGP) (± standard error of the mean)

	P-value				
	Days 0–7	**Days 7–14**	**Days 14–21**	**Days 21–28**	**Days 28–34**
Main effects					
CP	0.009	0.116	0.008	0.373	0.342
AGP	0.110	0.945	0.921	0.080	0.027
Bromelain	0.048	0.187	0.574	0.859	0.872
Interaction effects					
CP*AGP	0.643	0.363	0.100	0.325	0.301
CP*Bromelain	0.315	0.547	0.163	0.492	0.056
AGP*Bromelain	0.253	0.599	0.898	0.234	0.170
CP*AGP*Bromelain	0.029	0.092	0.514	0.783	0.781

Table 27 The main effect of crude protein (CP) concentration, bromelain supplementation, and antibiotic growth promoter (AGP) on the weekly mean feed conversion ratios (FCRs) of broilers (± standard error of the mean)

Treatment	**Days 0–7**	**Days 7–14**	**Days 14–21**	**Days 21–28**	**Days 28–34**
Crude protein					
Standard	1.40 (±0.02)	1.25 (±0.02)	1.52 (±0.02)	1.30 (±0.01) [a]	2.17 (±0.03)
High	1.45 (±0.02)	1.23 (±0.02)	1.51 (±0.02)	1.26 (±0.01) [b]	2.23 (±0.03)
AGP					
Without	1.38 (±0.02) [a]	1.21 (±0.02) [a]	1.51 (±0.02)	1.30 (±0.01) [a]	2.22 (±0.03)
With	1.47 (±0.02) [b]	1.27 (±0.02) [b]	1.53 (±0.02)	1.27 (±0.01) [a]	2.18 (±0.03)
Bromelain (g/kg)					
0	1.40 (±0.02) [a]	1.21 (±0.02) [a]	1.51 (±0.02)	1.30 (±0.01)	2.15 (±0.04)
0.125	1.48 (±0.02) [b]	1.27 (±0.02) [b]	1.53 (±0.02)	1.28 (±0.01)	2.26 (±0.04)
0.75	1.39 (±0.02) [a]	1.23 (±0.02) [ab]	1.52 (±0.02)	1.28 (±0.01)	2.19 (±0.04)

[a, b] Mean values within a column without a common superscript letter differ significantly ($P<0.05$)

Table 28 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on the weekly mean feed conversion ratios (FCR) of broilers (± standard error of the mean)

Treatment		Days 0–7	Days 7–14	Days 14–21	Days 21–28	Days 28–34
0 g/kg bromelain						
Standard CP	- AGP	1.36 (±0.05) ^{cd}	1.22 (±0.04) ^{bc}	1.52 (±0.04)	1.32 (±0.02) ^{acd}	2.20 (±0.08) ^{bc}
	+ AGP	1.33 (±0.05) ^{a}	1.18 (±0.04) ^{bc}	1.52 (±0.04)	1.30 (±0.02) ^{abc}	2.17 (±0.08) ^{bc}
High CP	- AGP	1.44 (±0.05) ^{bcd}	1.19 (±0.04) ^{c}	1.45 (±0.04)	1.33 (±0.02) ^{c}	2.15 (±0.08) ^{bc}
	+ AGP	1.45 (±0.05) ^{bcd}	1.26 (±0.04) ^{c}	1.54 (±0.04)	1.24 (±0.02) ^{b}	2.08 (±0.08) ^{b}
0.125 g/kg bromelain						
Standard CP	- AGP	1.36 (±0.05) ^{cd}	1.23 (±0.04) ^{bc}	1.51 (±0.04)	1.32 (±0.02) ^{ace}	2.16 (±0.08) ^{bc}
	+ AGP	1.60 (±0.05) ^{bc}	1.40 (±0.04) ^{a}	1.57 (±0.04)	1.29 (±0.02) ^{abc}	2.10 (±0.08) ^{b}
High CP	- AGP	1.44 (±0.05) ^{bcd}	1.23 (±0.04) ^{c}	1.53 (±0.04)	1.26 (±0.02) ^{bde}	2.42 (±0.08) ^{a}
	+ AGP	1.52 (±0.05) ^{ab}	1.24 (±0.04) ^{c}	1.49 (±0.04)	1.24 (±0.02) ^{b}	2.35 (±0.08) ^{ac}
0.75 g/kg bromelain						
Standard CP	- AGP	1.32 (±0.05) ^{d}	1.18 (±0.04) ^{bc}	1.50 (±0.04)	1.29 (±0.02) ^{abc}	2.25 (±0.08) ^{abc}
	+ AGP	1.40 (±0.05) ^{bcd}	1.26 (±0.04) ^{c}	1.53 (±0.04)	1.30 (±0.02) ^{abc}	2.12 (±0.08) ^{b}
High CP	- AGP	1.33 (±0.05) ^{d}	1.18 (±0.04) ^{c}	1.53 (±0.04)	1.26 (±0.02) ^{abc}	2.13 (±0.08) ^{b}
	+ AGP	1.49 (±0.05) ^{ac}	1.28 (±0.04) ^{abc}	1.52 (±0.04)	1.26 (±0.02) ^{bde}	2.27 (±0.08) ^{abc}

$^{a-e}$ Mean values within a column without a common superscript differ significantly (P<0.05)

Table 29 Sources of variation (P-values) in weekly feed conversion ratio (FCR) of broilers that received different levels of crude protein (CP) and bromelain, with and without an antibiotic growth promoter (AGP) (± standard error of the mean)

	***P*-values**				
	Days 0–7	**Days 7–14**	**Days 14–21**	**Days 21–28**	**Days 28–34**
Main effects					
CP	0.077	0.511	0.547	0.01	0.157
AGP	0.002	0.012	0.423	0.089	0.434
Bromelain	0.015	0.106	0.837	0.445	0.149
Interaction effects					
CP*AGP	0.870	0.893	0.661	0.453	0.446
CP*Bromelain	0.373	0.208	0.746	0.764	0.011
AGP*Bromelain	0.044	0.399	0.781	0.307	0.804
CP*AGP*Bromelain	0.178	0.078	0.235	0.396	0.288

Cumulative performance

The main and interaction effects of CP level, bromelain supplementation, and inclusion of an AGP on cumulative weight gain, feed intake, and FCR, along with the sources of variation of each category are shown in Tables 30–38.

For the first 14 d, the cumulative weight gain of the birds was significantly lower (P<0.05) with the inclusion of an AGP compared to without an AGP. From days 0-21 and 0-28, birds that received a standard level of CP experienced significantly less (P<0.05) weight gain than those that received a high level of CP (table 30).

The birds given a combination of 0.125 g/kg bromelain, a high CP level, and the inclusion of an AGP had significantly lower (P<0.05) cumulative body weight gain than birds that received a combination of 0.75 g/kg bromelain, a standard amount of CP and no inclusion of an AGP (table 31).

No significant interaction effects were noted between CP, bromelain and AGP inclusions. However, CP had a significant effect on weight gain in weeks 3 and 4. AGP was also seen to have a significant effect on weight gain in weeks 2 and 3 (table 32).

The feed intake of birds in days 0-7 and 0-14 was significantly lower (P<0.05) when given a standard level of CP, as opposed to a high level of CP. In days 0-28 and 0-34, cumulative feed intake without an AGP was significantly higher (P<0.05) than with an AGP (table 33).

In days 0-21, birds that received a combination of 0.75 g/kg bromelain, a high amount of CP and the inclusion of an AGP had significantly higher (P<0.05) feed intake than the birds that received the following three treatment diets: 0.75 g/kg bromelain, a standard amount of CP and the inclusion of an AGP; 0 g/kg bromelain, a standard amount of CP and no AGP, and 0 g/kg bromelain, a high amount of CP and no AGP (table 34).

A significant main effect of CP was seen on feed intake of broilers in weeks 1 and 2. A similar significant main effect of AGP was seen in weeks 4 and 5. A significant interaction effect was seen in weeks 1 and 2 between CP, AGP and bromelain on feed intake (table 35).

From day 0-7, 0-14 and 0-21, birds given a diet including an AGP had significantly higher (P<0.05) FCR than birds given a diet excluding an AGP. Moreover, birds given bromelain at 0.125 g/kg had a significantly higher (P<0.05) FCR, compared to birds given bromelain levels of 0 g/kg and 0.75 g/kg on days 0-7, 0-14 and 0-34 (table 36).

From days 0 to 14, birds that received a combination of 0.125 g/kg bromelain, a standard amount of CP and the inclusion of an AGP had significantly higher (P<0.05) cumulative feed intakes (table 37).

Crude protein had a significant effect on FCR in weeks 3 and 4. AGP and bromelain had significant main effects on FCR in weeks 1 and 5. Crude protein and bromelain had a significant interaction effect on FCR in week 2. AGP and bromelain had a significant interaction effect in weeks 1 and 5 (table 38).

Table 30 The main effect of crude protein (CP) concentration, bromelain supplementation, and inclusion of an antibiotic growth promoter (AGP) on the mean cumulative weight gain (g) of broilers (± standard error of the mean)

Treatment	Days 0–7	Days 0–14	Days 0–21	Days 0–28	Days 0–34
Crude protein					
Standard	128.1 (±1.35)	417.9 (±3.47)	899.9 (±5.90) [a]	1652 (±8.35) [a]	2319 (±12.44)
High	128.1 (±1.35)	424.3 (±3.47)	927.9 (±5.90) [b]	1706 (±8.35) [b]	2344 (±12.43)
AGP					
Without	130.4 (±1.35) [a]	427.2 (±3.46) [a]	918.8 (±5.57)	1686 (±8.33)	2346 (±12.40)
With	125.8 (±1.35) [b]	415.1 (±3.46) [b]	909.1 (±5.57)	1672 (±8.32)	2318 (±12.40)
Bromelain (g/kg)					
0	130.1 (±1.65)	424.5 (±4.23)	919.1 (±6.82)	1682 (±10.2)	2344 (±15.18)
0.125	126.3 (±1.65)	415.1 (±4.25)	907.9 (±6.85)	1671 (±10.24)	2310 (±15.25)
0.75	127.9 (±1.65)	423.8 (±4.24)	914.8 (±6.83)	1684 (±10.12)	2341 (±15.19)

[a, b] Mean values within a column without a common superscript differ significantly ($P<0.05$)

Table 31 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on the mean cumulative weight gain (g) of broilers (± standard error of the mean)

Treatment		Days 0–7	Days 0–14	Days 0–21	Days 0–28	Days 0–34
0 g/kg bromelain						
Standard CP	- AGP	127.6 (±3.31) ^{ab}	417.3 (±8.51) ^{abc}	899.0 (±13.71) ^{ab}	1665 (±20.49) ^{abcd}	2350(±30.50) ^{ab}
	+ AGP	131.6 (±3.31) ^{ad}	425.9 (±8.51) ^{c}	916.5 (±13.71) ^{a}	1650 (±20.48) ^{bc}	2308 (±30.50) ^{ab}
High CP	- AGP	132.5 (±3.31) ^{ad}	435.5 (±8.51) ^{ac}	931.4 (±13.71) ^{a}	1710 (±20.50) ^{acb}	2365 (±30.50) ^{a}
	+ AGP	128.7 (±3.31) ^{ab}	419.4 (±8.51) ^{abc}	929.6 (±13.71) ^{a}	1703 (±20.48) ^{bf}	2354 (±30.50) ^{ab}
0.125 g/kg bromelain						
Standard CP	- AGP	129.4 (±3.31) ^{ab}	419.3 (±8.51) ^{abc}	913.1 (±13.71) ^{a}	1659 (±20.48) ^{bcd}	2317 (±30.50) ^{ab}
	+ AGP	120.9 (±3.31) ^{b}	398.1 (±8.51) ^{b}	862.4 (±13.71) ^{b}	1615 (±20.48) ^{c}	2278 (±30.50) ^{b}
High CP	- AGP	131.0 (±3.31) ^{ad}	427.1 (±8.51) ^{ac}	927.4 (±13.71) ^{a}	1709 (±20.48) ^{df}	2360 (±30.50) ^{ab}
	+ AGP	123.9 (±3.32) ^{bd}	415.6 (±8.51) ^{abc}	928.8 (±13.73) ^{a}	1701 (±20.50) ^{bf}	2284 (±30.54 ^{ab}
0.75 g/kg bromelain						
Standard CP	- AGP	134.1 (±3.32) ^{a}	434.3 (±8.52) ^{ac}	914.9 (±13.72) ^{a}	1680 (±20.5) ^{abde}	2322 (±30.54) ^{ab}
	+ AGP	125.0 (±3.32) ^{ab}	413.1 (±8.51) ^{abc}	893.6 (±13.71) ^{ab}	1643 (±20.48) ^{ce}	2338 (±30.54) ^{ab}
High CP	- AGP	127.6 (±3.31) ^{ab}	429.7 (±8.51) ^{ac}	926.7 (±13.71) ^{a}	1693 (±20.48) ^{bfe}	2361 (±30.50) ^{ab}
	+ AGP	124.9 (±3.32) ^{ab}	418.3 (±8.51) ^{abc}	923.9 (±13.73) ^{a}	1722 (±20.50) ^{af}	2344 (±30.54) ^{ab}

$^{a-f}$ Mean values within a column without a common superscript letter differ significantly (P<0.05)

Table 32 Sources of variation (P-values) in cumulative weight gain (g) of broilers that received feed with different levels of crude protein (CP) and bromelain, with and without the inclusion of an antibiotic growth promoter (AGP) (± standard error of the mean)

	***P*-values**				
	Days 0–7	**Days 0–14**	**Days 0–21**	**Days 0–28**	**Days 0–34**
Main effects					
CP	0.992	0.204	0.001	<.0001	0.152
AGP	0.020	0.016	0.227	0.249	0.114
Bromelain	0.273	0.220	0.511	0.622	0.213
Interaction effects					
CP*AGP	0.998	0.861	0.289	0.130	0.710
CP*Bromelain	0.475	0.593	0.554	0.702	0.982
AGP*Bromelain	0.227	0.493	0.253	0.749	0.423
CP*AGP*Bromelain	0.312	0.261	0.120	0.595	0.680

Table 33 The main effect of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on the mean cumulative feed intake (g) of broilers (± standard error of the mean)

Treatment	**Days 0–7**	**Days 0–14**	**Days 0–21**	**Days 0–28**	**Days 0–34**
Crude protein					
Standard	177.6 (±1.94) [a]	536.1 (±3.33) [a]	1181 (±9.80)	2200 (±15.80)	3684 (±24.67)
High	185.0 (±1.94) [b]	548.2 (±3.33) [b]	1205 (±9.80)	2175 (±15.80)	3657 (±24.67)
AGP					
Without	179.1 (±1.94)	542.1 (±3.33)	1189 (±9.75)	2214 (±15.76) [a]	3731 (±24.60) [a]
With	183.5 (±1.93)	542.1 (±3.32)	1197 (±11.94)	2161 (±15.74) [b]	3610 (±24.60) [b]
Bromelain (g/kg)					
0	181.3 (±2.37) [ab]	538.8 (±4.07)	1182 (±12.00)	2191 (±19.29)	3659 (±30.11)
0.125	185.5 (±2.38) [a]	547.8 (±4.09)	1203 (±11.95)	2194 (±19.38)	3664 (±30.25)
0.75	177.1 (±2.37) [b]	539.7 (±4.09)	1194 (±11.95)	2177 (±19.30)	3687 (±30.13)

[a, b] Mean values within a column without a common superscript letter differ significantly ($P<0.05$)

Table 34 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on the mean cumulative feed intake (g) of broilers (± standard error of the mean)

Treatment		Days 0–7	Days 0–14	Days 0–21	Days 0–28	Days 0–34
0 g/kg bromelain						
Standard CP	- AGP	174.8 (±4.76) ^{bc}	538.8 (±8.17) ^{bd}	1151 (±24.00) ^{b}	2237 (±38.76)	3812 (±60.51) ^{a}
	+ AGP	174.5 (±4.76) ^{bd}	538.8 (±8.19) ^{b}	1200 (±24.00) ^{ab}	2159 (±38.76)	3599 (±60.51) ^{bc}
High CP	- AGP	189.7 (±4.76) ^{a}	538.8 (±8.19) ^{bd}	1159 (±24.00) ^{b}	2218 (±38.76)	3717 (±60.51) ^{c}
	+ AGP	186.2 (±4.76) ^{acd}	538.8 (±8.18) ^{abd}	1217 (±24.00) ^{ab}	2149 (±38.76)	3509 (±60.51) ^{b}
0.125 g/kg bromelain						
Standard CP	- AGP	176.6 (±4.76) ^{ab}	547.8 (±8.17) ^{bd}	1217 (±24.00) ^{ab}	2224 (±38.76)	3659 (±60.51) ^{abc}
	+ AGP	188.9 (±4.76) ^{a}	547.8 (±8.18) ^{abd}	1185 (±24.00) ^{ab}	2159 (±38.76)	3577 (±60.51) ^{bc}
High CP	- AGP	188.2 (±4.76) ^{ac}	547.8 (±8.18) ^{a}	1215 (±24.00) ^{ab}	2238 (±38.76)	3801 (±60.51) ^{ad}
	+ AGP	188.5 (±4.77) ^{a}	547.8 (±8.19) ^{abd}	1197 (±24.03) ^{ab}	2156 (±38.76)	3620 (±60.59) ^{bce}
0.75 g/kg bromelain						
Standard CP	- AGP	176.6 (±4.76) ^{ab}	539.7 (±8.18) ^{bd}	1184 (±24.03) ^{ab}	2216 (±38.80)	3702 (±60.58) ^{ac}
	+ AGP	174.1 (±4.76) ^{bd}	539.7 (±8.18) ^{b}	1151 (±24.03) ^{b}	2206 (±38.80)	3754 (±60.58) ^{ace}
High CP	- AGP	168.5 (±4.76) ^{b}	539.7 (±8.18) ^{b}	1212 (±24.00) ^{ab}	2151 (±38.76))	3695 (±60.51) ^{de}
	+ AGP	189.0 (±4.77) ^{a}	539.7 (±8.19) ^{ad}	1233 (±24.03) ^{a}	2136 (±38.76)	3598 (±60.59) ^{bce}

$^{a-e}$ Mean values within a column without a common superscript letter differ significantly (P<0.05)

Table 35 Sources of variation (P-values) in cumulative feed intake of broilers that received different levels of crude protein (CP) and bromelain, with and without an antibiotic growth promoter (AGP) (± standard error of the mean)

	***P*-value**				
	Days 0–7	**Days 0–14**	**Days 0–21**	**Days 0–28**	**Days 0–34**
Main effects					
CP	0.009	0.012	0.088	0.260	0.442
AGP	0.110	0.988	0.578	0.019	0.001
Bromelain	0.048	0.240	0.435	0.803	0.783
Interaction effects					
CP*AGP	0.643	0.343	0.364	0.924	0.251
CP*Bromelain	0.315	0.826	0.293	0.398	0.061
AGP*Bromelain	0.253	0.473	0.066	0.438	0.097
CP*AGP*Bromelain	0.029	0.022	0.767	0.974	0.652

Table 36 The main effect of crude protein (CP) concentration, bromelain supplementation, and inclusion of an antibiotic growth promoter (AGP) on the mean cumulative feed conversion ratio (FCR) of broilers (± standard error of the mean)

Treatment	**Days 0–7**	**Days 0–14**	**Days 0–21**	**Days 0–28**	**Days 0–34**
Crude protein					
Standard	1.39 (±0.02)	1.28 (±0.01)	1.31 (±0.01)	1.32 (±0.01) [a]	1.57 (±0.01) [a]
High	1.45 (±0.02)	1.29 (±0.01)	1.30 (±0.01)	1.26 (±0.01) [b]	1.53 (±0.01) [b]
AGP					
Without	1.37 (±0.02) [a]	1.27 (±0.01) [a]	1.29 (±0.01) [a]	1.30 (±0.01)	1.56 (±0.01)
With	1.47 (±0.02) [b]	1.30 (±0.01) [b]	1.32 (±0.01) [b]	1.29 (±0.01)	1.54 (±0.01)
Bromelain (g/kg)					
0	1.40 (±0.24) [a]	1.27 (±0.01) [a]	1.29 (±0.01) [a]	1.29 (±0.01)	1.54 (±0.01) [b]
0.125	1.48 (±0.24) [b]	1.32 (±0.01) [b]	1.33 (±0.01) [b]	1.30 (±0.01)	1.57 (±0.01) [a]
0.75	1.38 (±0.24) [a]	1.27 (±0.01) [a]	1.30 (±0.01) [ab]	1.28 (±0.01)	1.54 (±0.01) [b]

[a,b] Mean values within a column without a common superscript differ significantly ($P<0.05$)

Table 37 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and inclusion of an antibiotic growth promoter (AGP) on the mean cumulative feed conversion ratios (FCR) of broilers (± standard error of the mean)

Treatment		Days 0–7	Days 0–14	Days 0–21	Days 0–28	Days 0–34
0 g/kg bromelain						
Standard CP	- AGP	1.36 (±0.05) ^{bc}	1.28 (±0.03) ^{bcd}	1.28 (±0.02) ^{bcd}	1.31 (±0.02) ^{acd}	1.58 (±0.03) ^{a}
	+ AGP	1.33 (±0.05) ^{b}	1.20 (±0.03) ^{d}	1.31 (±0.02) ^{bc}	1.31 (±0.02) ^{ac}	1.56 (±0.03) ^{ab}
High CP	- AGP	1.44 (±0.05) ^{bce}	1.24 (±0.03) ^{cd}	1.25 (±0.02) ^{d}	1.28 (±0.02) ^{abc}	1.54 (±0.03) ^{ab}
	+ AGP	1.45 (±0.05) ^{bce}	1.31 (±0.03) ^{abc}	1.31 (±0.02) ^{bc}	1.25 (±0.02) ^{b}	1.49 (±0.03) ^{b}
0.125 g/kg bromelain						
Standard CP	- AGP	1.36 (±0.05) ^{bc}	1.28 (±0.03) ^{bcd}	1.33 (±0.02) ^{ce}	1.33 (±0.02) ^{a}	1.57 (±0.03) ^{ad}
	+ AGP	1.60 (±0.05) ^{ad}	1.38 (±0.03) ^{a}	1.38 (±0.02) ^{ae}	1.33 (±0.02) ^{a}	1.56 (±0.03) ^{ac}
High CP	- AGP	1.44 (±0.05) ^{bce}	1.32 (±0.03) ^{ab}	1.31 (±0.02) ^{bc}	1.29 (±0.02) ^{abc}	1.60 (±0.03) ^{a}
	+ AGP	1.52 (±0.05) ^{ae}	1.30 (±0.03) ^{abcd}	1.29 (±0.02) ^{bcd}	1.26 (±0.02) ^{bc}	1.55 (±0.03) ^{ab}
0.75 g/kg bromelain						
Standard CP	- AGP	1.32 (±0.05) ^{b}	1.23 (±0.03) ^{cd}	1.29 (±0.02) ^{bcd}	1.30 (±0.02) ^{abc}	1.58 (±0.03) ^{ad}
	+ AGP	1.40 (±0.05) ^{bce}	1.27 (±0.03) ^{bcd}	1.29 (±0.02) ^{bcd}	1.33 (±0.02) ^{a}	1.57 (±0.03) ^{ac}
High CP	- AGP	1.33 (±0.05) ^{b}	1.24 (±0.03) ^{cd}	1.31 (±0.02) ^{bc}	1.27 (±0.02) ^{bc}	1.50 (±0.03) ^{bc}
	+ AGP	1.49 (±0.05) ^{cde}	1.33 (±0.03) ^{ab}	1.33 (±0.02) ^{bc}	1.24 (±0.02) ^{b}	1.50 (±0.03) ^{bcd}

$^{a-e}$ Values within a column without a common superscript letter differ significantly (P<0.05)

Table 38 Sources of variation (P-values) in cumulative feed conversion ratio (FCR) of broilers that received different levels of crude protein (CP) and bromelain, with and without an antibiotic growth promoter (AGP) (± standard error of the mean)

	***P*-value**				
	Days 0–7	**Days 0–14**	**Days 0–21**	**Days 0–28**	**Days 0–34**
Main effects					
CP	0.069	0.266	<0.0001	0.010	0.069
AGP	0.002	0.082	0.404	0.136	0.002
Bromelain	0.013	0.031	0.358	0.137	0.013
Interaction effects					
CP*AGP	0.903	0.982	0.105	0.616	0.903
CP*Bromelain	0.354	0.037	0.861	0.108	0.354
AGP*Bromelain	0.045	0.370	0.827	0.632	0.045
CP*AGP*Bromelain	0.152	0.219	0.869	0.765	0.152

Performance of broilers during the different phases

The main and interaction effects of CP, bromelain supplementation, and inclusion of an AGP on weight gain, feed intake, and FCR, along with the sources of variation of each category during three phases of broiler rearing (starter, grower, and finisher) are shown in Tables 39–47.

Birds given a standard CP level in the grower phase exhibited significantly less (P<0.05) weight gain than those given a high CP level (table 39).

In the grower phase, within the 0.125 g/kg bromelain treatments specifically, birds given a high amount of CP and no AGP had significantly higher (P<0.05) weight gain than birds given a standard amount of CP and an AGP (table 40).

Crude protein had a significant effect on weight gain in the grower and finisher phases (table 41).

In the starter phase, birds that were given a standard CP level had a significantly lower (P<0.05) feed intake than birds given a high level of CP. In the finisher phase, birds given an AGP had a significantly lower (P<0.05) feed intake than birds not given an AGP (table 42).

In the grower phase, birds given 0.75 g/kg bromelain, a high amount of CP and an AGP had significantly higher (P<0.05) FIs than birds that received the following three treatments: 0.75 g/kg bromelain, a standard amount of CP and the inclusion of an AGP; 0.125 g/kg bromelain, a standard amount of CP and the inclusion of an AGP and 0 g/kg bromelain, a standard amount of CP and the inclusion of an AGP (table 43).

Crude protein had a significant effect on feed intake during the starter phase. Antibiotic growth promoter had a significant effect on feed intake during the finisher phase. Crude protein and AGP had a significant interaction effect on feed intake during the grower phase, and CP, AGP and bromelain had a significant interaction effect on feed intake during the starter phase (table 44).

Birds given an AGP in the starter phase had significantly higher (P<0.05) FCR than those not given an AGP. Birds given bromelain at a level of 0.125 g/kg had significantly higher (P<0.05) FCR compared to those given bromelain at levels of 0 g/kg and 0.75 g/kg (table 45).

In the starter phase, birds that received a combination of 0.125 g/kg bromelain, a standard amount of CP and the inclusion of an AGP had significantly higher (P<0.05) FCR. No significant interaction effects were seen in the grower phase (table 46).

Antibiotic growth promoter and bromelain had significant main effects on FCR in the starter phase. Crude protein and bromelain had a significant interaction effect on broiler FCR in the finisher phase (table 47).

Table 39 The main effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on mean weight gain (g) during three phases of broiler rearing (± standard error of the mean)

Treatment	Days 0–14 (Starter)	Days 14–28 (Grower)	Days 28–34 (Finisher)
Crude protein			
Standard	209.9 (±1.93)	616.7 (±3.56) [a]	667.0 (±9.75)
High	212.2 (±1.93)	640.8 (±3.56) [b]	638.1 (±9.75)
AGP			
Without	213.7 (±1.92)	629.1 (±3.55)	659.8 (±9.72)
With	208.4 (±1.92)	628.4 (±3.55)	645.4 (±9.72)
Bromelain (g/kg)			
0	212.2 (±2.35)	628.6 (±4.35)	662.4 (±11.90)
0.125	207.7 (±2.36)	627.8 (±4.37)	638.6 (±12.00)
0.75	213.3 (±2.35	629.9 (±4.35)	656.8 (±11.90)

[a-b] Values within a column without a common superscript letter differ significantly (P<0.05)

Table 40 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on mean weight gain (g) during three phases of broiler rearing (± standard error of the mean)

Treatment		Days 0–14 (Starter)	Days 14–28 (Grower)	Days 28–34 (Finisher)
0 g/kg bromelain				
Standard CP	- AGP	212.2 (±4.73) [abc]	623.7 (±8.74) [ab]	685.0 (±23.92) [abc]
	+ AGP	212.2 (±4.73) [ac]	611.9 (±8.74) [a]	658.1 (±23.92) [a]
High CP	- AGP	212.2 (±4.73) [ae]	637.0 (±8.74) [a]	655.4 (±23.92) [a]
	+ AGP	212.2 (±4.73) [ab]	641.7 (±8.74) [ac]	651.1 (±23.92) [a]
0.125 g/kg bromelain				
Standard CP	- AGP	207.7 (±4.73) [abc]	619.4 (±8.74) [ab]	658.2 (±23.92) [a]
	+ AGP	207.7 (±4.73) [bd]	608.1 (±8.74) [bc]	663.3 (±23.92) [bc]
High CP	- AGP	207.7 (±4.73) [ae]	640.8 (±8.74) [a]	650.4 (±23.91) [a]
	+ AGP	207.7 (±4.73) [bce]	642.8 (±8.75) [ac]	582.4 (±23.95) [a]
0.75 g/kg bromelain				
Standard CP	- AGP	213.3 (±4.73) [a]	622.7 (±8.75) [a]	641.9 (±23.95) [a]
	+ AGP	213.3 (±4.73) [ade]	614.4 (±8.75) [ac]	695.4 (±23.95) [ab]
High CP	- AGP	213.3 (±4.73) [ad]	631.2 (±8.74) [a]	667.9 (±23.92) [a]
	+ AGP	213.3 (±4.73) [ab]	651.5 (±8.75) [ac]	621.9 (±23.95) [a]

[a-e] Values within a column without a common superscript letter differ significantly (P<0.05)

Table 41 Sources of variation (P-values) in weight gain during three phases of broiler rearing according to varying levels of crude protein (CP) and bromelain, with and without an antibiotic growth promoter (AGP) (± standard error of the mean)

	P-values		
	Days 0–14 (Starter)	**Days 14–28 (Grower)**	**Days 28–34 (Finisher)**
Main effects			
CP	0.402	<0.0001	0.041
AGP	0.057	0.885	0.298
Bromelain	0.220	0.937	0.346
Interaction effects			
CP*AGP	0.655	0.060	0.078
CP*Bromelain	0.399	0.859	0.720
AGP*Bromelain	0.780	0.637	0.582
CP*AGP*Bromelain	0.448	0.805	0.171

Table 42 The main effect of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on mean feed intake (g) during three phases of broiler rearing (± standard error of the mean)

Treatment	**Days 0–14 (Starter)**	**Days 14–28 (Grower)**	**Days 28–34 (Finisher)**
Crude protein			
Standard	267.3 (±2.67) [a]	858.9 (±5.58)	1445 (±13.49)
High	275.2 (±2.67) [b]	872.3 (±5.58)	1426 (±13.49)
AGP			
Without	270.3 (±2.60)	868.9 (±5.56)	1457 (±13.45) [a]
With	272.3 (±2.60)	862.3 (±5.56)	1414 (±14.44) [b]
Bromelain (g/kg)			
0	267.8 (±2.77)	859.4 (±7.06)	1434 (±16.46)
0.125	275.5 (±2.78)	864.5 (±7.09)	1442 (±16.54)
0.75	270.5 (±2.77)	872.9 (±7.06)	1430 (±16.47)

[a, b] Mean values within a column without a common superscript differ significantly ($P<0.05$)

Table 43 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on mean feed intake (g) during three phases of broiler rearing (± standard error of the mean)

Treatment		Days 0–14 (Starter)	Days 14–28 (Grower)	Days 28–34 (Finisher)
0 g/kg bromelain				
Standard CP	- AGP	265.5 (±5.56) [bc]	875.6 (±14.18) [ab]	1483 (±33.08) [ac]
	+ AGP	259.6 (±5.56) [bd]	844.9 (±14.18) [b]	1431 (±33.10) [abc]
High CP	- AGP	273.9 (±5.56) [cda]	857.9 (±14.18) [ab]	1474 (±33.10) [ac]
	+ AGP	272.3 (±5.56) [abcd]	859.3 (±14.18) [ab]	1349 (±33.08) [bd]
0.125 g/kg bromelain				
Standard CP	- AGP	268.8 (±5.56) [bc]	869.3 (±14.18) [ab]	1428 (±33.08) [abc]
	+ AGP	278.6 (±5.56) [cae]	841.1 (±14.18) [b]	1408 (±33.08) [ac]
High CP	- AGP	280.3 (±5.56) [cae]	869.7 (±14.18) [ab]	1497 (±33.08) [a]
	+ AGP	274.2 (±5.57) [abcd]	877.9 (±14.20) [ab]	1435 (±33.12) [abc]
0.75 g/kg bromelain				
Standard CP	- AGP	269.2 (±5.57) [bc]	866.6 (±14.20) [ab]	1457 (±33.12) [ac]
	+ AGP	262.1 (±5.57) [bd]	856.0 (±14.20) [b]	1461 (±33.08) [ac]
High CP	- AGP	263.8 (±5.56) [bde]	874.3 (±14.18) [ab]	1403 (±33.08) [cd]
	+ AGP	286.8 (±5.57) [a]	894.5 (±14.20) [a]	1400 (±33.12) [bc]

[a-e] Values within a column without a common superscript letter differ significantly ($P<0.05$)

Table 44 Sources of variation (P-values) in feed intake during three phases of broiler rearing according to different levels of crude protein (CP) and bromelain, with and without an antibiotic growth promoter (AGP) (± standard error of the mean)

	***P*-values**		
	Days 0–14 (Starter)	**Days 14–28 (Grower)**	**Days 28–34 (Finisher)**
Main effects			
CP	0.016	0.109	0.342
AGP	0.527	0.420	0.027
Bromelain	0.153	0.403	0.872
Interaction effects			
CP*AGP	0.346	0.050	0.301
CP*Bromelain	0.629	0.428	0.056
AGP*Bromelain	0.338	0.601	0.170
CP*AGP*Bromelain	0.017	0.989	0.781

Table 45 The main effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on mean feed conversion ratios (FCR) during three phases of broiler rearing (± standard error of the mean)

Treatment	Days 0–14 (Starter)	Days 14–28 (Grower)	Days 28–34 (Finisher)
Crude protein			
Standard	1.32 (±0.02)	1.41 (±0.01)	2.17 (±0.03)
High	1.34 (±0.02)	1.39 (±0.01)	2.22 (±0.03)
AGP			
Without	1.29 (±0.02) [a]	1.40 (±0.01)	2.22 (±0.03)
With	1.37 (±0.02) [b]	1.40 (±0.01)	2.18 (±0.03)
Bromelain (g/kg)			
0	1.31 (±0.02) [a]	1.40 (±0.01)	2.15 (±0.04)
0.125	1.38 (±0.02) [b]	1.40 (±0.01)	2.26 (±0.04)
0.75	1.31 (±0.02) [a]	1.40 (±0.01)	2.19 (±0.04)

[a, b] Values within a column without a common superscript differ significantly (P<0.05)

Table 46 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on mean feed conversion ratios (FCR) during three phases of broiler rearing (± standard error of the mean)

Treatment		Days 0–14 (Starter)	Days 14–28 (Grower)	Days 28–34 (Finisher)
0 g/kg bromelain				
Standard CP	- AGP	1.29 (±0.04) [bc]	1.42 (±0.02)	2.20 (±0.08) [bc]
	+ AGP	1.26 (±0.04) [bd]	1.41 (±0.02)	2.17 (±0.08) [bc]
High CP	- AGP	1.31 (±0.04) [bc]	1.39 (±0.02)	2.15 (±0.08) [bc]
	+ AGP	1.36 (±0.04) [cd]	1.39 (±0.02)	2.08 (±0.08) [b]
0.125 g/kg bromelain				
Standard CP	- AGP	1.30 (±0.04) [cb]	1.42 (±0.02	2.16 (±0.08) [bc]
	+ AGP	1.50 (±0.04) [a]	1.43 (±0.02)	2.10 (±0.08) [b]
High CP	- AGP	1.34 (±0.04) [bc]	1.40 (±0.02)	2.42 (±0.08) [a]
	+ AGP	1.38 (±0.04) [c]	1.37 (±0.02)	2.35 (±0.08) [ac]
0.75 g/kg bromelain				
Standard CP	- AGP	1.25 (±0.04) [bd]	1.39 (±0.02)	2.25 (±0.08) [abc]
	+ AGP	1.33 (±0.04) [bc]	1.42 (±0.02)	2.12 (±0.08) [b]
High CP	- AGP	1.26 (±0.04) [bd]	1.42 (±0.02)	2.13 (±0.08) [b]
	+ AGP	1.39 (±0.04) [c]	1.41 (±0.02)	2.27 (±0.08) [ab]

[a-d] Within a column, values without a common superscript letter differ significantly (*P*<0.05)

Table 47 Sources of variation (P-values) in feed conversion ratio (FCR) during three phases of broiler rearing according to different levels of crude protein (CP) and bromelain, with and without an antibiotic growth promoter (AGP) (± standard error of the mean)

	***P*-values**		
	Days 0–14 (Starter)	**Days 14–28 (Grower)**	**Days 28–34 (Finisher)**
Main effects			
CP	0.414	0.113	0.157
AGP	0.001	0.986	0.434
Bromelain	0.012	0.998	0.149
Interaction effects			
CP*AGP	0.878	0.599	0.446
CP*Bromelain	0.195	0.585	0.011
AGP*Bromelain	0.071	0.736	0.804
CP*AGP*Bromelain	0.063	0.799	0.288

Morphology of the small intestine

The main and interaction effects of CP concentration, bromelain supplementation, and inclusion of an AGP on duodenal, ileal, and jejunal villi height, crypt depth, and villus height-to-crypt depth ratio, as well as the sources of variation of each treatment, are shown in Tables 48–56.

The duodenal crypt depth in birds with 0 g/kg bromelain supplementation was significantly higher (P<0.05) than that in birds who had received 0.125 g/kg or 0.75 g/kg bromelain. A significant decrease (P<0.05) can be seen in the duodenal villus height-to-crypt depth ratio in birds that received 0 g/kg bromelain compared to the levels 0.125 and 0.75 g/kg bromelain (table 48).

A combination of the effects of 0 g/kg bromelain, standard CP level, and no AGP produced a significant increase (P<0.05) in duodenal villi height. A converse effect is seen with the combination of 0.75 g/kg bromelain, high CP level, and an AGP, in which the villi height is significantly decreased (P<0.05) (table 49).

Bromelain had a significant main effect on crypt depth and villus height: crypt depth, and AGP and bromelain had a significant interaction effect on villus height: crypt depth ratio. Another significant interaction effect was seen on both crypt depth and villus height: crypt depth ratio by CP, AGP and bromelain (table 50).

There is a significant decrease (P<0.05) in the ileal villi heights of birds that received an AGP compared to birds that did not. There is a significant decrease (P<0.05) between the ileal crypt depths of birds that received a high level of CP and birds that received a standard level of CP. Birds

that received 0 g/kg bromelain had significantly longer (P<0.05) villi heights than those that received bromelain at 0.125 and 0.75 g/kg, and significantly lower (P<0.05) crypt depths were seen in the treatment containing 0.75 g/kg bromelain (table 51).

Crypt depth was significantly higher when birds received a combination of 0.125 g/kg bromelain, a standard amount of CP and no AGP compared to the combination of 0.75 g/kg, a high amount of CP and the inclusion of an AGP (table 52).

Antibiotic growth promoter and bromelain had a significant effect on villi height. Bromelain also had a significant effect on crypt depth. Crude protein, AGP and bromelain had a significant interaction effect on crypt depth (table 53).

Jejunal villi height of birds that received 0.125 g/kg bromelain was significantly higher (P<0.05) than those of the other two bromelain levels, whereas jejunal crypt depth was significantly higher (P<0.05) in birds that received 0 g/kg bromelain. Jejunal crypt depth was also significantly higher (P<0.05) in birds that received a high level of CP. Jejunal villus height-to-crypt depth ratio was significantly higher (P<0.05) in birds that received a high level of CP. Villus height: crypt depth ratio was significantly lower (P<0.05) in birds that received 0 g/kg bromelain (table 54).

Crypt depths were significantly lower (P<0.05) in birds that received these two treatments: a combination of 0.75 g/kg bromelain, a standard amount of CP and no AGP and 0.75 g/kg bromelain, a high amount of CP and the inclusion of an AGP (table 55).

Crude protein and bromelain had a significant interaction effect on both crypt depth and villus height: crypt depth. Crude protein, AGP and bromelain had a significant interaction effect on crypt depth (table 56).

Table 48 The main effect of crude protein (CP) concentration, bromelain supplementation and inclusion of an antibiotic growth promoter (AGP) on duodenal villi height, crypt depth, and villus height-to-crypt depth ratio means (± standard error of the mean)

	Villi height (µm)	Crypt depth (µm)	Villus height: crypt depth
Treatment: Crude protein			
Standard	1598 (±24.62)	69.50 (±2.31)	24.50 (±0.77)
High	1608 (±24.62)	63.43 (±2.31)	26.13 (±0.77)
AGP			
Without	1626 (±24.62)	68.28 (±2.31)	25.14 (±0.77)
With	1580 (±24.62)	64.65 (±2.31)	25.49 (±0.77)
Bromelain (g/kg)			
0	1623 (±30.16)	74.14 (±2.83)^{a}	22.99 (±0.94)^{b}
0.125	1631 (±30.16)	63.87 (±2.83)^{b}	26.42 (±0.94)^{a}
0.75	1556 (±30.16)	61.39 (±2.83)^{b}	26.52 (±0.94)^{a}

$^{a, b}$ Values within a column without a common superscript letter differ significantly (P<0.05)

Table 49 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on duodenal villi height, crypt depth, and villus height-to-crypt depth ratio means (± standard error of the mean)

Treatment		Villi height (µm)	Crypt depth (µm)	Villus height: crypt depth
0 g/kg bromelain				
Standard CP	- AGP	1679 (±60.31) ^{a}	84.96 (±5.65) ^{a}	21.26 (±1.89) ^{bc}
	+ AGP	1593 (±60.31) ^{ab}	68.96 (±5.65) ^{bc}	24.53 (±1.89) ^{cde}
High CP	- AGP	1615 (±60.31) ^{ab}	72.35 (±5.65) ^{ac}	22.68 (±1.89) ^{bcd}
	+ AGP	1604 (±60.31) ^{ab}	70.28 (±5.65) ^{ac}	23.52 (±1.89) ^{cf}
0.125 g/kg bromelain				
Standard CP	- AGP	1627 (±60.31) ^{ab}	72.75 (±5.65) ^{ac}	22.52 (±1.89) ^{bcd}
	+ AGP	1602 (±60.31) ^{ab}	63.19 (±5.65) ^{bc}	26.69 (±1.89) ^{adf}
High CP	- AGP	1671 (±60.31) ^{ab}	61.40 (±5.65) ^{bc}	27.97 (±1.89) ^{aef}
	+ AGP	1625 (±60.31) ^{ab}	58.15 (±5.65) ^{bc}	28.50 (±1.89) ^{aef}
0.75 g/kg bromelain				
Standard CP	- AGP	1537 (±60.31) ^{ab}	53.39 (±5.65) ^{b}	30.82 (±1.89) ^{a}
	+ AGP	1552 (±60.31) ^{ab}	73.76 (±5.65) ^{ac}	21.16 (±1.89) ^{bc}
High CP	- AGP	1626 (±60.31) ^{ab}	64.82 (±5.65) ^{bc}	25.59 (±1.89) ^{ac}
	+ AGP	1509 (±60.31)^{b}	53.57 (±5.65) ^{b}	28.53 (±1.89) ^{aef}

$^{a-f}$ Within a column, values without a common superscript letter differ significantly (P<0.05)

Table 50 Sources of variation (P-values) on duodenal villi height, crypt depth, and villus height-to-crypt depth ratio of broilers that received different levels of crude protein (CP) and bromelain, with and without an antibiotic growth promoter (AGP) (± standard error of the mean)

	***P*-value**		
	Villi height (µm)	**Crypt depth (µm)**	**Villus height: crypt depth**
Main effects			
CP	0.774	0.067	0.138
AGP	0.199	0.270	0.750
Bromelain	0.162	0.005	0.014
Interaction effects			
CP*AGP	0.704	0.563	0.322
CP*Bromelain	0.755	0.889	0.414
AGP*Bromelain	0.980	0.203	0.061
CP*AGP*Bromelain	0.477	0.012	0.005

Table 51 The main effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on ileal villi height, crypt depth, and villus height-to-crypt depth ratio means (± standard error of the mean)

Treatment	**Villi height (µm)**	**Crypt depth (µm)**	**Villus height: crypt depth**
Crude Protein			
Standard	468 (±16.62)	90.0 (±2.33) [a]	5.33 (±0.22)
High	429 (±16.62)	77.4 (±2.33) [b]	5.66 (±0.22)
AGP			
Without	472 (±16.62) [a]	85.8 (±2.33)	5.58 (±0.22)
With	425 (±16.62) [b]	81.6 (±2.33)	5.40 (±0.22)
Bromelain (g/kg)			
0	488 (±20.36) [a]	86.2 (±2.85) [a]	5.77 (±0.28)
0.125	454 (±20.36)	87.2 (±2.85) [a]	5.36 (±0.28)
0.75	403 (±20.36) [b]	77.8 (±2.85) [b]	5.35 (±0.28)

[a,b] Within a column, values without a common superscript letter differ significantly ($P<0.05$)

Table 52 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and an antibiotic growth promoter (AGP) on ileal villi height, crypt depth, and villus height-to-crypt depth ratio means (± standard error of the mean)

Treatment		Villi height (µm)	Crypt depth (µm)	Villus height: crypt depth
0 g/kg bromelain				
Standard CP	- AGP	571 (±40.71) ^{a}	93.3 (±5.71) ^{ac}	6.23 (±0.55)
	+ AGP	436 (±40.71) ^{bc}	92.0 (±5.71) ^{ad}	4.92 (±0.55)
High CP	- AGP	459 (±40.71) ^{ac}	79.8 (±5.71) ^{bcd}	5.81 (±0.55)
	+ AGP	485 (±40.71) ^{ac}	79.8 (±5.71) ^{bcd}	6.12 (±0.55)
0.125 g/kg bromelain				
Standard CP	- AGP	522 (±40.71) ^{ac}	107.0 (±5.71) ^{a}	4.97 (±0.55)
	+ AGP	424 (±40.71) ^{bc}	84.0 (±5.71) ^{bcd}	5.11 (±0.55)
High CP	- AGP	456 (±40.71) ^{bc}	80.3 (±5.71) ^{bcd}	5.71 (±0.55)
	+ AGP	414 (±40.71) ^{bc}	77.2 (±5.71) ^{bcd}	5.66 (±0.55)
0.75 g/kg bromelain				
Standard CP	- AGP	443 (±40.71) ^{bc}	75.9 (±5.71) ^{bd}	5.90 (±0.55)
	+ AGP	413 (±40.71) ^{bc}	88.0 (±5.71) ^{bcd}	4.85 (±0.55)
High CP	- AGP	383 (±40.71) ^{b}	78.6 (±5.71) ^{bcd}	4.88 (±0.55)
	+ AGP	375 (±40.71) ^{b}	68.5 (±5.71) ^{b}	5.77 (±0.55)

$^{a-d}$ Within a column, values without a common superscript letter differ significantly (P<0.05)

Table 53 Sources of variation (P-values) on ileal villi height, crypt depth, and villus height-to-crypt depth ratio of broilers that received feed with different levels of crude protein (CP) and bromelain, with and without an antibiotic growth promoter (AGP) (± standard error of the mean)

	***P*-value**		
	Villi height (µm)	**Crypt depth (µm)**	**Villus height: crypt depth**
Main effects			
CP	0.098	0.0002	0.305
AGP	0.046	0.207	0.579
Bromelain	0.016	0.043	0.473
Interaction effects			
CP*AGP	0.094	0.958	0.083
CP*Bromelain	0.955	0.584	0.667
AGP*Bromelain	0.651	0.173	0.766
CP*AGP*Bromelain	0.463	0.038	0.343

Table 54 The main effects of crude protein (CP) concentration, bromelain supplementation, and antibiotic growth promoter (AGP) on jejunal villi height, crypt depth, and villus height-to-crypt depth ratio means (± standard error of the mean)

Treatment	Villi height (µm)	Crypt depth (µm)	Villus height: crypt depth
Crude protein			
Standard	1077 (±21.19)	79.96 (±1.92) [a]	14.20 (±0.53) [b]
High	1060 (±21.19)	68.09 (±1.92) [b]	16.12 (±0.53) [a]
AGP			
Without	1067 (±21.19)	75.50 (±1.92)	14.94 (±0.53)
With	1069 (±21.19)	72.54 (±1.92)	15.38 (±0.53)
Bromelain (g/kg)			
0	1032 (±25.95) [b]	79.31 (±2.35) [a]	13.38 (±0.65) [b]
0.125	1111 (±25.95) [a]	75.90 (±2.35) [b]	15.59 (±0.65) [a]
0.75	1062 (±25.95)	66.86 (±2.35) [b]	16.50 (±0.65) [a]

[a, b] Within a column, values without a common superscript letter differ significantly (P<0.05)

Table 55 The interaction effects of crude protein (CP) concentration, bromelain supplementation, and antibiotic growth promoter (AGP) on jejunal villi height, crypt depth, and villus height-to-crypt depth ratio means (± standard error of the mean)

Treatment		Villi height (µm)	Crypt depth (µm)	Villus height: crypt depth
0 g/kg bromelain				
Standard CP	- AGP	995.1 (±51.91) [bc]	91.70 (±4.71) [a]	11.07 (±1.30)[c]
	+ AGP	1010 (±51.91) [bc]	82.66 (±4.71) [ad]	12.30 (±1.30) [bcd]
High CP	- AGP	1032 (±51.91) [bcd]	70.91 (±4.71) [bcd]	14.84 (±1. 30) [bde]
	+ AGP	1091 (±51.91) [ac]	71.96 (±4.71) [bdef]	15.32 (±1. 30) [bce]
0.125 g/kg bromelain				
Standard CP	- AGP	1183 (±51.91) [a]	90.71 (±4.71) [a]	11.07 (±1.30) [c]
	+ AGP	1074 (±51.91) [ac]	79.39 (±4.71) [ad]	14.04 (±1. 30) [bcd]
High CP	- AGP	1120 (±51.91) [ac]	69.58 (±4.71) [bcd]	16.93 (±1.30) [ad]
	+ AGP	1065 (±51.91) [ac]	63.94 (±4.71) [ce]	17.87 (±1. 30) [ae]
0.75 g/kg bromelain				
Standard CP	- AGP	1034 (±51.91) [bcd]	58.13 (±4.71) [c]	18.81 (±1. 30) [a]
	+ AGP	1163 (±51.91) [ad]	77.15 (±4.71) [bde]	15.42 (±1. 30) [bde]
High CP	- AGP	1040 (±51.91) [ac]	72.00 (±4.71) [bde]	14.46 (±1. 30) [be]
	+ AGP	1011 (±51.91) [bc]	60.15 (±4.71) [cf]	17.33 (±1. 30) [ae]

[a-f] Within a column, values without a common superscript letter differ significantly (P<0.05)

Table 56 Sources of variation (P-values) on ileal villi height, crypt depth and villus height-to-crypt depth ratio of broiler rearing that received feed with different levels of crude protein (CP) and bromelain, with and without the inclusion of an antibiotic growth promoter (AGP) (± standard error of the mean)

	***P*-values**		
	Villi height (µm)	**Crypt depth (µm)**	**Villus height: crypt depth**
Main effects			
CP	0.579	<0.0001	0.012
AGP	0.951	0.280	0.560
Bromelain	0.105	0.001	0.003
Interaction effects			
CP*AGP	0.738	0.358	0.190
CP*Bromelain	0.188	0.030	0.015
AGP*Bromelain	0.148	0.194	0.802
CP*AGP*Bromelain	0.271	0.005	0.132

4.5 Digestibility trial

The effect of different levels of bromelain, as well as a commercially used protease, AxtraPro, on the ileal CP and DM digestibility in broilers is summarised in Table 57 below.

Table 57 Effect of 0 g/kg bromelain, 0.75 g/kg bromelain, and 0.25 g/kg AxtraPro on the ileal CP digestibility (%) and ileal DM digestibility (%) (± standard error of the mean)

Treatment	**CP digestibility (%)**	**DM digestibility (%)**
Negative Control (NC) (standard diet, 0 g/kg bromelain)	83.62	71.73 [b]
NC + 0.75 g bromelain/kg feed (0.075%)	83.97	73.04 [a]
Positive Control (0.25 g/kg AxtraPro. DSM)	84.25	74.48 [a]

[a,b] Within a column, values without a common superscript letter differ significantly (P<0.05)

CP, crude protein; DM, dry matter

There were no significant difference between the CP digestibility of all three treatments. The DM digestibility of the negative control group was significantly lower (P<0.05) than the NC + 0.75 bromelain/kg feed and positive control groups, implying that the 0.75 g/kg bromelain treatment enhanced DM digestibility, compared to 0 g/kg bromelain (table 57).

CHAPTER 5: DISCUSSION

5.1 Performance trial

The aim of the performance trial was to determine whether bromelain at different levels of supplementation would have a significant effect on broiler health and therefore broiler performance, both alone and in combination with various CP and AGP inclusion levels.

5.1.1 Production parameters

Body weight and body weight gain

Birds that received a high level of CP, across all combinations of bromelain and AGP, had significantly higher body weights and body weight gains on days 21 and 28. This observation is conventional as the birds' protein requirement was met and they did not undergo nutrient deficiency-related stress, allowing them to grow more than the birds that received a lower level of CP (Xue *et al.,* 2016)

A trend (not significant) was observed throughout the entire 34 d whereby birds that received an AGP, regardless of CP level or bromelain supplementation, exhibited smaller body weights and body weight gains than birds that did not receive an AGP. This is contrary to literary findings, in which an AGP is seen to decrease health issues in broilers and therefore increase production and body weight gain (Nanduri *et al.,* 2006). This could be due to antibiotic resistance that may have occurred; however, this typically occurs later in life, not in the first 7 days. According to Untari *et al.* (2021), antibiotic resistance of bacteria in broilers can also result from chronic use of antibiotics in the form of AGPs due to pre-existing antibiotic-resistant bacteria in the environment. AGPs also work by altering the microbial populations within the chicken gut, to lower immune response, and thus provide more energy to the bird for growth. It is possible that the birds were already receiving enough energy from their diet, rendering the AGP futile, however a more likely explanation for this occurrence would be that the conditions in the University of Pretoria experimental facility are more hygienic than a commercial broiler house, and the birds in this trial might have experienced less stress than commercial birds, causing AGP to have no effect. The level of bromelain supplementation did not seem to have a significant effect on body weight gain when interacting with the different levels of AGP across all levels of crude protein and days of age. In the first 14 d, body weight and body weight gain were notably less when birds received a combination of

bromelain inclusion of 0.125 g/kg, a standard level of CP, and an AGP. The combination of standard CP and an AGP may have produced small body weights and low body weight gain if the antibiotic altered the gut microbiota to such an extent that the amount of protein the birds received was not adequately absorbed (Dibner & Richards, 2005).

The level of bromelain supplementation did not seem to have a significant effect on broiler body weight or body weight gain. Across all treatments at 34 d-of-age, broilers seemed to be more affected by the level of CP and AGP. It must be noted that the birds that received a high level of crude protein had sufficient amino acids to grow to genetic potential. The inclusion of additional amino acids would therefore not improve growth, even if the protease increased digestibility. Furthermore, bromelain did not assist in increasing broiler body weights when the birds received a combination of standard CP and an AGP. This could be due to inadequate efficacy of the exogenous protease, or perhaps the protease does not have beneficial qualities in terms of broiler health. Supposing that the broiler gut microbiome was altered by the consistent presence of an AGP and a standard level of CP, it can be inferred that bromelain did not assist in potent protein absorption. A study by Akit *et al.* (2019) shows that a level of 2 g/kg bromelain yielded the highest body weight gain and feed intake, when compared to levels of 0.5 g/kg, 1 g/kg, 2 g/kg and 5 g/kg bromelain. This indicates that the 0.75 g/kg level of bromelain (the highest level used in the trial) may not have been enough to yield the same results.

Feed intake

It is important to note that, cumulatively, the level of CP did not have a significant effect on broiler feed intake. Birds that did not receive an AGP had higher feed intakes than those that did, especially later in the trial. A high level of crude protein, the inclusion of an AGP and 0.75 g/kg bromelain showed a significant interaction effect during the first three weeks.

The findings on the effect of bromelain on feed intake are inconclusive. Over days 0–34, birds that received a combination of standard CP, no AGP and no bromelain had significantly higher (P<0.05) feed intakes, suggesting that bromelain had no effect on broiler feed intake. The same study by Akit *et al.* (2019) showed that feed intake was increased at the optimal bromelain level of 2 g/kg, meaning the low level of bromelain used in the trial might not have been enough to see substantial effects.

A high crude protein diet was given to the birds as a means to challenge their gut environments. High protein concentrations or imbalanced amino acid concentrations in the diet may alter the gut environment. This can create a build-up of protein in the gut and allow for amino acid fermenting bacteria to flourish. Diets high in animal proteins like fishmeal predispose chickens to necrotic enteritis, which may be ascribed to the higher zinc, glycine and methionine concentrations in animal proteins that encourage *C. perfringens* proliferation (M'Sadeq *et al.,* 2015). The aim of including a high crude protein diet was to note whether bromelain would be effective in reducing the negative effect of excess levels of protein in the gut. This result was not seen.

Feed conversion ratio

It is beneficial to have low FCR across the entire growth period to optimise broiler yield and minimise feed expenses. To achieve a low FCR, a broiler must maximise nutrient absorption from the smallest amount of feed possible (Singh, 2020). In the first 28 d, the FCR indicated that the broilers were converting the feed into nutrients optimally; however, in the last week, the birds ate a substantial amount without yielding corresponding body weights. This could be due to external stresses, such as environmental factors, or feed that was not nutritionally adequate.

The crude protein level did not seem to have a significant effect on FCR. In some cases, a high level of CP seemed to yield a lower FCR; however, in most cases the level of CP did not significantly affect FCR. One study by Chrystal *et al*. (2020) found that when a reduced amount of CP was given to male broilers, the FCR increased. This agrees with the above observation.
In conjunction with an AGP, a high CP level caused a low FCR cumulatively over days 0–28.

Throughout the trial, FCR were typically lower when the AGP was excluded. During the starter phase, birds that received an AGP had notably higher FCRs than those not given an AGP. When the microbiome balance in the gut is disrupted (potentially by the AGP) dysbiosis develops, and disease or reduced performance may occur as a consequence. Pathogenic bacteria can damage the intestinal tract, causing reduced feed conversion efficiency and therefore slower growth (Dibner & Richards, 2005).

Lastly, bromelain had no significant effect on FCR, which corresponds with a study by Akit *et al.* (2019).

5.2 Intestinal morphology

In the small intestine, the height of the villi, depth of the crypts, and the villus height-to-crypt depth ratio are standard indicators of digestive and absorbative function (Biasato *et al.,* 2018). According to Wang *et al.* (2020), villus height is directly related to performance. Villus height-to-crypt depth ratio is also a measure of broiler performance. In a study by Nguyen *et al.* (2021), a positive correlation was found between villus height-to-crypt depth ratio of the duodenum and the number of lactic acid bacteria in the chicken gut, which in turn allowed for increased broiler performance. Furthermore, crypt depth size can be a measure of the potency of intestinal epithelial cell renewal processes. The crypt can be likened to the villus factory, where a large crypt indicates fast tissue turnover and a high demand for new tissue and a short crypt depth indicates efficient tissue turnover and superior gut condition (Umar, 2010).

Duodenum

Birds that did not receive AGP had both longer duodenal villi heights and shallower crypt depths and, a higher villus height-to-crypt depth ratio. Shorter villi can indicate the presence of toxins (namely antibiotics) (Miles *et al.,* 2006), possibly explaining this association of smaller villi heights with the presence of the AGP.

Crude protein did not have a significant effect on duodenal parameters; however, it was noted that a standard level of CP in combination with 0 g/kg bromelain and no AGP produced the longest villi height in this study. Duodenal crypt depth in birds supplemented with 0.75 g/kg bromelain was significantly reduced compared to birds supplemented with 0 g/kg bromelain. The efficacy of this effect is also seen in a study by Duque-Ramirez *et al.* (2023). According to Xu *et al* (2017), a decreased crypt depth indicates efficient tissue turnover and good condition of the gut.

Duodenal crypt depth and villus height-to-crypt depth ratio in birds with 0 g/kg bromelain supplementation was significantly lower than birds that had received 0.125 g/kg and 0.75 g/kg bromelain. It can therefore be concluded that bromelain did not have a positive effect on villus height or villus height-to-crypt depth ratio, but it did have a positive effect on crypt depth.

Jejunum

Similar to the findings in the duodenum, jejunal crypt depth was significantly greater in birds that received 0 g/kg bromelain. Jejunal crypt depth was also deeper in birds that received a standard level of CP; conversely, a high level of CP produced the highest villus height-to-crypt depth ratio.

Intestinal crypts consist of Paneth cells, which secrete antimicrobial peptides and proteins and decreased crypt depth is an indicator of good gut health (Bowen, 2019). In this study, it was seen that birds supplemented with 0.75 g/kg bromelain had a decreased crypt depth compared to those supplemented with 0 g/kg bromelain. A study by Xu *et al.* (2017) shows that feeding an exogenous protease to Arbor Acre broilers also resulted in decreased crypt depth. This allows the conclusion that bromelain is an effective promoter of good gut health. Good gut health in turn allows the broiler an increase in nutrient digestion and therefore good overall health and growth (Wielsma, 2017).

Ileum

Ileal villi height was significantly longer in birds who did not receive an AGP. In this instance, this could be due to the damaging effect of prolonged antibiotic use (Miles *et al.,* 2006). When coupled with high crude protein, the same result is seen. This indicates that both an excess of protein and prolonged antibiotic use can damage the epithelium of the intestine, causing unhealthy gut conditions (Dittoe *et al.*, 2022).

Smaller crypt depths were observed in birds fed a high level of CP, and the combination of 0.75. g/kg bromelain and high level of CP induced both significantly shorter villi and decreased crypt depths.

It was noted that birds that received 0.75 g/kg bromelain had decreased crypt depths, which was also seen in the duodenum and jejunum.

5.3 Digestibility of dry matter and crude protein

The digestibility trial was conducted to determine the digestibility of DM and CP in the ileum of chickens when fed a diet supplemented with bromelain, compared to a negative control diet and a diet supplemented with a commercially used protease. A high value of DM digestibility indicates

that the DM is well digested by the broiler and in turn, the broiler is able to perform more efficiently (Chang'a *et al.,* 2019). Crude protein digestibility it is directly related to broiler performance (Belloir *et al*., 2017).

It is interesting to note that there were no significant differences (P<0.05) in crude protein digestibility between all three treatments. There was a significant difference (P<0.05) in DM digestibility, namely the 0.75 g/kg bromelain treatment had a significantly higher (P<0.05) DM digestibility than the negative control group (0 g/kg bromelain), suggesting that bromelain contributed to an increased DM digestibility. This corresponds to a study by Akit *et al.* (2019) showed that birds fed 0.05 and 0.1% bromelain had higher fat and protein digestibility than the control. A study by Nguyen *et al.* (2018) where pigs fed bromelain had higher DM digestibility's in the 6th week also substantiates this. The lowest CP digestibility was also observed in the 0 g/kg bromelain treatment, indicating that bromelain can assist with CP digestibility and overall feed efficiency.

CHAPTER 6: CONCLUSION AND CRITICAL REVIEW

6.1 Conclusion

The broiler industry has begun to search for natural alternatives to AGPs that will produce similar results while also limiting the problem of antibiotic resistance and appeasing the public's concern of antibiotic transferal to humans. Exogenous enzymes have the ability to increase feed efficiency and therefore lower feed costs, while maintaining or improving broiler production; however, certain conditions must be in place for an exogenous enzyme to function optimally.

The results from the performance trial indicate that bromelain had no synergistic or additive effect on broiler health or production. In the standard crude protein treatments, birds did not show a greater response to bromelain, as shown in previous research. Under these specific circumstances, AGP also did not improve performance. However, bromelain did show a significant effect on reducing crypt depth size, enabling good gut conditions. In conclusion, bromelain was seen to improve gut health (decreased crypt depths).

The digestibility trial results indicated that bromelain was as effective as a commercial protease in digesting dry matter, and therefore can be considered acceptable for commercial use. Bromelain had no significant effects on crude protein digestibility.

Further studies may help to determine bromelain's exact mode of action and identify the parameters which must be met in order for bromelain to successfully improve broiler health and performance.

6.2 Critical review

It is possible that bromelain possesses health and production benefits for broilers; however, further research is required to confirm this.
Certain parameters used in the performance trial did not exactly mimic the environment of commercial broiler farms. For example, the stocking rate was lower than commercial farm stocking rates. This means that the birds might not have experienced the same level of stress as in commercial houses, which could have been a possible factor that affected the results of bromelain efficacy.

Furthermore, the chickens kept in facilities on the University of Pretoria experimental farm could have been different from commercial conditions. Commercial conditions provide an environment more suitable to microbes as they can be less hygienic than facilities typically found at research institutions, and therefore AGPs or any antimicrobial (such as bromelain) are more efficient and necessary to support bird health and growth.

The high crude protein diets fed to the birds were not sufficiently high enough to create a negative effect on gut health, therefore the antimicrobial effect of bromelain could not be adequately tested.

The bromelain inclusion level could also have influenced chick performance. According to some studies, bromelain might be more effective at higher levels, however due to its unpalatability, this would be difficult to execute.

REFERENCES

Adil, S. and Magray, S. (2012). Impact and Manipulation of Gut Microflora in Poultry: A Review. *Journal of Animal Veterinary and Advances*, 11(1680-5593).

Ahiwe, E.U., Omede, A.A., Abdallh, M.B. and Iji, P.A. (2018). *Managing Dietary Energy Intake by Broiler Chickens to Reduce Production Costs and Improve Product Quality*. [online] *www.intechopen.com*. IntechOpen. Available at: https://www.intechopen.com/chapters/61373 [Accessed 25 Dec. 2021].

Akit, H., Zainudin, N., Wahid, A., Zakaria, S., Foo, H. and Loh, T. (2019). Dietary bromelain improves nutrient digestibility, digesta viscosity and intestinal villus height as well as reduces intestinal E. coli population of broiler chickens. *J. Anim. Sci*, [online] 22(1), pp.1–16. Available at: http://mjas.my/mjas-v2/rf/pages/journal/v22i1/1-AKIT.pdf [Accessed 27 Mar. 2023].

Alagawany, M., Elnesr, S.S. and Farag, M.R. (2018). The role of exogenous enzymes in promoting growth and improving nutrient digestibility in poultry. *Iranian journal of veterinary research*, [online] 19(3), pp.157–164. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6184034/ [Accessed 10 Dec. 2019].

Albawi, K. and Hussain, F. (2020). Immune and antioxidant effects of bromelain with ciprofloxacin in broiler chicks. *Plant Archives*, [online] 20(2), pp.8072–8076. Available at: http://plantarchives.org/20-2/8072-8076%20(6985).pdf [Accessed 27 Mar. 2023].

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002). Carrier Proteins and Active Membrane Transport. *Molecular Biology of the Cell. 4th edition*. [online] Available at: https://www.ncbi.nlm.nih.gov/books/NBK26896/.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2018). *Analyzing Protein Structure and Function*. [online] Nih.gov. Available at: https://www.ncbi.nlm.nih.gov/books/NBK26820/.

Angel, C.R., Saylor, W., Vieira, S.L. and Ward, N. (2011). Effects of a monocomponent protease on performance and protein utilization in 7- to 22-day-old broiler chickens. *Poultry Science*, 90(10), pp.2281–2286. doi:https://doi.org/10.3382/ps.2011-01482.

Apajalahti, J. and Vienola, K. (2016). Interaction between chicken intestinal microbiota and protein digestion. *Animal Feed Science and Technology*, 221, pp.323–330. doi:https://doi.org/10.1016/j.anifeedsci.2016.05.004.

Apata, D.F. (2009). Antibiotic Resistance in Poultry. *citeseerx.ist.psu.edu*, [online] 4(1682-8356). Available at: http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.486.28&rep=rep1&type=pdf.

Aruwa, C.E., Pillay, C., Nyaga, M.M. and Sabiu, S. (2021). Poultry gut health – microbiome functions, environmental impacts, microbiome engineering and advancements in characterization technologies. *Journal of Animal Science and Biotechnology*, 12(1). doi:https://doi.org/10.1186/s40104-021-00640-9.

Bailey, R. (2019). *Gut health in poultry: the world within - update*. [online] www.thepoultrysite.com. Available at: https://www.thepoultrysite.com/articles/gut-health-in-poultry-the-world-within-1.

Barekatain, M.R., Antipatis, C., Choct, M. and Iji, P.A. (2013). Interaction between protease and xylanase in broiler chicken diets containing sorghum distillers' dried grains with solubles. *Animal Feed Science and Technology*, [online] 182(1), pp.71–81. doi:https://doi.org/10.1016/j.anifeedsci.2013.04.002.

Bayat, S., Amiri, N., Pishavar, E., Kalalinia, F., Movaffagh, J. and Hashemi, M. (2019). Bromelain-loaded chitosan nanofibers prepared by electrospinning method for burn wound healing in animal models. *Life Sciences*, 229, pp.57–66. doi:https://doi.org/10.1016/j.lfs.2019.05.028.

Begum, M., Li, H.L., Hossain, M.M. and Kim, I.H. (2015). Dietary bromelain-C.3.4.22.32 supplementation improves performance and gut health in sows and piglets. *Livestock Science*, 180, pp.177–182. doi:https://doi.org/10.1016/j.livsci.2015.07.013.

Belloir, P., Méda, B., Lambert, W., Corrent, E., Juin, H., Lessire, M. and Tesseraud, S. (2017). Reducing the CP content in broiler feeds: impact on animal performance, meat quality and nitrogen utilization. *Animal*, 11(11), pp.1881–1889. doi:https://doi.org/10.1017/s1751731117000660.

Biasato, I., Ferrocino, I., Biasibetti, E., Grego, E., Dabbou, S., Sereno, A., Gai, F., Gasco, L., Schiavone, A., Cocolin, L. and Capucchio, M.T. (2018). Modulation of intestinal microbiota, morphology and mucin composition by dietary insect meal inclusion in free-range chickens. *BMC Veterinary Research*, 14(1). doi:https://doi.org/10.1186/s12917-018-1690-y.

Biga, L.M., Dawson, S., Harwell, A., Hopkins, R., Kaufmann, J., LeMaster, M., Matern, P., Morrison-Graham, K., Quick, D. and Runyeon, J. (2019). *26.4 Acid-Base Balance*. [online] open.oregonstate.education. Available at: https://open.oregonstate.education/aandp/chapter/26-4-acid-base-balance/.

Binet, A. (2013). *Revealing the 3D structure of proteins*. [online] www.reflexions.uliege.be. Available at: https://www.reflexions.uliege.be/cms/c_352385/en/revealing-the-3d-structure-of-proteins?part=3 [Accessed 28 Mar. 2023].

Bowen, R. (2019). *Life Cycle of Small Intestinal Enterocytes*. [online] www.vivo.colostate.edu. Available at: http://www.vivo.colostate.edu/hbooks/pathphys/digestion/smallgut/lifecycle.html#:~:text=Villi%20are%20projections%20into%20the.

Broom, A., Broom, J., Kirby, E., Gibson, A. and Davis, M. (2017). Antibiotic optimisation in 'the bush': Local know-how and core-periphery relations. *Health & Place*, 48, pp.56–62. doi:https://doi.org/10.1016/j.healthplace.2017.09.003.

Byerley, D. (2020). *APUS: An Introduction to Nutrition 2e (Byerley)*. [online] Medicine LibreTexts. Available at: https://med.libretexts.org/Courses/American_Public_University/APUS%3A_An_Introduction_to_Nutrition_(Byerley)/APUS%3A_An_Introduction_to_Nutrition_2e_(Byerley).

Callahan, A., Leonard, H., Powell, T. (2020). *Nutrition: Science and Everyday Application*. [online] *openoregon.pressbooks.pub*. OpenOregon. Available at: https://openoregon.pressbooks.pub/nutritionscience/.

Cardinal, K.M., Moraes, M.L. de, Andretta, I., Schirmann, G.D., Belote, B.L., Barrios, M.A., Santin, E. and Ribeiro, A.M.L. (2019). Growth performance and intestinal health of broilers fed a standard or low-protein diet with the addition of a protease. Revista Brasileira de Zootecnia, [online] 48. doi:https://doi.org/10.1590/rbz4820180232.

Chadha, G. (2022). *Precision health plans for a better you | wellOwise*. [online] wellowise.com. Available at: https://wellowise.com/blog/the-power-of-protein.

Chander, Y., Gupta, S.C., Goyal, S.M. and Kumar, K. (2007). Antibiotics: Has the magic gone? *Journal of the Science of Food and Agriculture*, 87(5), pp.739–742. doi:https://doi.org/10.1002/jsfa.2764.

Chang'a, E.P., Abdallh, M.E., Ahiwe, E.U., Al-Qahtani, M., Mbaga, S. and Iji, P.A. (2019). Energy utilization, nutrient digestibility and bone quality of broiler chickens fed Tanzania-type diets in different forms with enzymes. *Journal of Animal Science and Technology*, [online] 61(4), pp.192–203. doi:https://doi.org/10.5187/jast.2019.61.4.192.

Chrystal, P.V., Moss, A.F., Khoddami, A., Naranjo, V.D., Selle, P.H. and Liu, S.Y. (2020). Effects of reduced crude protein levels, dietary electrolyte balance, and energy density on the performance of broiler chickens offered maize-based diets with evaluations of starch, protein, and amino acid metabolism. *Poultry Science*, 99(3), pp.1421–1431. doi:https://doi.org/10.1016/j.psj.2019.10.060.

Cooper, G.M. and Sunderland, M. (2000). *The Central Role of Enzymes as Biological Catalysts*. [online] National Library of Medicine. Available at: https://www.ncbi.nlm.nih.gov/books/NBK9921/.

Cordeiro, C.M.M., Esmaili, H., Ansah, G. and Hincke, M.T. (2013). Ovocalyxin-36 Is a Pattern Recognition Protein in Chicken Eggshell Membranes. *PLoS ONE*, 8(12), p.e84112. doi:https://doi.org/10.1371/journal.pone.0084112.

Cowieson, A.J., Abdollahi, M.R., Zaefarian, F., Pappenberger, G. and Ravindran, V. (2018). The effect of a mono-component exogenous protease and graded concentrations of ascorbic acid on the performance, nutrient digestibility and intestinal architecture of broiler chickens. *Animal Feed Science and Technology*, [online] 235, pp.128–137. doi:https://doi.org/10.1016/j.anifeedsci.2017.11.018.

Cowieson, A.J., Lu, H., Ajuwon, K.M., Knap, I. and Adeola, O. (2017). Interactive effects of dietary protein source and exogenous protease on growth performance, immune competence and jejunal health of broiler chickens. *Animal Production Science*, 57(2), p.252. doi:https://doi.org/10.1071/an15523.

Daveson, A.J.M., Popp, A., Taavela, J., Goldstein, K.E., Isola, J., Truitt, K.E., Mäki, M., Anderson, R.P., Adams, A., Andrews, J., Behrend, C., Brown, G., Chen Yi Mei, S., Coates, A., Daveson, A.J., DiMarino, A., Elliott, D., Epstein, R., Feyen, B. and Fogel, R. (2020). Baseline quantitative histology in therapeutics trials reveals villus atrophy in most patients with coeliac disease who appear well controlled on gluten-free diet. *GastroHep*, 2(1), pp.22–30. doi:https://doi.org/10.1002/ygh2.380.

Dean, J. (2021). *Structural Protein Function | Protocol*. [online] www.jove.com. Available at: https://www.jove.com/science-education/11524/structural-protein-function.

Dharne, H. (2008). *Maintaining Gut Integrity*. [online] www.thepoultrysite.com. Available at: https://www.thepoultrysite.com/articles/maintaining-gut-integrity.

Diaz Carrasco, J.M., Casanova, N.A. and Fernández Miyakawa, M.E. (2019). Microbiota, Gut Health and Chicken Productivity: What Is the Connection? *Microorganisms*, 7(10), p.374. doi:https://doi.org/10.3390/microorganisms7100374.

Dibner, J.J. and Richards, J.D. (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Science*, [online] 84(4), pp.634–643. doi:https://doi.org/10.1093/ps/84.4.634.

Dittoe, D.K., Olson, E.G. and Ricke, S.C. (2022). Impact of the gastrointestinal microbiome and fermentation metabolites on broiler performance. Poultry Science, 101(5), p.101786. doi:https://doi.org/10.1016/j.psj.2022.101786.

Ducatelle, R., Goossens, E., De Meyer, F., Eeckhaut, V., Antonissen, G., Haesebrouck, F. and Van Immerseel, F. (2018). Biomarkers for monitoring intestinal health in poultry: present status and future perspectives. *Veterinary Research*, 49(1). doi:https://doi.org/10.1186/s13567-018-0538-6.

Duff, A.F., Vuong, C.N., Searer, K.L., Briggs, W.N., Wilson, K.M., Hargis, B.M., Berghman, L.R. and Bielke, L.R. (2019). Preliminary studies on development of a novel subunit vaccine targeting Clostridium perfringens mucolytic enzymes for the control of necrotic enteritis in broilers. *Poultry Science*, 98(12), pp.6319–6325. doi:https://doi.org/10.3382/ps/pez448.

Duque-Ramírez, C.F., Javierre, J.A., Peñuela-Sierra, L.M. and Diaz-Vargas, M. (2023). Effect of exogenus protease on performance, nutrient digestibility, intestinal histomorphometric, meat

quality characteristics, carcass yield in broilers fed low protein diets. Tropical Animal Health and Production, [online] 55(3). doi:https://doi.org/10.1007/s11250-023-03562-y.

Esmail, S., H (2016). *Understanding protein requirements*. [online] Poultry World. Available at: https://www.poultryworld.net/health-nutrition/understanding-protein-requirements/#:~:text=For%20broiler%20chickens%2C%20diets%20are%20often%20formulated%20to%20contain%2022.

Forthal, D.N. (2014). Functions of Antibodies. *Microbiology spectrum*, [online] 2(4), pp.1–17. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4159104/.

Fu, Y., Alenezi, T. and Sun, X. (2022). Clostridium perfringens-Induced Necrotic Diseases: An Overview. *Immuno*, 2(2), pp.387–407. doi:https://doi.org/10.3390/immuno2020024.

Fuller, R. (2001). The Chicken Gut Microflora and Probiotic Supplements. *The Journal of Poultry Science*, 38(3), pp.189–196. doi:https://doi.org/10.2141/jpsa.38.189.

Gabella, G. (1985). Chicken gizzard. *Anatomy and Embryology*, 171(2), pp.151–162. doi:https://doi.org/10.1007/bf00341409.

Hacker, J., Ott, M. and Hof, H. (1993). Effects of low, subinhibitory concentrations of antibiotics on expression of a virulence gene cluster of pathogenic Escherichia coli by using a wild-type gene fusion. *International Journal of Antimicrobial Agents*, [online] 2(4), pp.263–270. doi:https://doi.org/10.1016/0924-8579(93)90060-I.

Haurowitz, F. and Koshland, D.E. (2019). Protein | Definition, Structure, & Classification. In: *Encyclopædia Britannica*. [online] Available at: https://www.britannica.com/science/protein.

Huyghebaert, G., Ducatelle, R. and Immerseel, F.V. (2011). An update on alternatives to antimicrobial growth promoters for broilers. *The Veterinary Journal*, 187(2), pp.182–188. doi:https://doi.org/10.1016/j.tvjl.2010.03.003.

Jacob, J. (2019). *Avian Digestive System – Small and backyard poultry*. [online] Extension.org. Available at: https://poultry.extension.org/articles/poultry-anatomy/avian-digestive-system/.

Kapoor, G., Saigal, S. and Elongavan, A. (2017). Action and Resistance Mechanisms of antibiotics: a Guide for Clinicians. *Journal of Anaesthesiology Clinical Pharmacology*, [online] 33(3), p.300. doi:https://doi.org/10.4103/joacp.joacp_349_15.

Ketnawa, S. and Rawdkuen, S. (2011). Application of Bromelain Extract for Muscle Foods Tenderization. *Food and Nutrition Sciences*, 02(05), pp.393–401. doi:https://doi.org/10.4236/fns.2011.25055.

Kirchhelle, C. (2018). Pharming animals: a global history of antibiotics in food production (1935–2017). *Palgrave Communications*, [online] 4(1). doi:https://doi.org/10.1057/s41599-018-0152-2.

Kleyn, P (2014). *The Use of Anti-biotic Growth Promoters in South Africa: Some Perspectives | Spesfeed*. [online] Spesfeed Consulting. Available at: https://spesfeed.com/2014/04/the-use-of-anti-biotic-growth-promoters-in-south-africa-some-perspectives/ [Accessed 28 Mar. 2023].

Li, C.-Y., Lu, J.-J., Wu, C.-P. and Lien, T.-F. (2014). Effects of probiotics and bromelain fermented soybean meal replacing fish meal on growth performance, nutrient retention and carcass traits of broilers. *Livestock Science*, 163, pp.94–101. doi:https://doi.org/10.1016/j.livsci.2014.02.005.

Lopez, M.J. and Mohiuddin, S.S. (2022). *Biochemistry, essential amino acids*. [online] PubMed. Available at: https://www.ncbi.nlm.nih.gov/books/NBK557845/.

M'Sadeq, S.A., Wu, S., Swick, R.A. and Choct, M. (2015). Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. *Animal Nutrition*, 1(1), pp.1–11. doi:https://doi.org/10.1016/j.aninu.2015.02.004.

Macwhirter, P. (2009). *Basic anatomy, physiology and nutrition*. [online] ScienceDirect. Available at: https://www.sciencedirect.com/science/article/pii/B978070202874800002X [Accessed 6 Apr. 2022].

Manzoor, Z., Nawaz, A., Mukhtar, H. and Haq, I. (2016). Bromelain: Methods of Extraction, Purification and Therapeutic Applications. *Brazilian Archives of Biology and Technology*, 59(0). doi:https://doi.org/10.1590/1678-4324-2016150010.

Martins, B.C., Rescolino, R., Coelho, D.F., Zanchetta, B., Tambourgi, E.B. and Silveira, E. (2014). Characterization of Bromelain from Ananas Comosus Agroindustrial Residues Purified by Ethanol Factional Precipitation. *The Italian Association of Chemical Engineering*, [online] 37. doi:https://doi.org/10.3303/CET1437131.

Mavromichalis, I. (2016). *How excess protein robs broiler profitability*. [online] www.wattagnet.com. Available at: https://www.wattagnet.com/articles/25678-how-excess-protein-robs-broiler-profitability.

Miles, R.D., Butcher, G.D., Henry, P.R. and Littell, R.C. (2006). Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. *Poultry Science*, 85(3), pp.476–485. doi:https://doi.org/10.1093/ps/85.3.476.

Mohamed Tap, F., Abd Majid, F., Ismail, H., Wong, T., Shameli, K., Miyake, M. and Ahmad Khairudin, N. (2018). In Silico and In Vitro Study of the Bromelain-Phytochemical Complex Inhibition of Phospholipase A2 (Pla2). *Molecules*, [online] 23(1), p.73. doi:https://doi.org/10.3390/molecules23010073.

Mohammadigheisar, M. and Kim, I.H. (2018). Addition of a protease to low crude protein density diets of broiler chickens. *Journal of Applied Animal Research*, 46(1), pp.1377–1381. doi:https://doi.org/10.1080/09712119.2018.1512862.

Molnar, C. and Gair, J. (2019). *15.3 Digestive System Processes – Concepts of Biology-1st Canadian Edition*. [online] Opentextbc.ca. Available at: https://opentextbc.ca/biology/chapter/15-3-digestive-system-processes/.

Moore, D. (2019). *Antibiotic Classification & Mechanism - Basic Science - Orthobullets*. [online] Orthobullets.com. Available at: https://www.orthobullets.com/basic-science/9059/antibiotic-classification-and-mechanism [Accessed 9 Nov. 2019].

Moore, D.R. and Soeters, P.B. (2015). The Biological Value of Protein. *The Importance of Nutrition as an Integral Part of Disease Management*, pp.39–51. doi:https://doi.org/10.1159/000382000.

Munir, K. and Maqsood, S. (2013). A review on role of exogenous enzyme supplementation in poultry production. *Emirates Journal of Food and Agriculture*, 25(1), p.66. doi:https://doi.org/10.9755/ejfa.v25i1.9138.

Nanduri, B., Lawrence, M.L., Boyle, C.R., Ramkumar, M. and Burgess, S.C. (2006). Effects of Subminimum Inhibitory Concentrations of Antibiotics on the *Pasteurella multocida* Proteome. *Journal of Proteome Research*, 5(3), pp.572–580. doi:https://doi.org/10.1021/pr050360r.

Nguyen, D.T.N, Lee, S.Y, Cheong, J.H & Kim, I. H. (2018). Influence of low-protein diets and protease and bromelain supplementation on growth performance, nutrient digestibility, blood urine nitrogen, creatinine, and faecal noxious gas in growing–finishing pigs. Canadian Journal of Animal Science, 98(3), 488–497. https://doi.org/10.1139/cjas-2016-0116

Nguyen, D.T.N. (2021). Relationship between the ratio of villous height:crypt depth and gut bacteria counts as well production parameters in broiler chickens. *The Journal of Agriculture and Development*, 20(03), pp.1–10. doi:https://doi.org/10.52997/jad.1.03.2021.

Oirere, S. (2019). *Meat consumption rising in South Africa | 2019-07-24 | World Grain*. [online] www.world-grain.com. Available at: https://www.world-grain.com/articles/12371-meat-consumption-rising-in-south-africa [Accessed 28 Mar. 2023].

Pan, D. and Yu, Z. (2013). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*, 5(1), pp.108–119. doi:https://doi.org/10.4161/gmic.26945.

Park, J.H., Lee, S.I. and Kim, I.H. (2020). The effect of protease on growth performance, nutrient digestibility, and expression of growth-related genes and amino acid transporters in broilers. *Journal of Animal Science and Technology*, [online] 62(5), pp.614–627. doi:https://doi.org/10.5187/jast.2020.62.5.614.

Pavan, R., Jain, S., Shraddha and Kumar, A. (2012). Properties and Therapeutic Application of Bromelain: A Review. *Biotechnology Research International*, 2012, pp.1–6. doi:https://doi.org/10.1155/2012/976203.

Ponomarenko, E.A., Poverennaya, E.V., Ilgisonis, E.V., Pyatnitskiy, M.A., Kopylov, A.T., Zgoda, V.G., Lisitsa, A.V. and Archakov, A.I. (2016). The Size of the Human Proteome: The Width and Depth. *International Journal of Analytical Chemistry*, 2016, pp.1–6. doi:https://doi.org/10.1155/2016/7436849.

Pourabedin, M. and Zhao, X. (2015). Prebiotics and Gut Microbiota in chickens. *FEMS Microbiology Letters*, 362. doi:https://doi.org/10.1093/femsle/fnv122.

Rajaguru, R.W.A.S.B., Vohra, P. and Kratzer, F.H. (1966). The Effects of Feeding High Protein Diets to Chickens. *Poultry Science*, 45(6), pp.1339–1345. doi:https://doi.org/10.3382/ps.0451339.

Reddy, M.K. (2019). amino acid | Definition, Structure, & Facts. In: *Encyclopædia Britannica*. [online] Available at: https://www.britannica.com/science/amino-acid.

Rix, I., Nexøe-Larsen, C., Bergmann, N.C., Lund, A. and Knop, F.K. (2019). *Glucagon Physiology*. [online] Nih.gov. Available at: https://www.ncbi.nlm.nih.gov/books/NBK279127/.

Rogers, K. (2020). *proteolytic enzyme | Britannica*. [online] www.britannica.com. Available at: https://www.britannica.com/science/proteolytic-enzyme/additional-info#history [Accessed 28 Mar. 2023].

Roque, B., Appuhamy, J.A.D.R.N. and Kebreab, E. (2017). *Role of exogenous enzyme supplementation to improve nutrition and health of ruminants | Feedipedia*. [online] www.feedipedia.org. Available at: https://www.feedipedia.org/content/role-exogenous-enzyme-supplementation-improve-nutrition-and-health-ruminants [Accessed 27 Mar. 2023].

Salim, H.MD., Huque, K.S., Kamaruddin, K.M. and Haque Beg, A. (2018). Global Restriction of Using Antibiotic Growth Promoters and Alternative Strategies in Poultry Production. *Science Progress*, 101(1), pp.52–75. doi:https://doi.org/10.3184/003685018x15173975498947.

Selaledi, A.L., Mohammed Hassan, Z., Manyelo, T.G. and Mabelebele, M. (2020). The Current Status of the Alternative Use to Antibiotics in Poultry Production: An African Perspective. *Antibiotics*, 9(9), p.594. doi:https://doi.org/10.3390/antibiotics9090594.

Shahbandeh, M. (2020). *Poultry meat: per capita consumption in South Africa 2010-2017*. [online] Statista. Available at: https://www.statista.com/statistics/806994/poultry-meat-per-capita-consumption-south-africa/ [Accessed 28 Mar. 2023].

Singh, R. (2020). *Tips to improve feed conversion ratio (FCR) in poultry – Pashudhan praharee*. [online] Pashudhan Praharee. Available at: https://www.pashudhanpraharee.com/tips-to-improve-feed-conversion-ratio-fcr-in-poultry/.

Smith, Y (2018). *Protein Structure and Function*. [online] News-Medical.net. Available at: https://www.news-medical.net/life-sciences/Protein-Structure-and-Function.aspx.

Sun, P.D., Foster, C.E. and Boyington, J.C. (2004). Overview of Protein Structural and Functional Folds. *Current Protocols in Protein Science*, 35(1). doi:https://doi.org/10.1002/0471140864.ps1701s35.

Taylor, P.W., Gaunt, H. and Unger, F.M. (1981). Effect of subinhibitory concentrations of mecillinam on the serum susceptibility of Escherichia coli strains. *Antimicrobial Agents and Chemotherapy*, 19(5), pp.786–788. doi:https://doi.org/10.1128/aac.19.5.786.

Umar, S. (2010). Intestinal Stem Cells. Current Gastroenterology Reports, [online] 12(5), pp.340–348. doi:https://doi.org/10.1007/s11894-010-0130-3.

Untari, T., Herawati, O., Anggita, M., Asmara, W., Wahyuni, A.E.T.H. and Wibowo, M.H. (2021). The Effect of Antibiotic Growth Promoters (AGP) on Antibiotic Resistance and the Digestive System of Broiler Chicken in Sleman, Yogyakarta. *BIO Web of Conferences*, [online] 33, p.04005. doi:https://doi.org/10.1051/bioconf/20213304005.

Van den Bogaard, A. and Stobberingh, E.E. (2000). Epidemiology of resistance to antibiotics Links between animals and humans. *International Journal of Antimicrobial Agents*, [online] 14(4), pp.327–335. doi:https://doi.org/10.1016/s0924-8579(00)00145-x.

Van Harn, J., Dijkslag, M.A. and van Krimpen, M.M. (2019). Effect of low protein diets supplemented with free amino acids on growth performance, slaughter yield, litter quality, and footpad lesions of male broilers. *Poultry Science*, 10(4868-4877). doi:https://doi.org/10.3382/ps/pez229.

Walk, C.L., Juntunen, K., Paloheimo, M. and Ledoux, D.R. (2019). Evaluation of novel protease enzymes on growth performance and nutrient digestibility of poultry: enzyme dose response. *Poultry Science*, 98(11), pp.5525–5532. doi:https://doi.org/10.3382/ps/pez299.

Wang, M., Yang, C., Wang, Q., Li, J., Huang, P., Li, Y., Ding, X., Yang, H. and Yin, Y. (2020). The relationship between villous height and growth performance, small intestinal mucosal enzymes activities and nutrient transporters expression in weaned piglets. *Journal of Animal Physiology and Animal Nutrition*, 104(2), pp.606–615. doi:https://doi.org/10.1111/jpn.13299.

Wegener, H.C. (2012). *Antibiotic resistance- linking human and animal health*. [online] Nih.gov. Available at: https://www.ncbi.nlm.nih.gov/books/NBK114485/.

Wethli, E., Morris, T.R. and Shresta, T.P. (1975). The effect of feeding high levels of low-quality proteins to growing chickens. *The British Journal of Nutrition*, [online] 34(3), pp.363–373. doi:https://doi.org/10.1017/s0007114575000426.

Wielsma, G. (2018). *Webinar: Gut health in young broilers*. [online] www.youtube.com. Available at: https://www.youtube.com/watch?v=4GZdnC7DMmg&ab_channel=PerstorpGroup [Accessed 27 Mar. 2023].

Wilcox, G. (2005). Insulin and Insulin Resistance. *Clinical Biochemist Reviews*, [online] 26(2), pp.19–39. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1204764.

Xu, X., Wang, H.L., Pan, L., Ma, X.K., Tian, Q.Y., Xu, Y.T., Long, S.F., Zhang, Z.H. and Piao, X.S. (2017). Effects of coated proteases on the performance, nutrient retention, gut morphology and carcass traits of broilers fed corn or sorghum based diets supplemented with soybean meal. *Animal Feed Science and Technology*, [online] 223, pp.119–127. doi:https://doi.org/10.1016/j.anifeedsci.2016.10.015.

Xue, P.C., Ajuwon, K.M. and Adeola, O. (2016). Phosphorus and nitrogen utilization responses of broiler chickens to dietary crude protein and phosphorus levels. *Poultry Science*, 95(11), pp.2615–2623. doi:https://doi.org/10.3382/ps/pew156.

Yadav, S. and Jha, R. (2019). Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. Journal of Animal Science and Biotechnology, 10(1). doi:https://doi.org/10.1186/s40104-018-0310-9.

Yegani, M. and Korver, D.R. (2008). Factors Affecting Intestinal Health in Poultry. *Poultry Science*, 87(10), pp.2052–2063. doi:https://doi.org/10.3382/ps.2008-00091.

Yenice, G., Iskender, H., Dokumacioglu, E., Kaynar, Ö., Kaya, A., Hayirli, A. and Sezmiş, G. (2019). Dietary bromelain supplementation for improving laying performance, egg quality and antioxidant status Nahrungsergänzung mit bromelain zur Verbesserung der Legeleistung, der Eiqualität und des Antioxidanszustandes. *European Poultry Science*, [online] 83. doi:https://doi.org/10.1399/eps.2019.272.

Yıldız, D. (2021). *Pellet and mash preference in feed production & production technologies*. [online] Feed & Additive Magazine. Available at: https://www.feedandadditive.com/pellet-and-mash-preference-in-feed-production-production-technologies/ [Accessed 28 Mar. 2023].

Yu, B., Lee, T.T.T. and Chiou, P.W.-S. (2010). Effects of sources of protein and enzyme supplementation on protein digestibility and chyme characteristics in broilers. *British Poultry Science*, 43(3), pp.424–431. doi:https://doi.org/10.1080/00071660120103701.

Zhao, P.Y., Li, H.L., Hossain, M.M. and Kim, I.H. (2015). Effect of emulsifier (lysophospholipids) on growth performance, nutrient digestibility and blood profile in weanling pigs. *Animal Feed Science and Technology*, 207, pp.190–195. doi:https://doi.org/10.1016/j.anifeedsci.2015.06.007.

Zhou, Y., Xia, M., Ye, Y. and Hu, C. (2004). Antimicrobial ability of Cu-montmorillonite. *Applied Clay Science*, 27(3-4), pp.215–218. doi:https://doi.org/10.1016/j.clay.2004.06.002.

Zimmerman, M. and Snow, B. (2012). *An Introduction to Nutrition - Table of Contents*. [online] 2012books.lardbucket.org. Available at: https://2012books.lardbucket.org/books/an-introduction-to-nutrition/index.html.