EVALUATING THE EFFICACY OF EXOGENOUS COMPOSITE MICROBIAL ENZYMES IN MAIZE-SOYBEAN BASED BROILER CHICKEN FEEDS

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ABSTRACT

This research reported here was carried out to examine alternatives to antibiotic growth promoters as a result of their being banned in the animal feed industry. Four experiments were conducted to evaluate the efficacy of non-medicated feed additives as replacements for antibiotic growth promoters in broiler feeds. The additives used were enzymes (a new thermo-tolerant powder enzyme called TXAP, phytase, lipase and a new phytase enzyme derived from *E. coli* called phyzyme XP), organic acid (Acid Pak), prebiotic (Bio-Mos®) and probiotic (All-Lac XCL). Mashed maize-soya based feeds were used in all the experiments, which were conducted in litter-floor pens.

The first experiment was a dose-response trial. Broilers in eight replicate pens of 50 males and 50 females were fed unsupplemented feeds and five additional feeds containing increasing levels of TXAP, from 0.5 to 2.5 g/kg to 42 d. The second experiment used enzyme TXAP with two different enzymes (phytase and lipase), individually or in combination. Six replicate pens of 50 males and 50 females were fed either unsupplemented feeds or one of six additional feeds treated with TXAP, lipase, phytase, a combination of TXAP and lipase, a combination of TXAP and phytase or a combination of all the three enzymes. This trial continued for 42 d.

In the third experiment three types of TXAP (Lot 1, 2 and 3) were used, with fixed levels of xylanase and amylase but varying levels of protease activities (4000, 2000 and 1000 U/kg for Lot 1, 2 and 3, respectively) in combination with phyzyme XP for 35 d. The fourth experiment used mannan-oligosaccharide (Bio-Mos®), organic acid (Acid pak 2x), probiotic (All Lac XCL 5x), individually or in combination and an antibiotic growth

promoter (Zinc bacitracin) for 42 d. The chickens in this experiment were challenged with *Clostridium perfringens* (*CP*) at 21, 22 and 23 d to determine the efficacy of these additives for replacing antibiotics in hindering the effects of *CP* on the villus surface area.

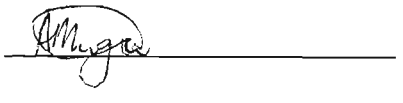
The dose-response trial did not show any significant improvement in broiler performance with any level of inclusion of enzyme TXAP. The results from this study showed some beneficial effects with the use of enzyme TXAP when fed alone and at a young age. Its use when combined with other enzymes and at later stages of growth needs further investigation. Feed additives in experiment 4 prevented the negative effects of *CP* as the treated chickens did not have lesions on their villus surfaces.

The conditions under which these trials were conducted appeared to be such that little benefit was derived from the use of any of the feed additives used. It is possible that under less-hygienic conditions such as those in commercial operations greater benefits from these additives may be realised.

DECLARATION

The experimental work described in this dissertation was carried out in the Discipline of Animal and Poultry Science, Faculty of Science and Agriculture, University of KwaZulu-Natal, Pietermaritzburg, from January 2004 to February 2006, under the supervision of Professor Rob Gous.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others this has been duly acknowledged in the text.



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CHAPTER ONE

GENERAL INTRODUCTION

The feed and animal production industry is faced with a number of challenges. Among them are the pressures to produce high quality products to satisfy consumer demand in a cost effective manner. To attain this there is a need to ensure that the feed supplied to animals is of a high quality.

Poultry feeds are composed mainly of cereal grains from which the nutrients essential for maintenance and production are derived. The quality of poultry feeds therefore depends largely on the digestibility and availability of nutrients from these cereal grains. For example, some of the phosphorus in these grains is in the form of phytic acid (phytate) (Lan *et al.*, 2002), which, because it cannot be digested by poultry, is not available to birds (Cowieson *et al.*, 2003).

In addition to nutrients in cereal grains that may not be available to poultry there are also anti-nutritional factors associated with these grains. These are elements that have the potential to bind other nutrients, thus rendering them unavailable to the bird. Phytate (Ravindran *et al.*, 2001) and fibre (Brenes *et al.*, 2003) are examples of such factors. They bind to minerals and amino acids and therefore create a shortage of these nutrients. There is a need therefore to provide additional nutrients to supplement those that are bound to these anti-nutritional factors.

Many strategies have been explored to improve the availability of nutrients to poultry. These include the addition of antibiotics (Edens, 2003) and more digestible, essential

nutrients like amino acids and inorganic phosphorus to supplement those nutrients that are unavailable in the feedstuffs used (Cowieson *et al.*, 2003; 2004). Lan *et al.* (2002) indicated that the supplementation of these more-digestible nutrients increases feed cost. This also, to some degree, contributes to environmental pollution, due to the undigested nutrients excreted with the faeces (Cowieson *et al.*, 2004). On the other hand, antibiotics have been found to decrease the level of activity of the immune system, and their routine use as growth promoters have had a negative impact on human medicine and may also have destroyed even the beneficial microbes (Percival, 1999). This has led to their ban by the European Union for use as growth promoters, and the likelihood that such a ban will soon be placed on their use in South Africa (Koster, 2004).

Therefore, safe alternatives to antibiotic growth promoters in the poultry industry have had to be considered. The ability of exogenous enzymes (Bedford and Morgan, 1996), organic acids (Patten and Waldroup, 1988; Mujdat *et al.*, 1999) and oligosaccharides (Iji and Tivey, 1998; Iji *et al.*, 2001) to act as growth promoters in broilers has been well documented. These are commonly used in the commercial feed industry for the full utilisation of feedstuffs that were under-utilised due to the anti-nutritional properties they possess.

The removal of antibiotic growth promoters from the animal feed industry has elevated the risk of diseases and lowered the growth rate of chickens. The objective of this study was thus to investigate alternatives to antibiotic growth promoters in broiler nutrition.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction

High levels of production and efficient feed conversion efficiency characterise the modern poultry industry, much of this efficiency having been attained by improved nutrition. Many strategies have been explored to improve nutrient availability to the birds, including supplementation with antibiotics (Edens, 2003) and readily available nutrients (Lee *et al.,* 2003) like phosphorus to supplement those that are unavailable from the feedstuffs (Cowieson *et al.,* 2003). Some of the strategies are expensive and to some degree contribute to environmental pollution, due to the undigested nutrients being excreted in the faeces (Bedford, 2000). Addition of exogenous enzymes to poultry feed is therefore seen as a better solution to reduce pollution associated with poultry manure (Rutherfurd *et al.*, 2004), improve feed efficiency and reduce feed costs (Classen and Bedford, 1999; Cowieson *et al.,* 2003; Cowieson *et al.,* 2004).

Enzyme preparations were used in foods long before there was any awareness of enzymes as such. The industrial use of microbial enzymes in the Western world started a number of years ago (Marquardt, 1997; Hong *et al.,* 2002; Bedford, 2003) with the patenting of a process for the production of alpha-amylase from fungi. Recently, genes encoding for different enzymes, including phytases, ß-glucanases and xylanases, have been cloned and expressed in different commercial systems (microorganisms and plants) (Marquardt, 1997; Hong *et al.,* 2002; Ghazi *et al.,* 2003).

Currently microbial enzymes are commonly used in commercial feeds, particularly in poultry feeds. This is mainly because cereal grains, which are the main components of poultry feeds, contain components that prevent or reduce the digestion of certain components of the feed by poultry. This results in some feedstuffs being under-utilized because of poor nutrient availability (Marquardt, 1997; Classen and Bedford, 1999).

During the early stages of development, enzymes were used only to target problem ingredients. These included cereal grains that are high in fibre and phytic acid (Classen and Bedford, 1999). Dietary fibre increases the viscosity of the digesta in the gastrointestinal tract (Jozefiak *et al.*, 2004), which renders other nutrients unavailable. On the other hand about two thirds of the phosphorus in cereal grains is in the form of phytic acid, which cannot be digested by poultry (Camden *et al.*, 2001; Cowieson *et al.*, 2003). These were the key problems with grains like wheat, barley, oats, rye and triticale (Classen and Bedford, 1999; Wu *et al.*, 2004).

Recent research on microbial enzymes has focussed on feed ingredients that were known to be of high quality. These include maize (Iji *et al.*, 2003) and soya (Ghazi *et al.*, 2003). The nutrient consistency and digestibility of maize/soya-based poultry feeds have generally been considered to be high, such that enzyme addition would have no benefits (Hong *et al.*, 2002). However, recent data indicate that there is room for improvement (Iji *et al.*, 2003), especially as a result of changes in composition during growth, harvesting and processing.

Enzymes are included in poultry feeds to reduce the concentration of antinutritional factors in the feeds, to increase nutrient digestibility and to supplement the enzymes secreted by the bird's own digestive system (Ferket, 1993; Jackson, 1998; Kim and Baker, 2003). Similar enzyme preparations have been promoted for swine feeds (Kim and Baker, 2003), but economic responses are less predictable.

Previous investigations involved the use of single enzyme supplements and not composite supplements (Jackson, 1998; Hong *et al.*, 2002; Rutherfurd *et al.*, 2004). For example, although Jackson (1998) compared five commercial enzymes, there was no trial in which enzyme mixtures were tested. However, Brenes *et al.* (2002) used a mixture of three enzymes and obtained good results.

This review concerns the nutrients required by broilers and the mechanisms by which the availability of those nutrients might be improved, thereby achieving maximum productivity.

2.2. Broiler production cycle

Broiler breeders have been selected for a combination of increased growth rate, decreased fatness and increased muscularity. Such selection has complex effects on nutritional and environmental requirements (Emmerson, 1997; Hermesch, 2004). Appropriate changes to the nutrition of the progeny as selection proceeds would help to avoid unwanted effects from occurring. Improved genotypes would, for example, require appropriate ratios of nutrients to energy.

The major costs in chicken production are those associated with feed and time. An obvious way to decrease these costs is to select those animals that take less time to reach market weight. The maintenance costs of such broilers is reduced (Gous, 2000; Hermesch, 2004). To produce these fast-growing broilers, breeders continue to select for rapid growth rate and improved feed conversion efficiency (Table 2.1).

Table 2.1. *Changes in the performance of broilers over the past 50 years (Gous, 2000)*

Period	Days to 1.8 kg	Food per unit gain
1950	84	3.25
1960	70	2.50
1970	59	2.20
1980	51	2.10
1990	42	1.93
2000	36	1.55

From an economic standpoint the optimum production cycle at present is 5.92 weeks. A further reduction of the growth period would allow a broiler producer to raise an additional broiler flock annually in every broiler house, whereas an increase in feed conversion will contribute to further reduction in production costs (Emmerson, 1997). A production cycle of six weeks is considered very short and therefore must be used efficiently to maximise bird performance. Therefore feed producers and chicken rearers must work towards a common goal to obtain high productivity within this short period.

2.3. Nutrition

If poultry are expected to remain healthy and productive, they must consume adequate amounts of all the essential nutrients. The quantity of each required nutrient varies depending on many variables like species of bird, age, productive state, environmental

conditions and disease status. Fortunately, many nutritional deficiency problems can be identified by the unique symptoms each exhibits. Various ingredients are blended together to ensure that the feed supplied contains all the essential nutrients required by the broilers.

2.3.1. Ingredients

2.3.1.1. Energy sources

The major energy sources in poultry feeds are cereal grains with their high carbohydrate content, and fat-containing ingredients. The widely used cereal grains, depending on location (tropics or temperate regions), are maize, barley, sorghum, oats, rye, wheat and triticale.

Numerous studies have reported that there is considerable variation in the apparent metabolisable energy (AME) of cereal grains (Annison, 1993; Pan *et al.*, 1998; Hughes and Choct, 1999; Bedford, 2000; Wu *et al.*, 2004). This variation was also noticed in cereals that were considered highly nutritious (maize and sorghum) (Hong *et al.*, 2002; Lan *et al.*, 2002; Iji *et al.*, 2003). This low energy phenomenon of cereal grains was thought to be a result of the presence of soluble non-starch polysaccharides (NSPSs) that are contained in cereals. AME was found to be negatively correlated to cereal NSPSs because of the inhibitory effect of these NSPSs on the digestion of starch, lipid and protein.

Cereal grains also possess anti-nutritional factors other than NSPS's. Among these are protease inhibitors, amylase inhibitors, tannins and saponins, which impair the digestion of nutrients in the gastrointestinal tract of the bird (Hughes and Choct, 1999).

Hughes and Choct (1999) discovered that the variation in the properties of cereal grains is caused by season, site of growth and stage of ripeness. Their study showed that weight gain and feed conversion efficiency of broilers fed barley-based feeds was reduced when this cereal was harvested early. Sorghum and maize were thought to be free of anti-nutritional factors until Lan *et al.* (2002) and Rutherfurd (2004) discovered that the phytic acid contained in these cereals also has an anti-nutritive activity by chelating other nutrients, thus hindering their digestion and absorption by the bird. Most unimproved varieties of sorghum also contain tannins (King *et al.*, 2000; Iji *et al.*, 2004).

2.3.1.2. Protein sources

Sources of dietary protein in the poultry industry are either of plant or animal origin. Animal protein sources include meat and bone meal, meat meal, poultry meal, feather meal, blood meal and fishmeal. These products are derived from the production of meat products for human consumption. They result from rendering of animal and poultry tissue (meat, viscera, bone, blood and feathers) to extract fat and remove moisture. The resulting residues contain from about 50% protein (meat and bone meal) to more than 80% protein (blood meal and feather meal).

Because of the occurrence of bovine spongiform encephalopathy (BSE) in Europe, the use of animal protein in animal feeds has been restricted (Pearl, 2002; Rubio *et al.*,

2003). Nowadays, vegetable protein sources are used in increased quantity due to high cost, non-availability and presence of pathogenic microorganisms like *E. coli* and Salmonella in animal protein sources. On the other hand, fishmeal is associated with gizzard and intestinal erosion, depressed growth and feed utilization efficiency and off-flavours in broilers, which are correlated to the deposition of highly unsaturated fatty acids (Elkin, 2002; Butcher, *et al.*, 2002).

Plant protein sources include cereals, cereal by-products, oilseed meals and grain legumes. Cereals make up the bulk of poultry feedstuffs but do not supply enough of the protein required by birds. Therefore, oilseed meals are the most widely used protein sources in poultry feeds. Oilseeds that are used include soya beans, cottonseed, peanuts, and sunflower. Anti-nutritional factors associated with legume grains (Balloun, 1980; Sainsbury, 2000) prevent them from being fully utilized by poultry species, especially chickens, as they lack enzymes to break down these anti-nutritional factors.

2.3.1.3. Minor ingredients

Apart from energy and protein there are other nutrients that need to be included in the feed to ensure that all the bird's requirements are supplied. Some of these are needed by poultry in small amounts, but their deficiencies are detrimental to the bird, e.g. vitamins and trace minerals, as they support optimal performance.

Grains are low in vitamins and minerals, so it is necessary to supplement the feed with synthetic forms of the vitamins and minerals. This ensures that the bird has the required amounts necessary for normal productive efficiency (Beyer and Wilson, 2000, Islam *et al.*, 2004).

2.3.2. Nutrients

2.3.2.1. Carbohydrates

Carbohydrates are compounds that are composed of carbon, hydrogen and oxygen with the latter two elements in the same proportion as in water. These make up the largest portion of the dry matter of plants and are the chief source of energy in poultry feeds (Damron and Sloans, 1998). They include starch, cellulose, sugars and other non-starch compounds. Starch and sugars are easily digested by poultry and have a high feeding value. However, cellulose, the complex carbohydrate that forms the woody fibre of plants, is poorly digested and has a low feeding value for poultry.

Green plants build simple carbohydrates from carbon dioxide from the air and water from the soil through the action of chlorophyll in the leaves. The energy to produce carbohydrates comes from the sunlight absorbed through the plant tissue. Carbohydrates arise from photosynthesis, the most important chemical reaction in nature, as follows:

$$6CO_2 + 6H_2O + ADP \longrightarrow C_6H_{12}O_6 + 6O_2$$

Starch is the form in which most plants store reserve energy, and is the only complex carbohydrate that chickens can readily digest. The chicken does not have the enzymes required to digest cellulose and other complex carbohydrates. Carbohydrates are major energy sources for poultry (Damron and Sloans, 1998; Beyer and Wilson, 2000).

2.3.2.2. Protein

Dietary protein is the most costly part of the feed. Dietary protein is important for animal growth, development and maintenance. Crude protein is a measurement based on the

nitrogen content of a feed ingredient. Digestible protein is the protein in a feed that can be digested and used by the animal. It is usually about 50-80% of crude protein.

However, not all protein is the same. The components (amino acids) that make up crude protein can differ a great deal in type and concentration from one source to the next. Therefore the quality of protein also varies. It is the amino acids that are required by the bird, not protein *per se*. For nutritional purposes amino acids are grouped into essential and non-essential amino acids (Beyer and Wilson, 2000). Essential amino acids must be provided in the feed, while the animal itself when necessary can produce non-essential amino acids. The balance of amino acids in the feed is of paramount importance, as the animal will not perform well if the ratio of one or more amino acids to the others is not appropriate (Leeson, 2004).

Amino acids are essential for the growth and repair of worn-out tissues and to produce antibodies to fight diseases, as a component of protoplasm of living cells and as the primary constituent of many structural and protective tissues. Amino acids can be found in most feedstuffs, but synthetic amino acid supplements are often used to meet amino acid requirements. Cereal grains alone do not provide sufficient of the essential amino acids or a balanced amino acid profile, so they must be supplemented with dietary protein sources.

2.3.2.3. Dietary fat

Fats are sources of energy and in some cases fat-soluble vitamins. Like carbohydrates, they are organic compounds made up of carbon, hydrogen and oxygen, which form fatty

acids. Because they are higher in hydrogen and lower in oxygen than carbohydrates, fats have a higher energy value (ME) than carbohydrates (Wiseman, 2002).

Fats are important for the absorption of fat-soluble vitamins A, D, E and K and sources of essential fatty acids such as oleic, linoleic and linolenic acids. These essential fatty acids are responsible for membrane integrity, hormone synthesis and reproduction. Fats also improve growth rate, feed efficiency (Schaible, 1970), aid in reducing the dustiness of the feed (Beyer and Wilson, 2000) and promote feed palatability (Wiseman, 2002).

2.3.2.4. Vitamins

Vitamins are organic compounds, which are required in very small amounts. Like all other nutrients, they are essential part of a good nutrition program (Starcevic *et al.*, 2004). They are important for most physiological processes, including normal growth, feathering and leg development. Vitamins also provide animals with the ability to fight stress, disease and to maintain good health. Vitamins are divided into two classes: fat-soluble, which include A, D, E and K, and water-soluble, which include all the B-complex vitamins and vitamin C (Cheeke, 1999).

Deficiencies of various vitamins may cause problems such as skin lesions, nervous disorders, muscle problems, reduced egg production in layers, reduced growth in meat birds, and improper chick development in eggs from breeding birds. The severity of any of these problems will depend on which vitamin(s) is inadequate, and how deficient the feed is (Beyer and Wilson, 2000).

2.3.2.5. Minerals

Minerals are inorganic compounds, and are often classified as macro- or trace minerals in reference to the concentrations required. Macro-minerals such as calcium and phosphorus are generally required in larger quantities than trace minerals (iron, copper, iodine, etc.). In some cases the ratio of one to the other is also important, for example calcium and phosphorus. Minerals are important for structural components (such as bones) and/or physiological processes. They also aid in the construction of muscles, blood cells, internal organs and enzymes (Cheeke, 1999; Damron and Sloans, 1998).

2.3.2.6. Water

Water is the most abundant compound worldwide and not often thought of as a nutrient itself, but it is indeed a very important one. Animals must have frequent intakes of water to remain alive as it provides the basis for all the fluid of the animal's body. Water provides cells with pressure to allow them to hold their shape. Its functions include regulation of body temperature, traNSPsort of other nutrients, and taking part in numerous chemical reactions in the body. It also flushes the animal's body of waste and toxic materials (Damron and Sloans, 1998).

Water requirements (volume) of poultry are often crudely estimated by multiplying the amount of feed eaten by two (for example 1 kg feed: 2 litres of water). Under hot conditions however, they will drink substantially more (up to twice as much) (NRC, 1984; Beyer and Wilson, 2000).

2.3.3. Relationships between nutrients

Chickens eat to satisfy their requirement for the first-limiting nutrient in the feed (Emmans and Fisher, 1986) therefore there is an advantage in attempting to supply all the nutrients in amounts close to their requirement, thereby providing a feed balanced with respect to all essential nutrients. When a feed is unbalanced, i.e. one or more of the nutrients is included at a level below the requirement, the bird will consume more of the non-limiting nutrients than are required by the bird in an attempt to consume sufficient of the most limiting nutrient(s) (Gous *et al.*, 1990).

Where an amino acid is marginally limiting in a feed there is a marked increase in carcass fatness associated with increased ME (Figure 2.1), leading to increased slaughter weight (Morris, 2003). However, according to consumer preferences fatter carcasses are not in demand, thus affecting monetary returns. Therefore it is better to increase the ratio of limiting amino acids (lysine and methionine) to energy.

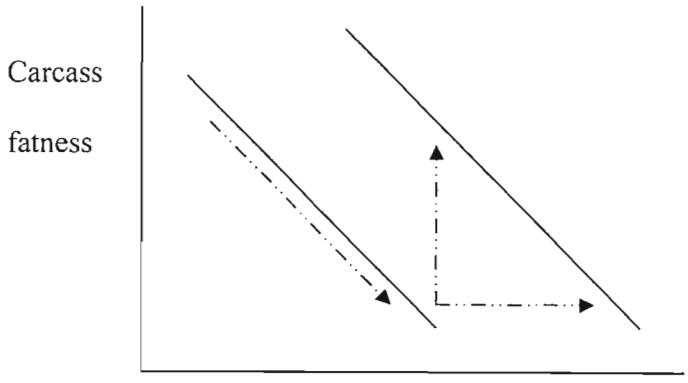


Figure 2.1. *The response of the carcass to increases in the ratio of limiting amino acids to ME (Morris, 2003).*

Generally there is an advantage in attempting to balance the nutrients according to the requirement of the bird because imbalances (of amino acids) and unbalances will result in the feed being utilised less efficiently. Where high protein feeds are used there is insufficient energy to efficiently utilise the excess protein, and the protein is therefore used with a lower efficiency than it would otherwise be, and the bird is very lean. The opposite is the case where excess energy is used; here the protein is used with maximum efficiency, but the excess energy is converted to lipid and the bird becomes excessively fat (Swatson *et al.*, 2002).

2.4. Enhancing productivity through the use of feed additives

2.4.1. Antibiotic growth promoters

The anti-nutritive activity of NSPSs is related to the gut microflora of the chicken and NSPS content in the feed (Annison and Choct, 1991). Addition of antibiotics to the feeds results in a marked increase in chick growth, feed conversion efficiency, feed intake and the retention of nutrients by the bird. Responses to antibiotic inclusion to feeds depend on the composition of the feed and the NSPS content in the cereal. Antibiotics work by suppressing the gut microflora (Percival, 1999), which competes with the bird for nutrients and depresses the productive performance of the bird.

Casewell *et al.* (2003) reported that the ban on the use of antibiotic growth promoters revealed that these additives had an important prophylactic activity. They reported that their withdrawal was associated with deterioration in animal health, including increased diarrhoea, weight loss and mortality due to *E. coli* in early post weaning pigs, and clostridial necrotic enteritis in broilers. NRC reports (1984) also ascribe the thinning of

the chick's intestinal wall to antibiotics, a mechanism by which they improve nutrient absorption.

Antibiotics were used worldwide as growth promoters until recently when they were reported to decrease the level of activity of the immune system in humans (Percival, 1999). Their routine use as growth promoters has been curtailed in certain regions because they may have a negative impact on human medicine. Overuse of antibiotics causes bacteria to mutate and develop resistance. Frei *et al.* (2001) reported a 100% resistance of *Enterococcus faecalis*, *E. faecium, Staphylococci* and *Lactobacilli* to bacitracin. Resistant bacteria may transfer the resistance to non-resistant bacteria, thus making them resistant. Antibiotics can also destroy the beneficial intestinal microflora, causing the proliferation of pathogenic microorganisms in humans (Percival, 1999). They are also associated with an increase in the viscosity of the digesta. As viscosity increases the rate of diffusion decreases, which causes decreased digestibility of all substrates. The undigested viscous digesta translates into very sticky manure, which causes wet litter problems (Bedford, 2000).

Antibiotic resistant *E. coli* were isolated from poultry industry workers, patients and chickens. The resistance was higher in chickens, thereby posing increased risk of transferring the pathogens to humans on consumption. *E. coli* is a common normal flora in the gastrointestinal tract of both man and animals and is also potentially pathogenic to both (Al-Ghamdi *et al.*, 1999). Control of intestinal pathogens during the earliest phases of broiler production may be the best strategy for the reduction of human pathogens in processed broiler carcasses. The recent ban on antibiotics in poultry feed has served to

focus much attention on alternative methods of controlling the gastrointestinal tract (GIT) microflora (Kleeseen *et al.*, 2003).

2.4.2. Readily available nutrients

The presence of anti-nutritive factors and a ban on antibiotics as growth promoters in poultry feeds in parts of the world has necessitated the addition of readily available nutrients to feeds. These include inorganic phosphates, essential oils and other nutrients that are rendered unavailable to the bird by the anti-nutritive factors.

Numerous authors have discovered that there are problems associated with the supplementation of readily available nutrients. Bedford (2000) and Lan *et al.* (2002) reported that supplementation of poultry feeds with these nutrients increases the feed formulation costs since all of these supplements are expensive.

Another problem associated with the supplementation of poultry feeds with readily available nutrients is that they increase the excretion of endogenous minerals and amino acids (Williams, 1995; Camden *et al.*, 2001; Cowieson *et al.*, 2003). These are responsible for environmental pollution problems, as too much nitrogen in the poultry manure causes poor air quality, while phosphorus pollutes the underground water.

2.4.3. Microbial enzymes

Enzymes are proteins, which function as catalysts in chemical reactions in the body where reactions are initiated or speeded up. Their primary function in the gut of the bird

is to activate the hydrolysis of large molecules into smaller ones that the bird can use (Chesson, 1993).

Starch is an important energy reserve in plants. The bird's enzyme system usually breaks down starch into simple sugars that are then absorbed. NSPs have a structural function and are found in the plant cell wall. They are not digested by birds and can interfere with digestion of other nutrients in the feedstuff. Enzymes are used to break down the cell wall polysaccharides into lower weight molecules, which can be absorbed by the bird (Choct, 1997).

Enzymes can be specific to cereals and are temperature, moisture and pH sensitive (Chesson, 1993). Poultry do not have the ability to digest fibre or NSPs because they lack the enzymes necessary to break down these large complex molecules. Ruminants, in contrast, have a large microbial population that synthesizes enzymes like cellulase that can digest fibre.

NSPs of grains are usually associated with the hull and outer layers and are inversely proportional to energy levels (Brenes *et al.*, 2003). Maize-soybean feeds have low NSPs and are assumed to be highly digestible. Therefore the use of enzymes for such feeds was thought not to be beneficial (Hong *et al.*, 2002). However, recent research (Iji *et al.*, 2003) has indicated that there is room for improvement.

Enzymes now manufactured specifically for feed use can be carbohydrases, proteases and lipases (Bedford, 2003). Enzymes that increase the digestibility of various carbohydrate fractions of cereals and plant proteins have received the most attention.

High fibre content of feed ingredients causes the bird to eat more, lowering feed conversion efficiency. Fibre is an anti-nutritional component, which limits feed intake, digestion of the food, and absorption of nutrients (Classen and Bedford, 1999; Jozefiak *et al.*, 2004).

Feed enzymes improve the digestion of NSPs availability and reduce the negative impact of these indigestible residues on gut viscosity. Enzymes have been developed that aid in the digestion of feeds containing wheat, barley and rye where improvements are seen in dry matter digestibility and consistency of excreta. Microbial enzymes work by removing the anti-nutritional factor, increasing the digestibility of existing nutrients that were formerly not accessible to endogenous enzymes, and by supplementing the bird's endogenous enzymes (Ferket, 1993; Jackson, 1998; Kim and Baker, 2003).

The NSPs are composed of hemicellulose, pectin and gum fractions whose components are more soluble than cellulose and interact with other nutrients in the gut, producing a viscous medium. Barley and wheat have cell walls containing building blocks (beta-glucans and arabinoxylans) that absorb water causing the intestinal digesta to become viscous (Annison and Choct, 1991; Campbell and Bedford, 1992).

In the normal digestion process there is unrestricted movement of enzymes, substrate and digesta throughout the gut, especially close to the gut wall where absorption of nutrients takes place (Figure 2.2) (Bedford, 2000).

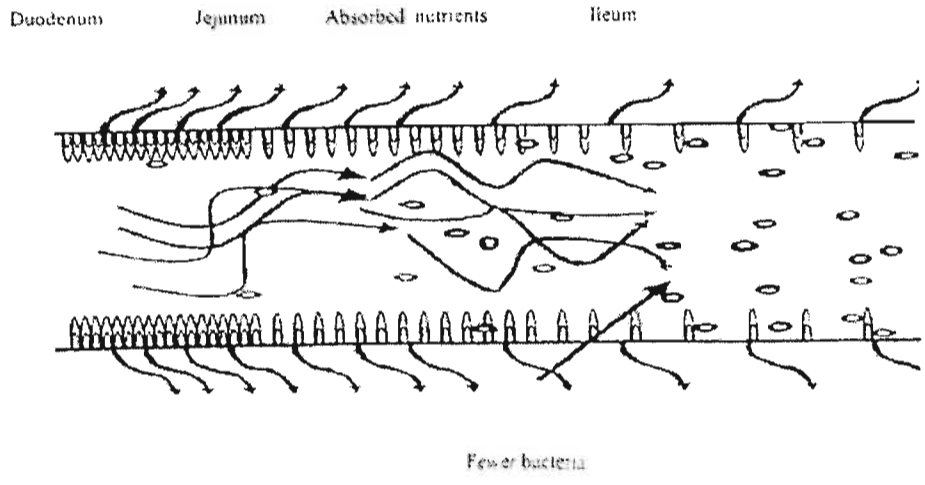


Figure 2.2. *Schematic representation of normal digestion where there are a limited number of gut microflora (Bedford, 2000).*

Viscous intestinal contents cause a decrease in the gut's ability to mix contents, prevent the action of enzymes in cells, and decrease contact of nutrients with gut wall and therefore reduce nutrient absorption. Undigested feed and water absorbed by NSPs are excreted, leading to poorer growth, decreased feed conversion efficiency and wetter litter. It also prolongs digesta passage rate, thereby limiting feed intake. Gut viscosity is decreased in direct relation with the enzyme's ability to digest substrates like beta-glucans (Bedford, 1995). Beta-glucans are the bulk of NSPs in oats and barley and arabinoxylans in wheat and rye.

For barley-based feeds the enzyme beta-glucanase is used, and for wheat-based feeds xylanase and arabinoxylanase are used. Some enzyme products may contain beta-glucanase, cellulase and xylanase for improved feed digestion. Phytase improves the utilization of phytate phosphorus in plant material. This enzyme liberates the

phosphorus, calcium and other minerals from the phytate complex (Pan *et al.*, 1998; Bedford, 2000; Camden *et al.*, 2001; Lan *et al.*, 2002; Selle *et al.*, 2003; Rutherfurd *et al.*, 2004; Wu *et al.*, 2004). Ration formulation must take this increased availability of phosphorus into account.

Bacterial counts in the intestine are higher with wheat, barley, and rye feeds than with maize-based feeds. The slower digesta passage rate with these cereals prevents major dislocation of bacterial populations. The viscous digesta provide a stable environment for microbial growth and proliferation, allowing for the establishment of bacteria in the upper small intestine. High intestinal bacterial populations irritate and thicken the gut lining by damaging microvilli and decreasing nutrient absorption (Bedford, 2000; Hong, 2002).

Broilers respond well to feed enzymes, as their rate of feed intake is more than that of other poultry. This consumption of feed overwhelms the broiler's ability to produce sufficient enzymes either from its own tissue or by the microflora in the gut. Feed enzymes used in wheat and barley feeds improve energy availability, improve nitrogen retention, and significantly affect protein digestion (feeds with enzymes require less crude protein). The greatest benefit is seen with low quality feeds (Bedford, 2000; Hong, 2002).

Enzyme supplementation therefore has the potential to improve the nutritive value of feedstuffs. The enzyme must be biologically active when reaching the GIT to improve nutritive value. The structure of the enzyme is significant to its activity and can be

altered by exposure to heat, extreme pH and certain organic solvents (Campbell and Bedford, 1992).

2.5. Nature of enzyme supplements

Consideration of the benefits of exogenous enzymes has focused mainly on cereals. Other dietary components of the feed are ignored. However, although cereals constitute a major part of the feed, there are other nutrient sources, such as plant-protein sources. Therefore considering only major ingredients with single enzyme supplements is not appropriate.

Bearing this in mind, enzyme manufacturers have developed enzyme supplements with multiple activities. An example of such a supplement is Avizyme^{TM}, which contains amylase, protease and xylanase activities. This was considered the best as it targeted most of the components of the feed. However, it might not be possible to have a single enzyme that can deal with all the substrates present in each feedstuff. Poultry feedstuffs are a combination of various components, each with their own particular properties. This results in a wide range of substrates in one feedstuff (Campbell and Bedford, 1992).

Another constraint to the use of exogenous enzymes in feeds is that enzymes are generally heat-labile (Jongbloed *et al.*, 1992). Activity is reduced or lost, especially when feeds are pelleted. This has led to the development of cold-pelleting machines, operating at moderately high temperature.

Recently, Finnfeeds have developed thermostable microbial enzymes that can withstand the effects of high temperature during feed processing. The studies reported in this thesis make use of some of these newly developed feed enzymes, the purpose being to provide information on their benefits in improving performance of broiler chickens. The study will also provide information on the use of composite microbial enzymes in improving nutrient availability. Some of the mechanisms underlying the response of birds to such microbial enzymes will also be investigated.

CHAPTER THREE

EFFECTS OF VARYING LEVELS OF THERMOSTABLE XYLANASE, AMYLASE AND PROTEASE (TXAP) COMPOSITE ENZYME SUPPLEMENT ON BODY GROWTH OF BROILER CHICKENS

3.1. Introduction

The nutrient consistency and digestibility of maize-soya poultry feeds have generally been considered as high and adequate. However, commercial maize and soybean meal samples have shown high degree of variability in terms of feeding value measured as metabolizable energy (ME) (Iji *et al.,* 2003; Ghazi *et al.,* 2003).

Research focusing on the bird's endogenous enzymes (Classen and Bedford, 1999) suggests that the young bird might be limited in the types and amounts of enzymes necessary to utilise a high carbohydrate and vegetable protein diet at an early age, thus decreasing nutrient digestibility.

Exogenous enzymes have been used commercially for a number of years to improve nutrient digestibility in maize-soya feeds and to supplement the bird's developing endogenous enzyme function. The enzyme's intolerance to heat has been discussed on the literature review and that had led to the development of thermostable, composite enzyme consisting of xylanase, amylase and protease (TXAP) by Finnfeeds. This enzyme is specifically intended for use in maize-soybean based poultry feeds.

Positive responses to the addition of Avizyme, a predecessor of TXAP, have been reported in a number of research studies with laying hens in terms of reduced diet cost (Cook *et al.*, 2000; Gonzales *et al.*, 2001), FCR improvement, liveability (Cook *et al.*, 2000); and egg weight (Gonzales *et al.*, 2001). In broilers and ducks, improvements in FCR, body weight, uniformity and nutrient digestibility (Zanella *et al.*, 1999; Hong *et al.*, 2002) were reported.

The aim of this study was to determine the effectiveness of the newly developed heat tolerant enzyme TXAP on the performance of broiler chickens fed maize-soybean based feeds. Furthermore, the rate of supplementation of this enzyme in broiler chicken feeds, for them to be fully beneficial in improving performance and diet quality, was to be determined.

3.2. Materials and methods

3.2.1. Experimental design

A completely randomised block design was used. There were three blocks, which were chosen down the length of the house, to account for variation in temperature that prevails in a longitudinally ventilated house. There were six dietary treatments each replicated eight times (six replicates being males and two being females), with each pen of 50 birds representing a replicate. The pens measured $5m^2$, yielding a stocking density of 10-birds/m^2. Temperature was reduced stepwise from 31^0C at day-old to 21^0C at day 21, and maintained there for the rest of the experimental period. The humidity in the house was dependent on exchange of air provided by the tunnel ventilation system.

3.2.2. Experimental animals and feeds

Two thousand four hundred sexed day-old Ross chicks were used for this study. The chicks were randomly allocated to 48 pens in groups of 50 and assigned to one of the six dietary treatments. The control diet (Treatment 1) contained no exogenous enzyme supplement while the five other feeds (Treatments 2 to 6) contained increasing rates of inclusion of enzyme TXAP, at 0.5, 1.0, 1.5, 2.0 and 2.5 g/kg, respectively. Two basal feeds (starter and finisher) were based on maize and soya bean meal (Table 3.1) and were formulated to contain low levels of energy and available phosphorus to see if there were any additive effects of TXAP on energy and phosphorus availability.

The enzyme, TXAP, was obtained from Danisco Animal Nutrition (Finnfeeds International, UK). It is a mixture of xylanase, amylase and protease with guaranteed minimum enzyme activities of 600 U/g endo-1,4 beta-xylanase (EC 3.2.1.8), 8000 U/g subtilisin (protease) (EC 3.4.21.62) and 800 U/g alpha-amylase (EC 3.2.1.1).

3.2.3. Assessment of performance variables

The chicks were weighed in groups (chicks in the same pen weighed together) to obtain their body weight at day-old and subsequently every 7^{th} day until the end of the trial. Feed consumption was recorded at the end of each week by calculating the difference between the feed supplied at the beginning of the week and the feed that was left in the feeders and in the bag at the end of the week. Feed conversion efficiency of the birds was calculated as the ratio of the weight gained by the birds to the feed that was consumed by the birds. Mortalities were also recorded weekly on pen basis as they occurred.

Table 3.1. *Ingredient and nutrient composition of the basal diet (g/kg)*

Ingredient	Starter	Finisher
Maize	614	636
Soybean oilcake meal	335	286
Sunflower oil	13.1	41.9
Salt	4.1	3.3
Lysine-HCl	6.0	9.0
DL Methionine	2.1	1.4
Limestone	11.9	11.5
Dicalcium Phosphate	14.8	14.2
Vit / Min premix[1]	5.0	5.0
Nutrients		
Crude Protein	215	193.6
ME MJ / kg	12.6	13.4
Calcium	9.0	8.6
Available Phosphorus	4.0	3.8
Methionine	5.5	4.5
TSAA	9.1	7.7
Lysine	12.0	10.8
Fat	41.1	68.8
Crude Fibre	21.6	25.0
Sodium	1.8	1.5

[1] Composition of the premix (g/kg): Vitamin B1 (2), B2 (6), B6 (5), K (3), Choline chloride (300), Niacin (70), Pantothenic acid (15), Oxiban, antioxidant (125). Other nutrients are (mg/kg): Vitamin B12 (20), Biotin (150), Cobalt (500), Selenium (300), Vitamin A (MIU) (12), D3 (4).

3.2.4. Statistical analysis

Data were analysed using both the general linear model (GLM) and regression of Genstat 6th edition (Genstat, 2002). The GLM analysis did not reveal any significant differences between the means of the tested variables. The data were regressed, using varying levels of the enzyme (TXAP) as the independent variable. The differences

between the mean values were determined by the use of least significant difference ($p<0.05$). Minitab software (Minitab, 2002) was used for the Chi-Squared test of the mortalities.

3.3. Results

3.3.1. Performance variables

The results of the effect of enzyme (TXAP) at different inclusion rates are presented in Table 3.2. The inclusion of TXAP did not affect ($p>0.05$) any of the performance variables as demonstrated by analysis of variance and regression analysis (Table 3.3).

Table 3.2. *The effects of varying inclusion rates of TXAP on body weight, feed intake, FCE and mortality of broiler chicks at 42 d of age*

Treatment	Weight gain (g/bd^{-1})	Feed intake (g/bd^{-1})	FCE (g/kg)	Mortality
1	49.8	103	484	9.3
2	50.6	103	495	9.3
3	50.7	100	506	9.3
4	51.0	100	511	9.3
5	49.6	104	482	9.4
6	51.2	102	502	9.3
P-value	0.230	0.819	0.250	0.442

3.3.2. Mortality

There was no effect of enzyme inclusion on mortality. The overall mortality was 2.3 %.

Table 3.3. *Regression of performance data of birds at 42 d of age against TXAP*

Variable	Regression coefficient	Significance

Weight gain (g/b d)	0.00251± 0.00258	NS
Feed intake (g/b d)	-0.0014 ± 0.0106	NS
FCE	0.0329 ± 0.0502	NS
Mortality	-0.00068 ± 0.00186	NS

NS = Not significant

3.4. Discussion

The results of this experiment did not show any significant improvements caused by the inclusion of the enzyme TXAP at any dosage rate. These results are in line with findings by Anonymous (2005), who reported an insignificant improvement in the performance traits of laying hens, but in contrast with findings by Hruby (2003), who reported dramatic improvements in the performance data through the addition of Avizyme, an enzyme that is similar to TXAP.

This lack of response may be attributed to the quality of maize and soybean used in this experiment. Significant improvements are expected when supplementation is made to poor quality ingredients (Bedford, 2000; Douglas, 2000; Dudley-Cash, 2001). Research by Douglas *et al.* (2000), using 12 different soybean meal sources, demonstrated that enzyme use is more beneficial in poor than in good quality ingredients.

Bedford and Apajalahti (2000) reported that if there were a healthy or beneficial microflora in the GIT, there would be no beneficial effects of the enzyme inclusion. The type of microflora present in the gastrointestinal tract (GIT) of the bird is therefore expected to play an important role in the response of the host to enzyme supplementation.

3.5. Conclusion

This study showed no response to the inclusion of the enzyme TXAP in broiler feeds. This lack of response might be due to the quality of the ingredients used in this study. Therefore further research using ingredients with different nutritional status has to be done.

CHAPTER FOUR

EFFECT OF SUPPLEMENTAL TXAP, LIPASE AND PHYTASE, INDIVIDUALLY OR IN COMBINATION, IN MAIZE-SOYA FEEDS FOR BROILER CHICKENS

4.1 Introduction

Enzymes widely used in the poultry feed industry are the glycanases (xylanase and beta-glucanases) that cleave the non-starch polysaccharides (NSPSs) in cereal grains such as wheat and barley thereby improving the digestibility of these cereals. Recently, maize and soybean meal have been the target for enzyme addition as a means of improving digestibility and eliminating antinutritional factors such as phytate (Ghazi *et al.*, 2003).

Finnfeeds recently established an enzyme cocktail for maize-soya feeds called TXAP, which is a combination of xylanase, amylase and protease. Several mechanisms have been proposed to explain the beneficial effects of TXAP in improving energy and nutrient utilisation of maize-soya feeds and these include: degradation of the phytate and xylose in the cell wall matrix and the release of encapsulated nutrients (Zanella *et al.*, 1999), increased accessibility of nutrients to endogenous digestive enzymes (Chesson, 1993), improved feed passage rate (Bedford, 2000), and supplementation of the enzyme capacity of young animals (Bedford, 2000).

Campbell and Bedford (1992) reported that the use of a combination of phytase and TXAP was beneficial in increasing the apparent metabolisable energy (AME) and nutrient digestibility in maize-soy feeds for broilers. Similar improvements, using

combinations of phytase and xylanase in wheat and wheat-based feeds, have been reported previously (Wu *et al.*, 2004). The aim of the present study was to examine the influence of thermostable xylanase, amylase and protease (TXAP), lipase and phytase, individually or in combination, on performance, AME, and digestive tract measurements in broilers fed maize-soya feeds.

4.2. Materials and methods

4.2.1. Experimental design

The experiment was designed as described in Chapter 3 except that there were seven dietary treatments, each treatment being replicated six times (three replicates of males and three of females).

4.2.2. Experimental animals and feeds

Two thousand one hundred sexed day-old Ross chicks were used for this study. The chicks were randomly allocated to 42 pens in groups of 50 and assigned to one of seven dietary treatments. The control diet contained no added enzymes whilst the seven other feeds contained enzymes. There were six replicates per treatment. The two basal feeds (starter and finisher) were based on maize and soya bean meal (Table 4.2) and were formulated to contain relatively low levels of energy and available phosphorus thereby improving the chances of measuring any additive effects of TXAP and phytase addition. The starter diet was fed for the first three weeks and the finisher diet was given for the remaining three weeks of the experimental period. Lipase was also used in this trial to determine whether there will be additional benefits on mineral and fat digestibility. A summary of the treatments applied is given in Table 4.1.

Enzymes were obtained from Danisco Animal Nutrition (Finnfeeds International, UK). These included TXAP (a mixture of xylanase, amylase and protease with guaranteed minimum enzyme activities of 600 U/g endo-1,4 beta-xylanase (EC 3.2.1.8), 8000 U/g subtilisin (protease) (EC 3.4.21.62) and 800 U/g alpha-amylase (EC 3.2.1.1)), phytase and lipase. Titanium dioxide was included in the feed as an insoluble marker to enable the assessment of nutrient digestibility.

Table 4.1. *Summary of the treatments applied in this trial*

Treatment	Enzymes added
1	Control – no addition
2	TXAP (200 g/kg)
3	Lipase (100 g/kg)
4	Phytase (100 g/kg)
5	TXAP (200 g/kg)+ Lipase (100 g/kg) (TL)
6	TXAP (200 g/kg) + Phytase (100 g/kg) (TP)
7	TXAP (200 g/kg) +Lipase (100 g/kg) + Phytase (100 g/kg) (TLP)

4.2.3. Assessment of performance

Performance of the chickens was assessed as described in Chapter 3. Visceral organs of one bird per pen, killed by carbon dioxide asphyxiation, were weighed at the end of the experiment.

4.2.4. Determination of ileal digestibility of nutrients

The digestibilities of protein, gross energy (GE), calcium and phosphorus in each of the experimental feeds were determined at the ileum, using the change in concentration of the indigestible marker (titanium dioxide) between the feed and the ileal contents. Six

birds were sacrificed from each pen at the end of the experiment and their ileal contents were collected and freeze-dried prior to further chemical analysis.

The marker was analysed using a method described by Short *et al.* (1996). The samples were dried in an oven at 70^0C overnight. They were ashed by placing them in a cold muffle furnace, with the temperature set to rise slowly to 580^0C for 13 hours. The crucibles were removed from the furnace and allowed to cool. 10 ml of 7.1M H_2SO_4 was poured into the crucibles and boiled for about 60 minutes until the digesta was completely dissolved. The solution was poured into small beakers each containing 25 ml distilled water (ROH). It was then filtered into a 100 ml volumetric flask through Whatman 541 filter paper. Twenty (20) ml H_2O_2 was added to the filtrate in each flask and diluted to 100 ml with ROH. The resulting aliquot was measured on a spectrophotometer at 410 nm.

The standard solution was prepared by dissolving 250 g titanium dioxide in 100 ml concentrated sulphuric acid in a heatproof beaker by heating to just below boiling (it may take up to 2.5h to dissolve). The solution was placed in a volumetric flask (500 ml) containing 200 ml ROH with rinsings. One hundred (100) ml concentrated H_2SO_4 was added to the flask and the mixture diluted to 500 ml with ROH. Graded volumes (0-10 ml for example) of a standard TiO_2 (5 mg/ml) were placed in 100 ml volumetric flasks. Then 7.4 M H_2SO_4 was added to each flask to make 10 ml and 20 ml H_2O_2 solution was added and made up to 100 ml with ROH. The results were measured as for the samples.

4.2.5. Determination of nutrient concentrations

Crude Protein was measured by the method described by Dennison and Gous (1980), and gross energy, phosphorus and calcium were analysed using the AOAC (1990) methods of analysis. Calcium was analysed by wet ashing the samples using concentrated sulphuric acid, followed by atomic absorption spectophotometer. Gross energy was determined with a DDS isothermal CP500 bomb calorimeter.

Table 4.2. *Ingredient and nutrient composition of the basal feeds (g/kg)*

Ingredient composition	Starter	Finisher
Maize	575	644
Soybean oilcake meal	384	314
Sunflower Oil	10.0	10.0
Salt	4.1	3.3
Lysine-HCl	0.5	0.8
DL Methionine	2.1	1.4
Limestone	12.4	14.3
Dicalcium Phosphate	7.0	6.1
Vit / Min premix[1]	5.0	5.0
Titanium Dioxide[2]	-	1.0
Nutrient composition		
CP	219	195
ME MJ/kg	12.1	12.4
Ca	7.5	7.8
Av P	2.5	2.2
Met	5.6	4.5
TSAA	9.2	7.8
Lys	12.3	10.8
Fat	37.1	39.1
Fibre	26.8	26.1
Sodium	1.8	1.5

[1] Composition of the premix (g/kg): Vitamin B1 (2), B2 (6), B6 (5), K (3), Choline chloride (300), Niacin (70), Pantothenic acid (15), Oxiban, antioxidant (125). Other nutrients are (mg/kg): Vitamin B12 (20), Biotin (150), Cobalt (500), Selenium (300), Vitamin A (MIU) (12), D3 (4)

[2] Indigestible marker.

4.2.6. Sample collection and preparation for biochemical assays

The activities of the pancreatic and intestinal enzymes were measured using biochemical techniques. Each sacrificed bird was dissected to expose the visceral organs. A piece of the jejunum (part of the intestinal tract extending from the ligament of Treitz to Meikel's diverticulum) and the pancreas were removed for analysis. The tissues were flushed with ice-cold saline water and the jejunum was slit longitudinally. The mucosa was rinsed of digesta and wrapped in aluminium foil. The tissues were then snap frozen in liquid nitrogen for use in the determination of the activities of digestive enzymes.

The brush-border membrane vesicles were isolated from the intestinal tissue using the method described by Shirazi-Beechey *et al.* (1991). Fresh frozen intestinal samples were cut into small pieces and defrosted in buffer (100 mM mannitol, 2 mM *N*-[2-hydroxyethyl] piperazine-*N*- [2-ethanesulfonic acid] (HEPES)/Tris, pH 7.1). About 25 ml of buffer was used for 1-2 g tissue. The mixture was vibromixed for 2 x 30 seconds and filtered through a Buchner funnel, 1 mm pore size. The filtrate was then blended with a homogeniser for 30 seconds at high speed. One ml of homogenate was taken for assessment of protein and brush-border membrane enzyme activity.

The pancreas was fractionated in accordance with the method of Bradford (1976). Fresh frozen pancreatic tissues were weighed and cut into small pieces and defrosted in ice-cold distilled water. The mixture was homogenised at moderate speed for 1 minute. The

mixture was then centrifuged using Beckman J2-HS centrifuge at 27200 g for 10 minutes to obtain a crude homogenate. One ml of the homogenate was taken for assessment of protein and pancreatic enzyme activity.

4.2.7. Measurement of the digestive enzymes

4.2.7.1. Alkaline phosphatase (EC.3.1.3.1)

Alkaline phosphatase was assayed in line with modified methods described by Forstner *et al.* (1968) and Holdsworth (1970). The assay system consists of 50 mM $MgCl_2$, 50 mM Tris (pH 10.1) and the substrate, 10 mM paranitrophenol phosphate (PNP Sigma 104). The standard was paranitrophenol (Sigma 104-1). The reaction was initiated by incubating 25 μ l of homogenate with 0.8 ml of 50 mM Tris buffer, pH 10.1; 0.1 ml of 25 mM $MgCl_2$; and 0.1 ml of PNP, at 39^0C for 20 minutes. The reaction was terminated with 0.1 ml 40% trichloroacetic acid (TCA). Further colour development was accomplished by adding 2.0 ml of 0.4 M NaOH to 0.1 ml of the primary mixture, which was then vibromixed and read at 410 nm with an UV-Vis spectrophotometer.

4.2.7.2. Chymotrypsin (EC. 3.4.2.1.1)

The substrate was 0.1 mM solution of succinyl-$(ALA)_2$-Pro-Phe-*p*-nitroanilide (SAPNA) in 50 mM Tris-HCl/20 mM $CaCl_2$ buffer (pH 7.5). For the standard, graded concentrations of *p*-nitroaniline were used. About 30 μ l of sample were added to 1.5 ml fresh substrate solution and incubated at 39^0C for 10 minutes; the reaction was terminated with 250 μ l of 30 % acetic acid and the absorbance was read at 410 nm against a water blank (Serviere-Zaragoza *et al.*, 1997).

4.2.8. Statistical analysis

The data were analysed for significant differences using the regression analysis of Minitab (Minitab, 2002). The differences between the mean values were determined by the use of least significant difference ($p<0.05$).

4.3. Results

4.3.1. Performance variables

The effects of microbial enzyme supplementation on broiler performance during the growing period (1-21 days), the finisher period (22-42 days) and the overall period (1-42 days) are presented in Table 4.3.1. The mean body weight gain (BWG) was increased by TXAP ($P<0.05$), and phytase ($P<0.001$) during the growing period. Phytase decreased ($P<0.001$) feed intake (FI), but TXAP did not have any ($P>0.05$) effect on FI. Feed conversion efficiency (FCE) was not ($P>0.05$) improved by any of the supplemented enzymes. Lipase did not have any ($P>0.05$) effect on the performance of the chicks. The combination of enzymes did not have any ($P>0.05$) effect on the performance of the chicks during this period.

There was an increase in BWG with inclusion of TXAP ($P<0.01$) and phytase ($P<0.001$), while lipase did not have any ($P>0.05$) effect. The combination of TXAP and lipase increased BWG ($P<0.01$) of the chicks. Phytase increased FI ($P<0.001$) during the last period (22-42 d). TXAP and lipase did not have any ($P>0.05$) effect on FI. FCE was not affected ($P>0.05$) by enzyme supplementation.

There was an increase in BWG with the inclusion of TXAP ($P<0.01$), phytase ($P<0.001$) and the combination of TXAP and lipase ($P<0.01$) for the overall experimental period.

FI was decreased (P<0.001) by phytase inclusion only. TXAP and lipase did not have any effect on FI (P>0.05) overall.

Table 4.3. *Mean body weight gain (g/d), food intake (g/d) and feed conversion efficiency (FCE) (g gain/kg feed) of broilers on seven feed treatments*

	Weight gain (g/b d)			Feed intake (g/b d)			FCE (g gain/ kg feed)		
Treatment	1-21d	22-42d	1-42d	1-21d	22-42d	1-42d	1-21d	22-42d	1-42d
Control	27.6	94.5	45.3	48.7	191.0	88.0	571	499	518
TXAP	28.3	99.0	47.2	45.4	195.8	87.9	627	505	536
Lipase	28.6	100.6	47.8	47.1	201.6	90.8	609	499	527
Phytase	30.4	107.5	51.0	49.3	211.9	95.3	616	507	535
TL	26.1	89.0	42.7	51.3	180.8	85.9	507	501	505
TP	27.4	91.0	44.0	52.3	184.6	87.7	523	501	508
TLP	28.1	92.8	45.0	47.9	192.1	88.0	594	488	515
SOV	P	P	P	P	P	P	P	P	P
TXAP	0.247	0.341	0.295	0.033	0.565	0.992	0.033	0.678	0.194
Lipase	0.113	0.196	0.158	0.288	0.212	0.378	0.129	0.996	0.509
Phytase	0.000	0.009	0.003	0.648	0.018	0.024	0.077	0.598	0.213
TL	0.112	0.414	0.318	0.220	0.402	0.645	0.081	0.916	0.497
TP	0.825	0.598	0.613	0.090	0.603	0.958	0.181	0.928	0.588
TLP	0.570	0.806	0.906	0.723	0.918	1.000	0.511	0.603	0.894
R^2	59.4	19.5	30.6	22.1	14.7	20.0	2.9	0.0	0.0

SOV = Source of variation, TL = TXAP + Lipase, TP = TXAP + Phytase, TLP = TXAP + Lipase + Phytase.

There was an increase in body weight gain of all birds supplied with enzymes (Table 4.3), the increase being greater to day 21 (P<0.001) than from 22 to 42 d (P<0.01). Phytase increased weight gains (P<0.01) whether fed individually or in combination with lipase and TXAP (Table 4.3).

There were no changes in FCE with the addition of any of the enzymes (Table 4.3). Although the effect of enzyme addition was not significant on FCE, there was a slight increase of FCE of the birds assigned to the feeds with added enzymes.

4.3.2. .Visceral organ weights

There were no ($p>0.05$) effects of enzymes (individually or in combination) on the visceral organ weights of the birds (Table 4.4).

4.3.2. Ileal digestibility of nutrients

The ileal digestibilities of nutrients are presented in Table 4.5. Nutrient digestibility was higher with lipase inclusion. Individual supplementation performed better that the enzyme combination. Enzyme combination decreased the digestibility of nutrients compared to the control. Lipase increased the digestibility of protein ($P<0.01$), GE ($P<0.01$), phosphorus ($P<0.01$) and calcium ($P<0.05$), while phytase increased only the phosphorus digestibility ($P<0.01$). TXAP had no effect on nutrient digestibility.

Table 4.4. *Visceral organ weights of birds on seven dietary treatments (g/100g body weight)*

Treatment	Provent./ Gizzard	Small Intestine	Pancreas	Liver	Caeca/Colon	Spleen
Control	3.5	4.6	0.3	2.6	1.2	0.2
TXAP	3.4	4.9	0.3	2.7	1.1	0.1
Lipase	2.9	5.0	0.3	2.6	1.1	0.1
Phytase	3.6	5.0	0.2	2.8	1.0	0.2
TL	3.9	4.6	0.3	2.7	1.3	0.2
TP	3.3	4.5	0.3	2.3	1.4	0.2
TLP	4.1	4.1	0.4	2.3	1.4	0.2
SOV	P	P	P	P	P	P
TXAP	0.773	0.528	0.883	0.837	0.623	0.219
Lipase	0.107	0.270	0.386	0.964	0.600	0.325
Phytase	0.793	0.343	0.238	0.378	0.357	0.580
TL	0.507	0.901	0.304	0.779	0.347	0.964
TP	0.589	0.769	0.236	0.272	0.236	0.271
TLP	0.439	0.482	0.132	0.418	0.424	0.214
R^2	0.0	0.0	1.7	0.0	0.0	4.9

SOV = Source of variation, TL = TXAP + Lipase, TP = TXAP + Phytase, TLP = TXAP + Lipase + Phytase.

Table 4.5. *Digestibilities (g/g) of protein, gross energy (GE), phosphorus and calcium in the seven feeds used in the trials*

Treatment	Protein	GE	Phosphorus	Calcium
Control	0.76	0.72	0.42	0.32
TXAP	0.82	0.78	0.55	0.53
Lipase	0.79	0.76	0.47	0.54
Phytase	0.82	0.77	0.61	0.53
TL	0.71	0.64	0.22	0.27
TP	0.75	0.69	0.46	0.34
TLP	0.70	0.61	0.29	0.05
SOV	P	P	P	P
TXAP	0.006	0.033	0.037	0.014
Lipase	0.125	0.124	0.406	0.013
Phytase	0.007	0.056	0.003	0.016
TL	0.037	0.009	0.002	0.532
TP	0.833	0.306	0.552	0.755
TLP	0.023	0.001	0.038	0.000
R^2	55.6	60.2	58.2	63.4

SOV = Source of variation, TL = TXAP + Lipase, TP = TXAP + Phytase, TLP = TXAP + Lipase + Phytase.

4.3.3. Tissue protein content and endogenous enzyme activities

The mean tissue protein contents and endogenous enzyme activities measured in birds sacrificed at the end of the trial are presented in Table 4.6. There were no effects of enzyme inclusion on either jejunum or pancreas protein content or enzyme activities in this study.

Table 4.6. *Effect of enzyme supplementation on jejunum and pancreas tissue protein and their digestive enzyme activities measured in 42-day-old broiler chickens*

Treatment	Jejunum		Pancreas	
	Protein	Alkaline phosphatase	Protein	Chymotrypsin
Control	21.3	8.7	30.9	11.8
TXAP	19.5	14.0	34.7	12.4
Lipase	20.9	7.8	26.6	15.9
Phytase	19.3	7.5	34.3	12.3
TL	22.8	5.9	29.5	13.5
TP	22.9	9.3	25.0	14.0
TLP	26.9	8.9	30.5	13.2
	P	P	P	P
TXAP	0.775	0.112	0.391	0.659
Lipase	0.949	0.799	0.323	0.010
Phytase	0.743	0.725	0.432	0.732
TL	0.670	0.119	0.884	0.156
TP	0.539	0.445	0.039	0.639
TLP	0.429	0.633	0.702	0.209
R^2	0.0	4.2	6.8	10.3

SOV = Source of variation, TL = TXAP + Lipase, TP = TXAP + Phytase, TLP = TXAP + Lipase + Phytase.

4.4. Discussion

4.4.1. Performance variables

The performance of broiler chicks in the present study was not affected by TXAP or lipase inclusions, whether these were included individually or in combination. Phytase, however, increased mean weight gain and feed intake but did not have any effect on FCE. Therefore the increase in body weight gains by the chicks is explained by their increased feed intake and therefore higher feed costs. These results are in contrast with

findings by Cowieson *et al.* (2004), who reported improved performance traits of chicks with enzyme addition. There are no available data on TXAP, but its predecessor (Avizyme^{TM}) was reported to have improved the performance traits of broilers. The results of this study are in contrast with our expectations. We expected to measure improvements, especially when the enzymes were included in combination. Campbell and Bedford (1992) reported that because poultry feeds are composed of a wide range of ingredients providing different substrates for enzymes, it would be beneficial to include different enzymes or composites in such feed.

The lack of response in this trial may perhaps be the result of the high quality of the maize and soybean oilcake meal used in the basal feeds. A useful study in the future would be to ascertain under what conditions a feed would benefit from the addition of feed enzymes. Certainly, there are positive reports on the use of enzymes, so there are instances where the substrate benefits from such additions. A better understanding of the chemistry of carbohydrates might lead to the identification of substrates that will or will not benefit from the use of enzymes. Such understanding would be greatly beneficial to companies selling feed enzymes.

4.4.2. Visceral organ weights

The results from this study showed no significant changes in visceral organ weights with enzyme inclusion, individually or in combination. These results are in line with reports by Alam *et al.* (2003), who reported no differences in liver and gizzard weights due to enzyme inclusion. These results are in contrast with our expectations that enzyme supplementation would reduce visceral organ weights thereby increasing digestion and absorption of nutrients.

4.4.3. Nutrient digestibilities

In this study phytase improved the digestibility of the phosphorus contained in the feed. Its action is to release the phosphorus bound in the phytate molecule (Augspurger *et al.*, 2003). Lipase increased the digestibility of all measured nutrients in this study (protein, phosphorus, gross energy and calcium). This is in line with findings by Santos *et al.* (2004). On the other hand, TXAP had no effect on the digestibility of any of the measured nutrients. This is in contrast with our expectations. This lack of response to TXAP supplementation might be attributed to the quality of maize used in this experiment. Higher quality ingredients are unlikely to benefit from enzyme inclusion, in contrast with the case in poor quality ingredients (Douglas *et al.*, 2000).

4.4.4. Endogenous enzyme activities

Exogenous enzyme inclusion did not influence endogenous enzyme activities in this study. This might be because the maize used in this study was low in antinutritional factors. These results are in contrast with our expectations, but are in line with findings with Avizyme by Khumalo (2002).

Conclusion

The results of this study showed that phytase inclusion is beneficial in maize-soybean based feeds. TXAP inclusion was not effective. Lipase inclusion was beneficial in improving the utilization of nutrients, while phytase improved only the phosphorus utilization. Therefore, phytase should be used in combination with lipase if the full benefits of exogenous enzyme addition are to be obtained on maize-soybean based feeds. Further research needs to be done on the effects of TXAP.

CHAPTER FIVE

EFFECT OF A COMBINATION OF THERMOSTABLE XYLANASE, AMYLASE AND PROTEASE (TXAP) AND PHYZYME XP (PHYTASE) IN MAIZE-SOYA FEEDS ON THE PERFORMANCE OF BROILER CHICKENS

5.1. Introduction

Broiler feeds are composed predominantly of plant materials, mainly cereals and vegetable proteins. Most of these feed ingredients contain an indigestible fraction, for example, cellulose, and some also contain anti-nutritional factors, which inhibit feed utilization and performance. Phytic acid is one of these anti-nutritional factors, being able to bind to certain minerals, such as zinc, and to amino acids, thus rendering them unavailable for digestion. Lan *et al.* (2002) and Rutherfurd *et al.* (2004) confirmed that it is this binding effect that exerts its anti-nutritional activity. The anti-nutritional effect is manifested by depressed nutrient utilization accompanied by poor growth.

These adverse effects can be overcome by supplementation of the feed with exogenous phytase enzymes, which have been shown to break down the phytic acid molecule, thus releasing the bound nutrients and improving digestibility of starch, protein, fat and AME in broilers fed feeds containing maize (Cowieson, 2003; 2004).

Another of the anti-nutritional factors in some feed ingredients is a group of polyphenolic compounds known as tannins, which bind with protein, inhibit digestive enzymes and reduce feed intake by virtue of their astringent effect (Alam *et al.*, 2003).

Therefore supplementation of feeds with protease enzymes has been suggested as a means of compensating for the digestibility losses due to enzyme inhibition.

Whilst these various enzymes have been shown to exert beneficial effects when used singly in broiler feeds, there may be an advantage in using more than one simultaneously. Finnfeeds (Finnfeeds International) is already producing composite enzymes such as TXAP, which is a combination of xylanase, amylase and protease. The aim of this experiment was to determine the beneficial effects of three types of TXAP (with fixed levels of amylase and xylanase but varying levels of protease) when used with phyzyme XP (that contains phytase).

5.2. Materials and methods

5.2.1. Experimental design

The experiment was designed as described in Chapter 3 except that there were eight dietary treatments each replicated six times (three replicates being males and three being females), with each pen of 50 birds representing a replicate.

5.2.2. Experimental animals and feeds

Two thousand four hundred sexed day-old Ross chicks were used for this study. The chicks were randomly allocated to 48 pens in groups of 50 and assigned to one of the eight dietary treatments. Two basal feeds (a negative and a positive control) with no added enzymes were used, while the remaining six feeds contained enzymes. The feeds were based on maize and soya bean meal (Table 5.1). The negative control diet was formulated to contain low levels of energy and available phosphorus to ascertain whether there were any additive effects of TXAP to energy and phyzyme XP to

phosphorus. The positive control diet contained conventional levels of available phosphorus and ME. Starter feeds were used from day old to 21d, while finisher feeds were used thereafter (Table 5.1).

Enzymes were obtained from Danisco Animal Nutrition (Finnfeeds International, UK). TXAP (a mixture of xylanase, amylase and protease with guaranteed minimum enzyme activities of 650 U/kg endo-1,4 beta-xylanase (EC 3.2.1.8), 1650 U/kg alpha-amylase (EC 3.2.1.1) and Phyzyme XP were used in all treatments that included enzymes, while variable levels of protease (EC 3.4.21.62) were used in the various treatments, viz. 4000 U/kg in Lot 1 (Treatments 2 and 6), 2000 U/kg in Lot 2 (Treatments 3 and 7) and 1000 U/kg in Lot 3 (Treatments 4 and 8). Phyzyme XP is a new phytase enzyme that is derived from *Escherichia coli* and expressed in *Schizosaccaromyces pombe*, with an enzyme activity of 500 U/kg (Dilger *et al.*, 2004). Titanium dioxide (2 g/kg) was also included in each feed as an insoluble marker to enable the assessment of nutrient digestibility.

5.2.3. Assessment of performance

Performance of the chickens was assessed as described in Chapter 3 and visceral organs weights were determined as described in Chapter 4.

5.2.4. Determination of ileal digestibility of nutrients

The ileal digestibility of the starter and finisher feeds was determined as described in Chapter 4.

Table 5.1. *Ingredient and nutrient composition of the basal feeds (g/kg)*

	Positive Control		Negative Control	
	Starter	Finisher	Starter	Finisher
Maize	555	615	603	670
SBM 44	357	286	346	271
Soybean Oil	46.0	58.0	14.0	23.0
Salt	4.1	3.6	4.1	3.6
Lysine-HCl 20%	1.1	1.8	1.2	2.1
DL Methionine	3.0	2.7	2.9	2.6
Threonine	0.0	1.3	0.1	1.3
Limestone	11.7	12.8	12.0	13.2
Dical Phosphate	14.9	11.7	9.5	6.1
Vit / Min premix[1]	5.0	5.0	5.0	5.0
Titanium Dioxide[2]	2.0	2.0	2.0	2.0
Nutrient (g/kg)				
CP	222	195	221	192
ME MJ/kg	12.8	13.4	12.2	12.7
Calcium	9.0	8.5	7.8	7.3
Available Phosphorus	4.0	3.3	3.0	2.2
Methionine	6.4	5.7	6.3	5.6
TSAA	10.1	9.0	10.0	8.9
Lysine	12.9	11.5	12.8	11.4
Fat	70.4	83.4	41.6	52.0
Fibre	25.5	24.5	26.2	25.3
Sodium	1.8	1.6	1.8	1.6

[1] Composition of the premix (g/kg): Vitamin B1 (2), B2 (6), B6 (5), K (3), Choline chloride (300), Niacin (70), Pantothenic acid (15), Oxiban, antioxidant (125). Other nutrients are (mg/kg): Vitamin B12 (20), Biotin (150), Cobalt (500), Selenium (300), Vitamin A (MIU) (12), D3 (4)

[2] Indigestible marker.

5.2.5. Determination of nutrient concentrations

The nutrient concentrations in the feed and in the digesta were determined as described in Chapter 4.

5.2.6. Statistical analysis

The data were analysed for significant differences using the General Linear Model procedure (Genstat, 2002). Fisher's pairwise comparison was used to compare the treatment means. The differences between the mean values were determined by the use of least significant difference ($p<0.05$).

5.3. Results

5.3.1. Performance variables

The effects of microbial enzyme supplementation (additive), energy level and their interaction on broiler performance during the growing period (1-21 days of age) are presented in Table 5.2. Results showed that the average values of body weight gain (BWG), and feed conversion efficiency (FCE) were not ($P>0.05$) affected by enzyme supplementation. Feed intake (FI) increased ($P<0.05$) with enzyme inclusion with a greater increase being observed for the TXAP Lot-3 enzyme. There were interactions in BWG and FCE between additive and sex ($P<0.05$). Males on TXAP Lot-1 were 1.3 g heavier than the control group, while females on TXAP Lot-3 were 1.4 g heavier, the males being heavier than females. There were no differences in performance between the two energy levels ($P>0.05$).

The effects of microbial enzyme supplementation (additive), energy level and their interaction on broiler performance for the overall period (1-35 days of age) are presented

in Table 5.3. Results showed that mean BWG, FI and FCE were again not (P>0.05) affected by enzyme supplementation or energy level. There was an interaction in FCE between additive and ME (P<0.05) and between ME and sex (P<0.05). On the low energy feed, enzyme supplementation increased FCE, with TXAP Lot-1 showing the highest increase. At high energy levels supplementation of this enzyme decreased FCE. Females on high-energy feeds had higher FCEs than those on low energy feeds, while the opposite was the case with males.

Table 5.2. *The effects of feed additives (enzymes) and dietary energy level on broiler performance at 21 days of age*

	Weight gain (g/bird d)				Feed intake (g/bird d)				FCE (g gain/kg feed)			
	LME		HME		LME		HME		LME		HME	
Add	F	M	F	M	F	M	F	M	F	M	F	M
0	25.6	26.7	29.3	30.5	43.4	44.1	44.6	45.6	589	604	656	668
1	25.7	31.6	27.7	28.2	44.2	46.1	43.9	44.1	582	684	629	638
2	28.1	25.8	27.1	24.5	43.6	43.3	43.8	42.7	646	595	616	572
3	27.3	27.9	30.3	24.7	43.9	44.7	47.1	45.0	621	623	645	549
SOV	**P**				**P**				**P**			
ME	0.301				0.313				0.790			
Add	0.571				0.044				0.407			
Sex	0.811				0.783				0.638			
ME*Sex	0.061				0.153				0.087			
Add*Sex	0.027				0.351				0.024			
Me*Add	0.107				0.083				0.066			
ME*Add*Sex	0.323				0.618				0.357			

LME – Low energy; HME – High energy; Add – Additive

Table 5.3. *Effects of feed additives (enzymes) and dietary energy level on broiler performance at 35 days of age*

	Weight gain (g/bird d)				Feed intake (g/bird d)				FCE (g gain/kg feed)			
	LME		HME		LME		HME		LME		HME	
Add	F	M	F	M	F	M	F	M	F	M	F	M
0	38.5	39.5	40.7	41.0	69.5	70.5	68.1	67.6	555	561	598	607
1	38.1	44.0	39.9	40.3	67.0	70.8	66.9	73.2	569	621	596	555
2	40.9	36.7	38.3	36.2	70.1	64.5	66.0	64.7	584	570	579	559
3	38.2	37.4	41.2	36.6	66.0	65.3	69.5	69.9	577	573	592	524

SOV	**P**	**P**	**P**
ME	0.918	0.847	0.994
Add	0.234	0.486	0.418
Sex	0.614	0.786	0.249
ME*Sex	0.337	0.607	0.023
Add*Sex	0.107	0.290	0.218
Me*Add	0.591	0.443	0.036
ME*Add*Sex	0.552	0.921	0.146

LME – Low energy; HME – High energy; Add – Additive

5.3.2. Visceral organs

The effects of microbial enzyme supplementation (additive), energy level and their interaction on visceral organ weights at 21d are presented in Table 5.4. The average weights of pancreas, proventriculus/gizzard and small intestine were not ($P>0.05$) influenced by enzyme supplementation or by the energy level of the diet. However, pancreas weight was almost significantly decreased ($P<0.055$) by the enzyme inclusion, the greatest decrease occurring with TXAP Lot-1 enzyme. There was an interaction in pancreas weight between additive and sex ($P<0.05$). Males on TXAP Lot-1 had their pancreas reduced by 0.120g/100g body weight while females on TXAP Lot-3 had their pancreas reduced by 0.06 g/100g body weight.

Table 5.4. *Effects of feed additives (enzymes) and dietary energy level on visceral organ weights (g/100g body weight) at 21 d*

	Pancreas				Proventr/Gizzard				Small intestines			
	LME		HME		LME		HME		LME		HME	
Add	F	M	F	M	F	M	F	M	F	M	F	M
0	0.5	0.6	0.5	0.5	6.1	7.1	6.8	6.5	7.5	9.9	8.1	8.9
1	0.5	0.4	0.4	0.4	6.0	6.1	6.4	6.7	7.8	8.9	9.3	8.6
2	0.50	0.4	0.4	0.5	6.2	6.1	6.9	7.1	8.6	8.2	8.6	8.8
3	0.4	0.6	0.4	0.5	6.0	6.8	5.3	6.8	7.8	8.6	8.5	9.6

SOV	**P**	**P**	**P**
ME	0.301	0.304	0.106
Add	0.055	0.501	0.920
Sex	0.157	0.067	0.078
ME*Sex	0.087	0.988	0.439
Add*Sex	0.012	0.311	0.379
Me*Add	0.870	0.274	0.851
ME*Add*Sex	0.394	0.464	0.460

LME – Low energy; HME – High energy; Add – Additive

The effects of microbial enzyme supplementation (additive), energy level and their interaction on visceral organ weights at 35d are presented in Table 5.5. The average weights of pancreas and small intestines were ($P<0.05$) decreased by enzyme supplementation. TXAP Lot-1 and Lot-2 caused the greatest decrease. There was an interaction in pancreas weight between sex, additive, and ME ($P<0.05$). At low energy level males on TXAP Lot-2 had their pancreas reduced by 0.1g/100g body weight, while those of females on TXAP Lot-1 were reduced by 0.06 g/100g body weight.

Table 5.5. *The effects of feed additives (enzymes) and dietary energy level on visceral organ weights (g/100g body weight) at 35d*

	Pancreas				Proventr/Gizzard				Small intestines			
	LME		HME		LME		HME		LME		HME	
Add	F	M	F	M	F	M	F	M	F	M	F	M
0	0.3	0.3	0.3	0.3	3.7	3.7	4.4	4.3	6.2	6.9	6.4	7.2
1	0.2	0.3	0.3	0.3	4.1	3.4	3.9	3.5	5.9	5.2	4.9	5.7
2	0.3	0.2	0.3	0.3	4.0	3.5	3.9	3.9	5.0	5.5	5.8	5.3
3	0.3	0.2	0.3	0.3	4.2	3.7	4.0	4.1	6.9	5.5	6.3	6.7

SOV	P	P	P
ME	0.658	0.090	0.662
Add	0.018	0.418	0.047
Sex	0.102	0.064	0.808
ME*Sex	0.435	0.183	0.449
Add*Sex	0.422	0.541	0.740
Me*Add	0.958	0.209	0.945
ME*Add*Sex	0.044	0.828	0.504

LME – Low energy; HME – High energy; Add – Additive

5.3.2. Ileal digestibility of nutrients

The effects of microbial enzyme supplementation (additive), energy level and their interaction on ileal digestibility of nutrients at 21d are presented in Table 5.6. Digestibilities of calcium, gross energy (GE), phosphorus and protein were not ($P>0.05$) affected by enzyme supplementation. However, calcium and phosphorus digestibilities were influenced by the dietary energy level ($P<0.01$ and $P<0.05$, respectively). Chickens on low energy feeds showed higher calcium and phosphorus digestibilities than those on high energy feeds. There were no interactions ($P>0.05$).

The effects of microbial enzyme supplementation (additive), energy level and their interaction on ileal digestibility of nutrients at 35d are presented in Table 5.7. Digestibility of GE, phosphorus and protein were not ($P<0.05$) affected by enzyme supplementation. TXAP supplementation enhanced calcium digestibility with 0.177, 0.245 and 0.231% increase observed for TXAP Lot 1, Lot 2 and Lot 3, respectively. There was an interaction between sex and ME on gross energy ($P<0.01$) and protein ($P<0.05$) digestibilities. Males on low energy diet have digested more energy and protein than males on high energy diet. On the other hand females on high energy diet have digested more energy and protein than those on low energy diet. Phosphorus digestion was not enhanced ($P>0.05$) by either enzyme supplementation or energy level.

Table 5.6. *Effect of feed additives (enzymes) and dietary energy level on nutrient digestibilities (%) at 21d*

	Calcium				Gross energy				Phosphorus				Protein			
Add	**LME**		**HME**		**LME**		**HME**		**LME**		**HME**		**LME**		**HME**	
	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
0	0.74	0.80	0.71	0.74	0.80	0.78	0.77	0.77	0.79	0.79	0.73	0.73	0.87	0.85	0.85	0.86
1	0.83	0.82	0.72	0.61	0.80	0.77	0.75	0.74	0.76	0.76	0.71	0.66	0.86	0.82	0.82	0.81
2	0.76	0.76	0.74	0.64	0.81	0.79	0.75	0.73	0.74	0.79	0.71	0.67	0.88	0.87	0.82	0.83
3	0.72	0.81	0.71	0.72	0.71	0.72	0.79	0.75	0.70	0.76	0.77	0.72	0.81	0.83	0.85	0.81

SOV	**P**	**P**	**P**	**P**
ME	0.003	0.320	0.039	0.240
Add	0.939	0.510	0.705	0.324
Sex	0.885	0.409	0.864	0.516
ME*Sex	0.134	0.988	0.181	0.818
Add*Sex	0.270	0.993	0.956	0.933
Me*Add	0.396	0.137	0.504	0.517
ME*Add*Sex	0.957	0.886	0.855	0.531

SOV – Source of variation, LME – Low energy; HME – High energy; Add – Additive

Table 5.7. *Effect of feed additives (enzymes) and dietary energy level on nutrient digestibility (%) at 35d*

	Calcium				Gross energy				Phosphorus				Protein			
Add	**LME**		**HME**		**LME**		**HME**		**LME**		**HME**		**LME**		**HME**	
	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
1	0.23	0.29	0.50	0.58	0.69	0.66	0.71	0.74	0.99	0.98	0.99	0.99	0.77	0.72	0.80	0.81
2	0.56	0.58	0.60	0.57	0.76	0.70	0.66	0.78	0.99	0.99	0.99	0.99	0.82	0.75	0.71	0.85
3	0.76	0.58	0.57	0.67	0.74	0.65	0.72	0.73	0.99	0.99	0.99	0.99	0.78	0.79	0.80	0.79
4	0.65	0.59	0.60	0.68	0.72	0.73	0.71	0.78	0.99	0.99	0.99	0.99	0.82	0.78	0.81	0.83

SOV	**P**	**P**	**P**	**P**
ME	0.262	0.176	0.268	0.234
Add	0.029	0.376	0.096	0.367
Sex	0.871	0.569	0.712	0.884
ME*Sex	0.402	0.008	0.087	0.021
Add*Sex	0.919	0.357	0.760	0.635
Me*Add	0.209	0.691	0.691	0.451
ME*Add*Sex	0.764	0.506	0.215	0.087

SOV – Source of variation, LME – Low energy; HME – High energy; Add – Additive

5.4. Discussion

5.4.1. Performance variables

The results from this study have demonstrated that males and females respond differently to feeds differing in ME content and supplemented with various feed enzymes. Females performed better on feeds supplemented with TXAP Lot-1 while males responded better with TXAP Lot-3. The results of enzyme supplementation in this trial were disappointing. An improved performance was expected with enzyme inclusion because of their action in breaking the cell wall, and thereby releasing the enclosed nutrients (Rutherfurd *et al.*, 2004). The lack of response in this trial to the addition of any of the enzymes used is in contrast with findings by Dilger *et al.* (2004), and may be due to variability of maize quality (D'Alfonso, 2002). Further research needs to be done using maize from different regions or differing in nutritional quality.

5.4.2. Visceral organs

The results from this study, which showed no significant changes in visceral organ weights with enzyme inclusion, are in line with reports by Alam *et al.* (2003), who also reported no difference in liver or gizzard weight due to enzyme inclusion.

However, pancreas responded to enzyme inclusion as it had a significant decrease with TXAP Lot-1. The sexes also responded positively on different supplements: Lot-1 decreased the pancreas weight of males while Lot-3 decreased that of females. However, over the entire period broilers in this study showed a positive response to TXAP Lot-1 and Lot-2. The ability of these two enzymes to reduce the thickness of

the small intestines is beneficial for improving the contact of the substrate and the digestive/absorptive site (Bedford, 2000).

5.4.3. Ileal digestibility of nutrients

Difficulty was experienced in collecting digesta from the ilea of the birds sampled, which might be due to the high temperatures experienced at the time of sampling. Hai *et al.* (2000) reported that high temperatures suppress the movement of the digesta from the crop to the small intestines and thereby decrease enzyme activity. This in turn adversely affects nutrient utilization by the birds.

Enzyme supplementation in this trial significantly improved calcium but not phosphorus digestibility at 35 d, which is in contrast to what was expected. Dilger *et al.* (2004) reported a dramatic increase in nutrient digestibility due to phyzyme XP inclusion in maize-soya feeds. However, Classen and Bedford (1999) reported that exogenous enzymes are more effective at a young age.

Conclusion

The results of the present study suggest that feeds for male and female broilers should be supplemented with different enzymes. This is because males in this study had a tendency to respond positively to TXAP Lot 3, while females improved performance with TXAP Lot 1 supplementation. Further research needs to be done on this aspect, taking close consideration to phyzyme XP. The lack of improvement in phosphorus digestibility was disappointing.

CHAPTER 6

EFFECT OF ALL-LAC XCL 5X, ACID-PAK 2X, BIO-MOS® AND ZINC BACITRACIN ON NUTRIENT DIGESTIBILITY AND GASTROINTESTINAL MORPHOLOGY OF BROILER CHICKENS

6.1. Introduction

The chicken's gastro-intestinal tract is composed of the mouth, oesophagus, crop, proventriculus and gizzard, small intestine, and large intestine. Digestion of feedstuffs must be completed before reaching the ileo-caecal junction and the nutrients must be absorbed across the villi in the small intestine to satisfy the nutritional needs of the animal. Any undigested feed residue passing into the colon will be used as a substrate by the microflora (Bedford, 2000; Hong, 2002). This will decrease the ratio of beneficial to pathogenic microflora, which in turn will result in problems such as necrotic enteritis (NE), caused by *Clostridium perfringens* (Wilson *et al.* 2005). NE suppresses chick growth by interfering with the absorptive process in the intestine and is responsible for high mortalities in the broiler industry. The removal of antibiotic growth promoters in the animal industry has aggravated the risk of this disease.

Therefore, there is a clear need for safe alternatives to antibiotic growth promoters in the poultry industry. The ability of enzymes (Bedford and Morgan, 1996), organic acids (Patten and Waldroup, 1988; Mujdat *et al.*, 1999) and oligosaccharides (Iji and Tivey, 1998; Iji *et al.*, 2001) to act as growth promoters in broilers has been well documented. However, their effects when added in combination have been less studied. Owens *et al.* (2003) showed that when yeast extract, organic acids and

enzyme were added in combination they behaved synergistically. The aim of this study was to evaluate the effect of All-Lac XCL 5x (a probiotic), Acid-Pak 2x (an organic acid) and Bio-Mos® (a prebiotic), either individually or in combination, and compare them with a commonly used antibiotic (Zinc Bacitracin), using the visceral organ weights and gut morphology of the birds as measures of activity.

Because previous experiments in this series have failed to show significant improvements in broiler performance to the use even of Zinc Bacitracin, possibly because of the hygienic conditions in the broiler houses used in these trials, it was decided to inoculate the birds in this trial with *Clostridium perfringens* (*CP*) to ensure that performance was compromised. In this way it was hoped that the addition of Zinc Bacitracin and its alternatives would significantly overcome the harmful effects of this organism.

6.2. Materials and methods

6.2.1. Experimental design

The experiment was designed as described in Chapter 3 except that there were four blocks in the house.

6.2.2. Experimental animals and feeds

Two thousand eight hundred and eighty sexed day-old Ross chicks were used for this study. The chicks were randomly allocated to 48 pens in groups of 60 and assigned to one of the six feeds. The control diet contained no feed supplements whilst the five other feeds contained supplements (Table 6.2). There were eight replicates per treatment. The three basal feeds (starter, grower and finisher) were based on maize

and soya bean meal (Table 6.1). The test materials were obtained from Alltech, South Africa. Celite (2 %) was also included in the diet as an insoluble marker to enable the assessment of nutrient digestibility.

Table 6.1. *Ingredient and nutrient composition of the basal feeds (g/kg)*

Ingredient	Starter	Grower	Finisher
Maize	542	540	600
Soybean full fat	171	281	261
Soybean oilcake meal	105	23.0	21.6
Sunflower oil	78.5	98.5	65.6
Salt	1.3	2.7	1.6
Lysine-HCl	0.7	2.2	1.4
DL Methionine	2.0	2.0	1.9
Limestone	18.0	16.8	12.0
Dicalcium Phosphate	8.8	12.0	10
Vit / Min premix[1]	4.0	4.0	4.0
Celite	-	-	2.0
Nutrients			
Crude Protein	230	207	185
ME MJ / kg	12.1	12.6	12.6
Calcium	12.2	10.2	7.8
Available Phosphorus	3.8	3.1	2.7
Potassium	7.9	8.1	7.3
Methionine	6.2	5.4	4.9
TSAA	10.0	9.1	8.4
Lysine	13.2	12.1	10.3
Fat	61.5	77.3	72.4
Crude Fibre	43.3	47.7	51.0
Sodium	2.2	1.4	1.1

Table 6.2. *Composition of treatments*

Treatment	Description
1	Broiler mash without additives (Control)
2	Control + All-Lac XCL 5x (All Lac)
3	Control + Acid-Pak 2x (Acid Pak)
4	Control + Bio-Mos® (2.0g/kg/ 1g/kg /0.5g/kg) (Mos)
5	Control + Zinc Bacitracin (0.333g/kg) (Zn Bac)
6	Control + combination of treatment 2, 3 and 4 (LAM)

6.2.3. Inoculation with *Clostridium perfringens*

Clostridium perfringens was cultured by Dr Roger Horner of Allerton Veterinary Regional Laboratories, and used to challenge the birds at 21d of age. The feed consumption of birds on the day prior to the first inoculation was measured (feeders in six pens were weighed) and 85% of that quantity of feed for each inoculation day was used. Inoculation was performed twice a day, three days in a row, via the feed (inoculation at 09H30 and at 14H00). Food was withdrawn from the feeders at least one hour before inoculation, so that all the chickens ate simultaneously. Half the calculated daily feed quantity was used for each inoculation.

The feed and broth were blended manually into plastic buckets. Blending was rapidly done for a maximum time of five minutes and the feed was delivered to the relevant pens quickly, to ensure that the bacterium was still active when consumed by the birds. The quantity of each feed was weighed out ahead of time, to minimise blending time. In the evening the feeders were topped up with sufficient food to ensure an *ad libitum* supply during the night.

For each feed inoculation approximately 20ml of a *C. perfringens* culture containing 1.1 x 10 to the 10 bacteria per ml (to allow 20 x 4 twice daily) was used. The 20ml was diluted in an isotonic saline (0.9% Na) to bring it to 1200 ml and each of the pens was inoculated with 100ml of that solution.

6.2.4. Determination of visceral organ weights

Visceral organ weights were determined as described in Chapter 4.

6.2.5. Determination of ileal digestibility of nutrients

The ileal digestibility of the finisher feeds was determined, using the changes in concentration of acid insoluble ash (AIA), a component of Celite. Five birds from each pen were slaughtered at the end of the experiment and their ileal contents were collected and freeze-dried.

The concentration of AIA in the diet and digesta was measured as described by Choct and Annison (1992). Feed and digesta samples were weighed, ashed at 480^0C for 8 hours and thereafter treated twice with boiling 4 M hydrochloric acid. The residue was washed, dried at 180^0C for 6 hours and collected as acid insoluble ash. The nutrient digestibility was calculated as follows:

Digestible nutrient = $[((\text{nutrient/AIA})_{\text{diet}} - (\text{nutrient/AIA})_{\text{ileal digesta}})/(\text{nutrient/AIA})_{\text{diet}}]$

Where: $(\text{nutrient/AIA})_{\text{diet}}$ = ratio of nutrient to acid insoluble ash in a diet and $(\text{nutrient/AIA})_{\text{ileal digesta}}$ = ratio of nutrient to acid insoluble ash in ileal digesta.

6.2.6. Determination of nutrient concentrations

The nutrient concentrations in the feed and in the digesta were determined as described in Chapter 4.

6.2.7. Determination of the gastrointestinal morphology

At the end of the experiment one chick was sacrificed and dissected to expose the visceral organs. A piece of the jejunum (part of the intestinal tract extending from the ligament of Treitz to Meikel's diverticulum) was removed for analysis. The tissue was cut into pieces of 3 cm and fixed in gluteraldehyde. The tissues were further cut into pieces of 3 mm and were transferred into cacodylate buffer twice for 30 minutes. Thereafter the tissues were fixed in 2% osmium for 2 h and thereafter rinsed twice with a cacodylate buffer for 30 minutes. The tissues were dehydrated successively with 30%, 50%, 70%, 80%, 90% and three times with 100% ethanol for 10 minutes each. The tissues were divided and one half used for the measurements of cell proliferation and the other half for microbial assessment. For microbial assessment tissues went for critical drying for 1.5 h and thereafter mounted on slides and viewed on a microscope and digitised.

6.2.8. Statistical analysis

The data were analysed for significant differences using the analysis of variance of Minitab (Minitab, 2002). The differences between the mean values were determined by the use of least significant difference ($p<0.05$).

6.3. Results

6.3.1. Visceral organs

The effects of All-Lac, Acid Pak, Bio-Mos®, zinc bacitracin and the combination of the first three additives on visceral organ weights are presented in Table 6.3. Results indicate that the average weights of pancreas, proventriculus/gizzard and small intestines were not (P>0.05) affected by any of the feed additives used.

Table 6.3. *Mean visceral organ weights (g/100g body weight) at 42 d of age as influenced by feed additives used*

Treatment	Small Intestines	Liver	Spleen	Pancreas
Control	5.13	2.44	0.12	0.27
All-Lac	4.33	2.49	0.11	0.25
Acid Pak	4.28	2.65	0.18	0.30
Mos	5.35	2.58	0.14	0.22
Zn Bac	4.62	2.66	0.15	0.26
LAM	5.85	2.53	0.12	0.24
SOV	P	P	P	P
All-Lac	0.375	0.853	0.482	0.501
Acid Pak	0.347	0.471	0.009	0.434
Mos	0.811	0.628	0.228	0.108
Zn Bac	0.568	0.444	0.094	0.652
LAM	0.429	0.764	1.000	0.373

SOV = source of variation, LAM = a combination of All-Lac, Acid Pak and Bio-Mos®

6.3.2. Ileal digestibility of nutrients

The effects of All-Lac, Acid Pak, Bio-Mos®, zinc bacitracin and the combination of the first three additives on ileal digestibility of nutrients are presented in Table 6.4.

Results show that the digestibilities of protein, GE, phosphorus and calcium were unaffected (P>0.05) by any of the additives supplemented onto the feeds.

Table 6.4. *Mean ileal digestibilities of protein, gross energy (GE), calcium and phosphorus (%) at 42 d of age as influenced by feed additives used*

Treatment	Protein	GE	Phosphorus	Calcium
Control	0.88	0.84	0.75	0.67
All-Lac	0.88	0.84	0.72	0.66
Acid Pak	0.87	0.84	0.70	0.62
Mos	0.90	0.87	0.78	0.74
Zn Bac	0.88	0.85	0.74	0.68
LAM	0.88	0.86	0.78	0.74
RMS	0.0006667	0.001167	0.003378	0.005739
LSD	0.04593	0.06076	0.1034	0.1348

LSD = least significant difference, RMS = residual mean square

6.3.3. Gastrointestinal morphology

The surface areas of the villi at 21 and 42 d of age are illustrated in Figures 6.1 and 6.2, respectively. There were no lesions observed before the challenge by *Clostridium perfringens* (21 d) but after the challenge (42 d) the control group showed lesions on the surface area, which were not observed on the other treatments. At the younger age the villus is bent and thin but at the later age it is thicker and straight. The surface area of the villi of birds supplemented with Bio-Mos® (Figure 6.2 C) and zinc bacitracin (Figure 6.2 E) were thin whilst the others were thick.

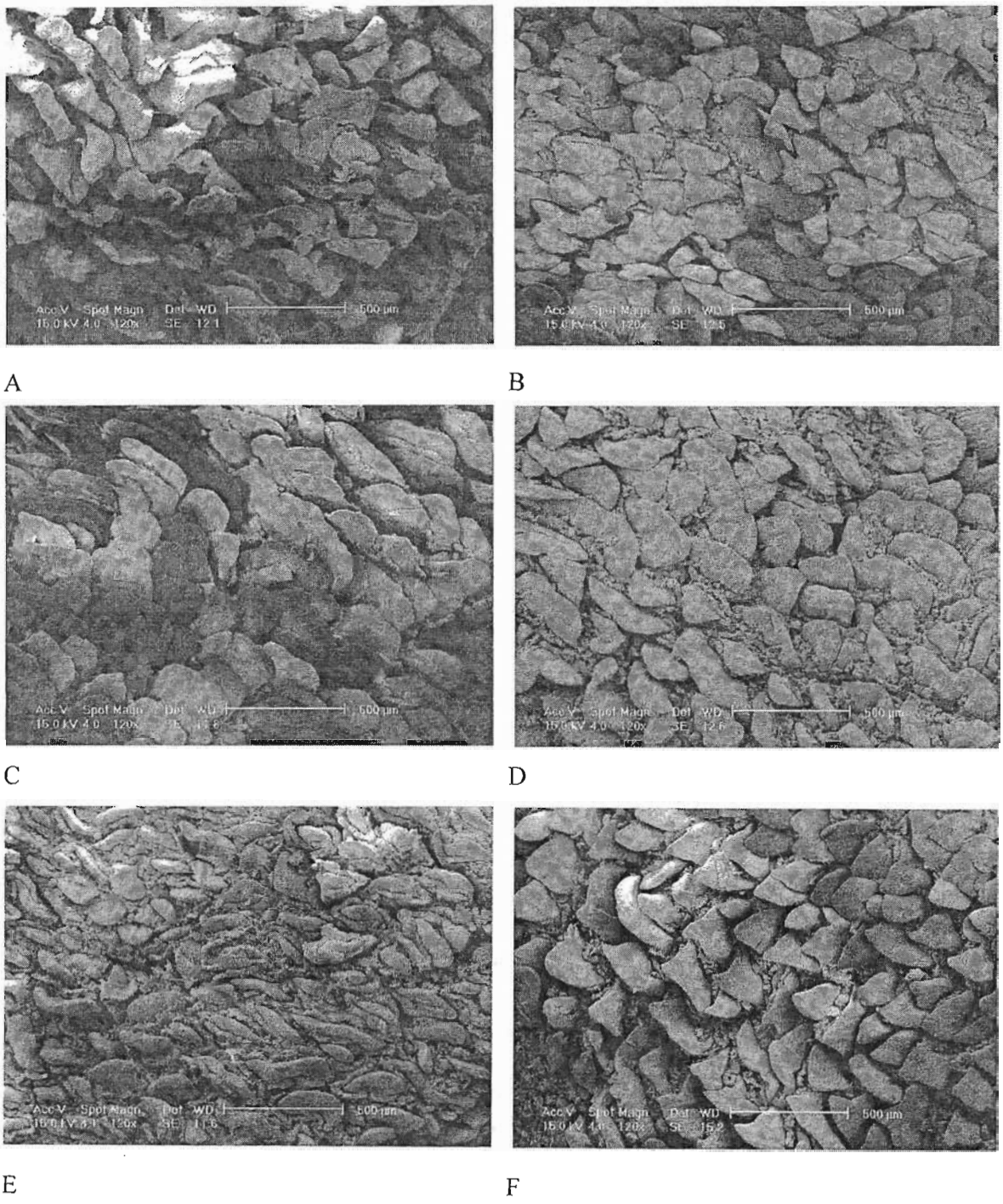
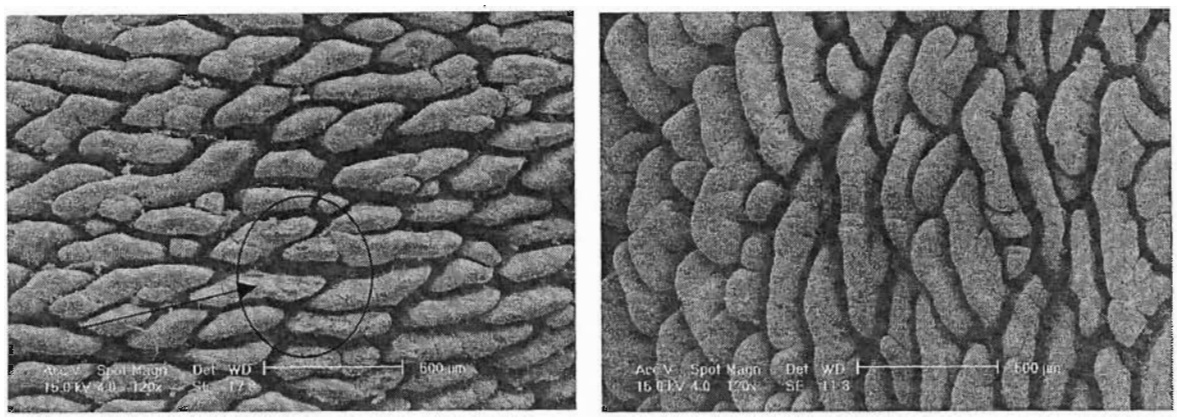
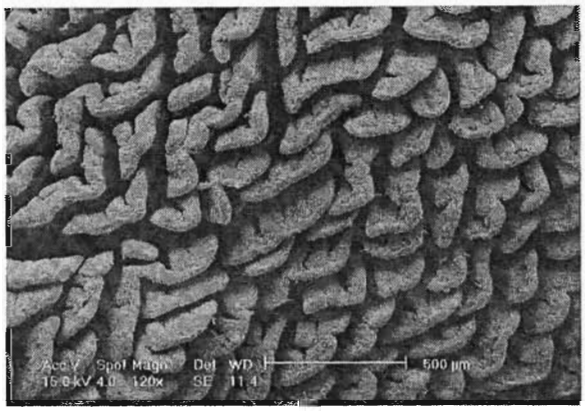


Figure 6.1. *Illustrations of the surface area of jejunal villi of chicks on control diet (A), and of those treated with All-Lac (B), Acid pak (C), Bio-Mos® (D), zinc bacitracin (E) and LAM, combination of B, C, D, (F) at 21 d.*

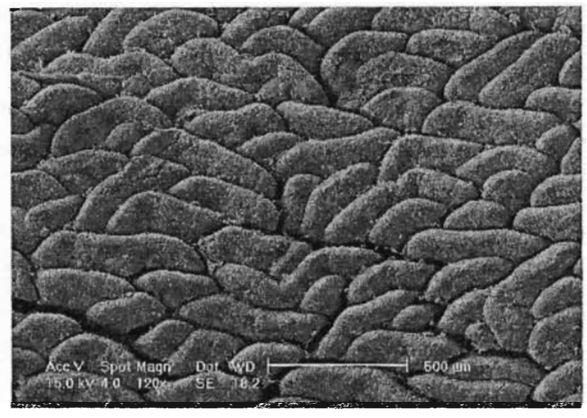


A

B



C



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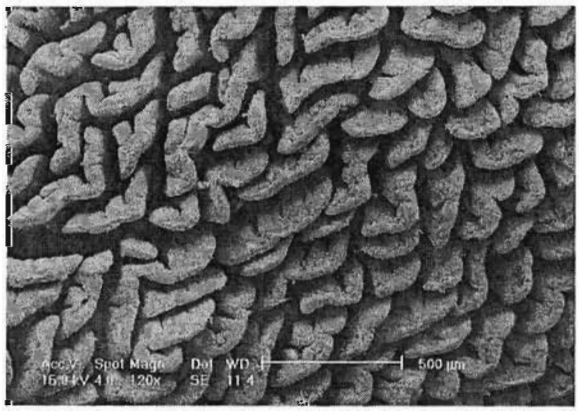
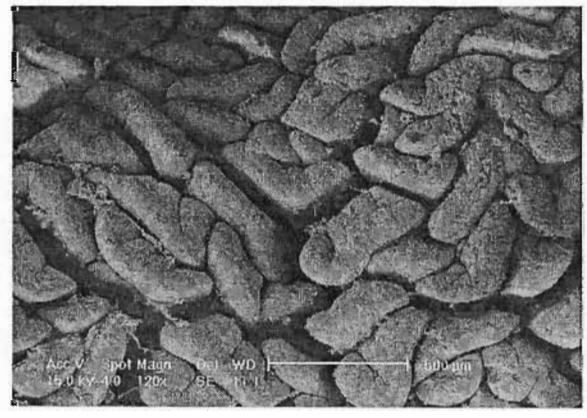


E



F

Figure 6.2. *Surface area of the jejunal villi of chicks on control diet (A), and those treated with All-Lac (B), Acid Pak (C), Bio-Mos® (D), zinc bacitracin (E) and LAM, combination of B, C, D, (F) at 42 d. Note the lesions on the villi in A.*

6.4. Discussion

6.4.1. Visceral organs

The results from this study showed no changes in visceral organ weights, except for the size of the spleen, which was increased by Acid Pak inclusion. These results are in line with reports by Kocher *et al.* (2003), who reported an increase in spleen weight with Acid Pak 4-way inclusion. Kocher *et al.* (2003) assumed that the increase in the weight of spleen was associated with an increase in the cell-mediated immune response. Whereas Al-Marzooqi and Leeson (2000) reported an increase in liver weight due to enzyme inclusion and attributed this to an increase in the metabolic activity related to increased nutrient utilization, no such increase was observed in this study.

6.4.2. Nutrient digestibilities

Where lesions occur on the villi of the small intestine it might be expected that the digestibility of nutrients be compromised, which would be overcome by the elimination of this damage through the use of feed additives such as those used in this trial. However, feed additives, individually or in combination, in this trial did not improve the digestibility of nutrients. There are no published data on the effects of these additives on the digestibility of nutrients so these results could not be compared with previously published data.

6.4.3. Gut morphology

The present study was designed to test whether the feed additives used as replacements for antibiotic growth promoters can effectively hinder or eliminate the negative effects of *Clostridium pefringens* on the GIT of the chicken. After the broilers had been challenged with *CP*, only those chickens on unsupplemented feed showed severe damage to their villi. Therefore the feed additives (Bio-Mos®, All Lac

XCL, Acid Pak and zinc bacitracin) were effective in preventing the ulcerating effect of *CP* on the intestines of the chickens. These results are in agreement with findings by Hofacre *et al.* (2003). The bending of the villi that was observed in broilers at a young age might be due to them not yet having matured. The thinning of the villi surface is good for digestion and absorption purposes (NRC, 1984).

Conclusion

The results from this study demonstrated that Bio-Mos®, Acid Pak and All-Lac are as effective as Zinc Bacitracin in preventing the ulcerating effects of *Clostridium perfringens*. Bio-Mos® had the same effect as an antibiotic in thinning the villi so as to improve the digestive/absorptive site, although the expected improvement in digestibility was not observed in this study. Further research on this subject is warranted.

CHAPTER SEVEN

GENERAL DISCUSSION

The objective of the research reported in this thesis was to investigate alternatives to antibiotic growth promoters in broiler nutrition. Viable alternatives are being sought because of the ban that has been placed on the use of antibiotic growth promoters by the EU, and the likelihood that such a ban will soon be placed on their use in South Africa. Four experiments were conducted, in which various alternatives were evaluated, as well as using different criteria to evaluate these alternatives. Instead of using only performance measures as the criteria, use was made in this study of changes to endogenous enzyme secretions, and to changes in the morphology of the villi using scanning electron microscopy.

Various replacements for antimicrobial growth promoters have been suggested as a means of controlling growth and body weight gain in broiler chickens. Based on the results obtained in Experiment 2, the enzyme TXAP appears to be an effective alternative at a young age. This is in agreement with findings by Classen and Bedford (1992) that exogenous enzymes are more beneficial at a young age because they supplement the bird's poorly developed endogenous enzymes at that stage. TXAP decreased FI without negatively affecting weight gains. This result was obtained when TXAP was used alone. When used in combination with other enzymes (phytase, lipase and Phyzyme XP in Experiment 3) bird performance was not improved, thus disagreeing with the suggestion that these may complement each other (Campbell and Bedford, 1992)

TXAP had no effect on visceral organ weights of the chickens whether used alone or in combination with other enzymes (phytase and lipase) (Experiment 2). In Experiment 3 TXAP Lot 1 and 2 reduced small intestine weights, thereby improving contact between the feed and the digestive/absorptive sites, as suggested by Bedford (2000). The study with other alternatives to antimicrobial growth promoters (Bio-Mos®, Acid Pak and All-Lac XCL) (Experiment 4) also showed no effects of these to visceral organ weights, except for the spleen, which was increased by the inclusion of Acid Pak (Kocher *et al.* 2003).

TXAP and phytase, individually, had a positive effect on nutrient utilisation as they increased the digestibility of nutrients (Experiment 2). In Experiment 3 a higher energy level improved calcium and phosphorus utilization at a young age (21 d), and TXAP improved calcium utilization at a later stage of growth (35 d). The other additives tested did not have any effect on nutrient utilization.

Lipase increased chymotrypsin activity in Experiment 2. Li *et al.* (2004) assumed that endogenous enzymes would be less necessary when feeds are supplemented with exogenous enzymes, which is contrary to the result in this experiment. Exogenous enzymes are assumed to suppress the secretion of enzymes because there will be less or no substrate for them to digest.

In the first three experiments very little change to performance was noted, not only to the use of the various alternatives to antibiotic growth promoters, but to the growth promoters themselves. This suggests that the conditions in the broiler houses where these experiments were conducted were so hygienic that the performance of the birds

was close to the potential. For this reason, birds were challenged with *Clostridium perfringens* in Experiment 4, and this challenge did cause lesions on the small intestines. Prior to the challenge (21d) there were no lesions observed on the villi surfaces. However, after the challenge (42d) the birds given unsupplemented feeds had lesions on the villi surfaces (Figure 6.2 A). This illustrates that Bio-Mos®, Acid Pak and All Lac can be used as replacements for antibiotic growth promoters to prevent the negative effects of *CP*.

The most important conclusion from the work reported here is that the enzyme TXAP may be used to enhance feed conversion efficiency in broiler chickens, due to its ability to reduce feed intake without apparently having any adverse effect on weight gain. The effect of combining this enzyme with other enzymes needs to be studied further as there appeared to be no benefit in using such combinations in this study. However, it should be borne in mind that the conditions under which these trials were conducted were such that little benefit was derived from the use of any of the feed additives used, calling into question the value of conducting trials of this nature in hygienic conditions. It may be more beneficial to test the various alternatives to antibiotic growth promoters under field conditions, where it is common for performance to be severely constrained by *Clostridium perfringens* and other harmful organisms.

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