Chapter-I

INTRODUCTION

Broiler poultry farming is a lucrative business across the globe. Broiler chickens are known to efficient converter of feed to meat. The main trend of all broiler farmers is to gain optimum production with low investment. This trend is pressing the poultry geneticists and nutritionists to find out alternative strategies for profitable poultry production. Feed is the single variable unit accounted for up to 70 % of the total production cost. It is noteworthy that the cost for protein requirement of poultry is always higher than that of carbohydrates The increasing cost of protein feedstuffs and concern over environmental pollution in animal agriculture, have resulted in a greater interest to supplement exogenous enzymes in poultry diets (Law *et al.,* 2015). Microbial enzymes are used as "pro-nutrient", which improves the nutrient digestibility and growth performance of poultry (Rosen, 2006). The inclusion of enzymes could reduce the feed cost by reducing crude protein and amino acids when it is supplemented with the dietary ingredients (Romero and Plumstead, 2013).

Protein is very important for growth and the most exorbitant item of any livestock diet. Now-a-days it is a great challenge for us, to utilize protein more efficiently for the reduction of feed and production costs without affecting animal health. Animals are required proteolytic enzymes say protease for digestion of protein. Supplementation of protease enzymes can enhance protein digestibility and protein utilization by the animal. As it is reported that large amount of undigested and unabsorbed protein is passed through the digestive tract of poultry, which result in increased excretion of nitrogen and environmental pollution (Applegate *et.al*, 2008; Angel *et.al,* 2011). Besides, the cost of

protein feedstuffs such as soybean meal is increasing rapidly since 2000. Application of protease enzyme in the ration accelerates protein ingestion and reduces environment pollution by lowering nitrogen emission, and thereby enhance protein digestion to an extend, It fortifies natural proteases in feed, and reasonably ameliorates amino acid and peptide for gearing up broiler performance. It saves feed cost considerably by increasing the digestibility of numerous protein sources and cereal grains (Leinonen, I., & Williams, A. G., 2015).

Monogastric animals or poultry produces enzymes inherently for executing the digestion process of feed materials. However, they can not digest high fibrous diet or have paucity of enzyme to break down fibre completely. For this why, microbial or exogenous enzymes are needed to digest the feed. Enzymes are composed of amino acids with vitamins and minerals, and it acts as a biological catalyst and performs many biochemical reactions or functions inside the body mechanisms. The benefits of using enzymes in poultry diets are to increase performance and feed efficiency and less environmental problems (Angel et al., 2011; Aureli et al., 2010; FruNji et al., 2011)

Major nutritional advances in the last fifty years is the feeding enzymes to poultry. It is the culmination of something that nutritionists realized for a long time, but it was out of their reach until 1980's. . The enzymes are retrieved from microorganisms that are carefully selected for the task and grown under controlled conditions (Wallis, 1996). Inclusion of protease along with phytase increase broiler performances consistently, but variable results are found when protease is used singly in the diet. Nitrogen retention was increased reported by Ghazi *et al.(*2010) when broiler chickens fed soybean meal diet

supplemented with protease. Protease supplementation lowers the dietary protein level without affecting broiler performance.

Xylanase and -glucanase enzymes have therefore been widely used and have shown an improvement in broiler chicken performance (Bedford and Morgan, 1996). Among the other enzymes, protease and -amylase are also commonly used to improve protein and starch digestion in non-ruminant animals (Burnett, 1996). The great variation in quality and composition of maize and the anti-nutritional factors present in soybean meal such as allergenic proteins which are not always entirely neutralized by heat treatment could lead to reduced feed digestibility in pigs and poultry (Tamminga et al., 1995). The use of protease is capable of increasing the solubility of soybean meal protein, decreasing the effect of trypsin inhibitors (Caine et al., 1998) and improving amino acid digestibility (Bernard and McNab, 1997). Ghazi et al. (2002) found that adding different sources of protease could increase true metabolizable energy (TME) and true nitrogen digestibility in chickens, with large differences among protease sources. Another experiment by Zanella et al. (1999) demonstrated that chickens receiving a lower energy diet supplemented with a cocktail of amylase, protease and xylanase achieved the same growth performance compared to a maize–soybean basal diet and increased protein digestibility by 2.9%. Researchers in the past have mostly focused on the energy improvement with much less emphasis on the protein and amino acid of maize–soybean-based broiler diets. Protease enzyme supplementation can possibly be employed to decrease dietary protein with the same broiler performance, hence less protein waste and nitrogen excretion into the environment

The protein requirement of modern broiler chicken is fulfilled mostly from vegetable sources say soybean meal, gluten meal, canola meal, cotton seed meal, sunflower meal along with other sources like animal by-products (Buttin et.al, 2016). The most common source of vegetable protein is soybean meal used for broiler diets, and the price of this protein is increasing rapidly since 2000 (Buttin *et.al*, 2016).. It has been reported that protease enzymes have many advantageous role over decreasing undigested protein, protein need in the diet, proteolytic fermentation, biogenic amines and bacterial toxins (Buttin *et.al*, 2016). Therefore, protease enzymes could be a great interest for many poultry industries to be used as an alternative and important feed supplement digestive enzyme for broiler and other animal production. The finding of the research study will help the poultry integrators to boost up their broiler meat production through reducing production cost and environmental hazard.

Objectives of the study:

i) To ascertain the live weight, feed consumption, feed conversion ratio (FCR) and viability of broilers fed protease supplemented diet.

ii) To evaluate the carcass traits and gastro-intestinal development of the broiler diet fed test diets

iii) To assess the intestinal tissue morphology (villi length, breadth, crypt length and breadth) and blood metabolites of broilers fed enzymes -supplemented diet.

iv) To appraise the cost benefit analysis of raising broiler chickens fed test diet.

Chapter-II

REVIEW OF LITERATURE

2.1 Background

Higher production with a minimum cost and environmental impact is the main goal for any broiler producer. The demand for broiler meat is growing rapidly around the world. Poultry production has been increasing sharply since the sixties and shows the highest rate of increase followed by pigs at a substantially lower rate (Raney *et al*., 2009). This indicates that production of poultry is one of the main reasons for increased production of agricultural feedstock and nitrogen fertilizers. Production of nitrogen fertilizers is very energy demanding. In addition, this production means that non-reactive molecular nitrogen (N_2) is converted into reactive forms of nitrogen compounds (e.g. NH_3, N_2O, NO_3, NO), which either act as greenhouse gases or cause of atmosphere and water pollution.The pollution issue is further emphasized by the development towards industrial production systems, where return of the manure to agricultural land as fertilizer is becoming increasingly difficult. Overall this means that, there is a need to improve feed utilization efficiency and to reduce harmful nitrogen emissions to the environment from broiler production. Using a protease in the feed can help to increase the digestibility of protein and utilization of feed by broiler chickens and thus represents a means to address the emerging need and challenges of the poultry industry.

The biggest single expense in poultry production is feed responsible for up to 70% of total production cost. Protein is the second major nutrient item and the most expensive in the broiler diet. The prices of protein sources in modern meat chicken diets are increasing consistently since 2000 (Buttin *et al*, 2016). Besides these, a good amount (18-20 %) of

protein passes through the gastrointestinal tract of poultry without being completely digested and absorbed (Angel et.al, 2011, Applegate et.al, 2008), which causes a great losses for poultry production. This undigested protein represents a scope for the use of supplemental exogenous proteases in broiler feeds to enhance protein digestibility. Poultry can produce some enzymes to improve the digestion of feed nutrients. However, poultry do not have much enzyme to digest fibre completely so exogenous enzymes are needed in feed to help digestion. The wide range of endogenous proteases, that synthesized and released in the gastrointestinal tract (**GIT**) is generally considered to be enough to optimize feed protein utilization (Le Heurou-Luron et al., 1993; Nir et al., 1993).

The first commercial protease was available in the poultry feed market in the 1990s which was the combination with other enzymes, with the aim to enhance the energy and protein digestibility of oilseed meal and grain based diets (Simbaya *et al.,* 1996). Whereas some data shows inconsistent results (Simbaya *et al.,* 1996; Marsman *et al.*, 1997; Naveed e*t al.*, 1998), other publications show increases in broiler live performance as well as in nitrogen energy and utilization when proteases were added to diets (Ghazi *et al*., 2003).

Supplementation of enzyme in poultry diets is justified nutritionally, economically, and environmentally (Kamel *et. al*, 2015). Enzymes are used to enhance the energy value of feed ingredients, and increase the use of protein, carbohydrates, fats, and phosphorus from plant materials. Which cause to a lower excretion rate of undigested nutrient elements into the environment and, hence, reduced environmental pollution. The most

important function of proteases is digestion of nitrogenous compounds in feed materials, which is necessary for reducing nitrogen (N) excretion – a major pollutant worldwide (Kamel *et. al*, 2015).

Studies have shown that response to exogenous enzymes ranges from adverse to beneficial (Campbell and Bedford, 1992, Smits and Annison, 1996, Madrid *et. al*, 2010, and Oxenboll et. al, 2011). Some studies has pointed out that protein is less digestible (80-85%) compared to starch (90%) in corn-soy diets (Kamel *et.al*, 2015). Also, some amounts of protein excreted through the gastrointestinal tract without being completely digested. Hence, the nitrogen content in the undigested protein is released into the environment, and this protein is of no use. Thus using proteases is very important to enhance protein utilization and reduce protein waste (Kamel *et. al,* 2015).

2.2 Sources of protease enzymes

Owing to the high demand of proteases in the global market, the search for proteases has greatly increased, as they are found everywhere in nature, namely, in plants, animals, and microbes. However, production of plant proteases is much time-consuming (Rani et al., 2012). The animal proteases, such as pancreatic, trypsin, pepsin, chymotrypsin, and renin are produced and prepared in pure form in large quantities (Weaver *et al.*, 1977; Boyer and Krebs, 1986). The production of proteases from animal sources is insufficient to fulfill the worldwide demand; therefore, scientists are working with their research of producing protease from bacterial sources Owing to the broad-spectrum biochemical variety and easy genetic manipulation, microbes produce an exceptionally promising number of proteases (Kuhad *et al.*, 2011). Among different sources, such as plants, animals, and microbes, proteases are generally produced by microbial sources. Among

microbes, *Bacillus* sp. are greatly researched for protease production in a large scale, and they are exploited in various industries like leather, detergent, pharmaceuticals, and textile; some fungal species like *Aspergillus* sp. have been studied thoroughly for the production of alkaline protease (Singhal *et al.*, 2012; Singh et al., 2016; Rehman *et al.*, 2017). Halophilic enzymes are getting more priority in biotechnological applications due to their thermal stability and ability to retain activity under high stress from organic solvents except for pyridine, which inhibits protease activity. The enzyme activities remain same upto 80% even at 50, 55, and 60°C for at least 30 min (Madern *et al*., 2000; Xue *et al*., 2012).

2.3 Types of protease enzymes:

The main enzyme types are exo-proteases, which act at or near the ends of the peptide chains, delineated as aminopeptidases and carboxypeptidases to indicate their action is at the N- or C-terminals of the peptide substrates. These enzymes may be further classified depending on the size of the moiety that is cleaved off, be it, for example, an amino acid, a dipeptide, or a tripeptide. Industrial organisms known to produce aminopeptidases include *Aspergillus oryzae, Bacillus licheniformis, B otulinum stearothermophilus, and Escherichia coli*. Carboxypeptidases, are produced by species such as *Aspergillus*, *Penicillium*, and *Saccharomyces* species, are classified further into three groups based on the presence of certain amino acid substituents at their active sites, that are- the serine carboxy proteases, the metallocarboxyproteases, and the cysteine carboxyproteases.

Endoproteases, which attack internal peptide bonds in the peptide chain remote from the C- or N-terminal, are further classified into the following subgroups based on their specific mechanism of action (Butler, M., & Moo-Young, M., 2011).

1. Serine endoproteases, having a serine residue at their active sites that participates in the catalytic reaction, have broad specificities and indeed these enzymes catalyze hydrolytic reactions involving esters and amides as well as peptides. Important well-known enzymes of this subgroup include the chymotrypsins and the subtilisins. Many serine proteases have high pH optima in the range 7–12, with those in the pH range 9–10 being known as serine alkaline proteases or the first generation of detergent proteases and those with pH optima of 11–12 being known as the high alkaline proteases, the second generation of detergent proteases. The subtilisins are an important family of serine proteases produced by *Bacillus* species. These enzymes were also the starting material for development of a third generation of detergent enzymes, resistant to oxidation by bleach and related detergent oxidants, which involved substitution of an oxidation-sensitive amino acid near the enzyme‘s active site with an oxidation-resistant amino acid. These enzymes are further discussed under commercial *Bacillus* proteases below.

2. Aspartic endoproteases have low pH optima (3–4),it contain a pair of aspartic acid residues at their active sites that are sorted into two groups: pepsins and pepsin-like enzymes (e.g., produced by *Aspergillus, Penicillium,* and *Rhizopus* species) and rennet and rennet-like enzymes (e.g., produced by *Mucor pusillus, M. Miehei,* and *Endothia* species).

3. Cysteine/thiol endoproteases contain a cysteine–histidine dyad at their catalytic sites and generally require reducing agents for retention of catalytic activity and are denatured or inhibited by sulfhydryl reagents. Subgroups include papain and papain-

like enzymes (including clostripain and streptopain from *Clostridium histolyticum* and *Streptomyces* species, respectively).

4. Metalloendoproteases. Biocatalysis requires the presence of a bound divalent cation. These enzymes are inhibited by ethylenediaminetetraacetic acid but not by sulfhydryl reagents or diisopropylfluorophosphate. The highly thermostable neutral zinc protease produced by *B. stearothermophilus*, thermolysin, is a well-known member of this subgroup. Protein stability is enhanced by four calcium atoms. Other group members include the metalloprotease collagenases (microbial example from *C . histolyticum*), the neutral metalloprotease elastase (microbial example from *P. aeruginosa*), and the alkaline cell wall lytic protease I from *Myxobacter* sp.

5. Glutamic acid and threonine endoproteases represent recently characterized new enzyme families having reaction mechanisms that involve specific participation of active-site glutamic acid and threonine, respectively.

2.4 Chemistry of protease enzymes

Proteolytic enzymes hydrolyze peptide bonds and are also known as peptidases, proteases, or proteinases (Mótyán *et al.,* 2013). The physiological advantages of proteases are essential for all living organisms, and proteolytic enzymes can be differentiated based on their origin: microbial (bacterial, fungal, and viral), plant, animal and human (Mótyán *et al.,* 2013). Proteolytic enzymes are in the hydrolase class of enzymes, and are grouped into the subclass of the peptide hydrolases or peptidases. On the basis of the site of enzyme action the proteases can also be subdivided into exopeptidases and endopeptidases. Endopeptidases split peptide

bonds within and distant from the ends of a polypeptide chain. The hydrolysis of the peptide bonds near the *N*- or *C*-terminal ends of the substrate is catalyzed by exopeptidase. Single amino acids, dipeptides (dipeptidyl peptidases) or tripeptides (tripeptidyl peptidases) from the N-terminal end of their substrates can be liberated by Aminopeptidase. Single amino acids can be released from dipeptide substrates by dipeptidases or from polypeptides by carboxypeptidases, while dipeptides can be freed from the C-terminal end of a polypeptide chain by peptidyl dipeptidases (Figure 1) (Mótyánet al., 2013).

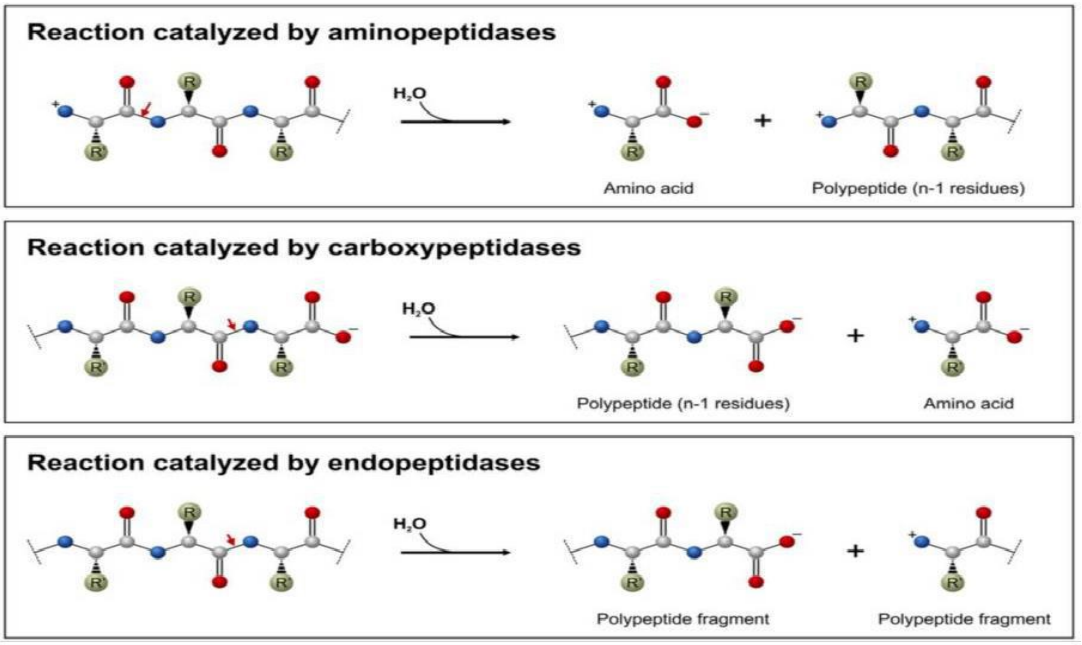

Figure 1: Action of aminopeptidases and carboxypeptidases removing the terminal amino acid residues as well as endopeptidases on a polypeptide substrate (having n residues). Red arrows show the peptide bonds to be cleaved (Mótyánet al., 2013)

There has been a a number of research about using protease in broiler diets. Some of research shows that most the broilers which are tested by adding protease in their diet have shown improvement in feed efficiency especially in birds fed low protein diets

(Buttin et al., 2016). However, many studies have reported improvement of crude protein digestibility by the addition of protease enzyme (Kamel et al., 2015). Furthermore, other studies have concluded that protein and energy digestibility enhanced by exogenous serine protease enzyme supplementation (Gitoee et al., 2015).

2.5 Role of protease enzymes on the protein digestion

Proteins are any of a class of naturally occurring, extremely complex nitrogenous compounds made up of amino acids subunits which are comprised of carbon, hydrogen, oxygen, nitrogen, and sometimes sulfur. A protein molecule consists of one or more chains of amino acids. Proteins are essential components of all body cells (such as enzymes, hormones, and antibodies) that are necessary for essential body functions. They are essential in the animal's diet for growth, tissue repair, and reproduction and can be found in many feedstuffs such as meat and fish meals, cereal grains, and legume byproducts such as soybean meal (Bailey *et.al,* 2016*).*

After consuming protein, the digestive tract breaks down the protein into amino acids by extracting protein degradation oxygenated enzymes such as protease, pepsin, and trypsin. The amino acids are then absorbed by the blood and carried to cells that change the individual amino acids into the specific proteins required by the animal. Proteins are used in the construction of body tissues such as muscles, nerves, cartilage, skin, feathers, and beak, and so on. Egg white is also high in protein. Proteins have major roles in poultry production because They are necessary for growth, maintenance of body, production, and reproduction (Dale, 2009). some studies has shown that the rate and efficiency of growth is decreased, and carcass composition is inferior when the crude protein (CP) level is

decreased by more than 3%, even when all the other nutrient requirements are met (Bregendahl *et al.,* 2002).

In case of monogastric animal,the digestion of protein is controlled mainly by endogenous protease. Two stages of the digestion process is there (Bedford et al., 2014). The first stage is the gastric stage, which is a low pH environment. During this stage pepsin breaks certain chemical bonds in proteins, producing smaller molecules called peptides and initiate protein digestion. The second stage is the small intestinal stage, it's a neutral phase where trypsin, chymotrypsin, elastase, and several other exo-proteases are present to complete the process of protein digestion (Bedford et al., 2014). The pancreas synthesizes trypsin and chymotrypsin, and these enzymes are released into the small intestine through the pancreatic duct. When food, that are digested partialy moves from the stomach into the intestine, trypsin, and chymotrypsin complete protein digestion, producing simple amino acids that are absorbed into the blood (Rogers, 2015).

The secreted proteases are essential for degrading dietary proteins and, are potentially dangerous as the animal's gastrointestinal (GI) tract and the cells in which they are made they could be digested by them (Bedford *et al*., 2014). However, this problem is solved since the enzymes are secreted in an inactive form and can only activated by pH or enzymes in lumen. In addition,a layer of mucus protect the gastrointestinal (GI) tract which is relatively resistant to proteolytic destruction. Generally, this system works well but protein digestion may be compromised, and certain amounts of protein pass through the gastrointestinal tract without being completely digested. Thus, the nitrogen content in the undigested protein is going into the environment. Several factors are responsible for influence protein digestion rate including (Kamel *et al.,* 2015): protease inhibitors used in

feed ingredients, intestinal damagee, increased transit time through the gastrointestinal tract, and insufficient endogenous proteases secretion

impediments like viscous non-starch polysaccharides (NSPs) also reduce the transformation rate of proteases, which resulting in insufficient proteases secretion to complete digestion (Bedford et al. 2014). Young and sick animals also have limited ability to produce or secrete digestive enzymes. In many cases the animal is found with one or more of the above situations. Under such circumstances, supplementation of the diet with enzymes can treat one or more of the factors limiting digestion increase more complete protein digestion and causes more efficient growth (Kamel *et al.*, 2015).

Recent studies has shown significant increase in digestibility of protein when proteases are used, but the performance improvement is not always clear (Angel *et al*., 2011). However, in the work of Liu *et al*.,2013) the protease effectiveness was correlated to protein level in the diet. The efficacy of a protease may also dependent on the ingredients which are used in the ration (Kocher *et al*., 2003). The efficacy of a protease may also depend on the availability of other enzymes, for example the benefit is lost or reduced when the protease is tested with a xylanase and/or phytase (Kalmendal, 2012). However, according to Yan *et al*. (2012) it was clear that the advantage of the protease was higher in the starter diet compared with the finisher diet, which indicate that the young animal may be more reactive to protease. A link between protein and protease was seen in which digestibility of CP and energy were more when protease was added to high-protein diets as compared with the low-protein diets. Another study shows that interaction between energy and protease was associated with an increase in energy digestibility when protease

was added to high-energy diets, as compared with the low-energy diets (Freitas *et al.*, 2011).

Kamel et al. (2015) showed that addition of protease has a significant effect on increasing the level of digestibility CP. The results were compatible with (Freitas *et al.*,2011) who showed an increase of 1.8% in crude protein digestibility in high-protein diets when the protease was added to it, while an improvement of only 1% was in diets with low protein. In addition, Angel et al. (2011) showed an improvement of digestibility of crude protein and amino acid in diets supplemented with graded levels of protease fed to 22-day old broiler chickens. Moreover, Fru-Nji et al., (2011) concluded that protein and energy digestibility enhanced by exogenous protease enzymes. the effects of multi-enzyme (ME) including protease dietary treatments on feed intake (FI), body weight (BW) and feed conversion ratio (FCR) at 10, 24 and 49 days of age was pointed out by Gitoee et al., (2015) . Results showed that the Multi enzyme main effects and their interaction had no significant role on Feed intake of broilers at 10 days and 24 days. Although, no enzyme effects or interaction could be detected in 49 days, the multy enzyme affect the feed intake of birds in the finisher diet (49 days) significantly. On the other hand, other research indicate no effect of protease alone or in combination with other enzymes on body weight and FCR (Kocher et al., 2003). Marsman et al. (1997) showed no beneficial effects of protease inclusion in a maize-soybean diet on broiler performance. Some other research showed that the source of the protease is valuable in the effectiveness of the enzyme to improve broiler performance by including a specific protease P2 (isolated from *Aspergillus* strains) in a SBM diet.

2.6 Role of protease on amino acids utilization:

Amino acids are used as the building blocks of structural proteins (muscle, skin, ligaments), metabolic proteins, enzymes, and precursors of several body components. Because body proteins are constantly being synthesized and degraded, an adequate amino acid supply is critical to support growth or egg production. In poultry, 22 amino acids are needed to form body protein, some of which can be synthesized by the bird (non-essential), whereas others cannot be made at all or in sufficient quantities to meet metabolic needs (essential). Hence, Amino acids are divided into two categories, essential and nonessential. Example of essential amino acid such as arginine, glycine, histidine, leucine, isoleucine, lysine, methionine, cystine, phenylalanine, threonine, tryptophan, and valine are those that cannot be made in the body to cover the necessity of the animal. The non essential amino acids are those that the body can produce if materials are available. About ten of them are essential and these must be supplied in the feed. Poultry diets typically consist of a variety of feedstuffs because no single ingredient can supply all the required amino acids at the optimum levels (Dale, 2009).

Breakdown of amino acids is also responsible for higher nitrogenous excretions in the fecal matter (Applegate et.al, 2008).

The best way to decrease nitrogen in poultry manure is reduce the amount of CP that is fed to the broiler by supplementing diets with amino acids.Decreasing the non essential amino acid amount, combined with increasing more essential amino acids in the diet, can reduce the efficacy of total N retention by the bird (Applegate et.al, 2008). Formulation based on birds requirements of amino acid not on CP requirement can minimize N excretion because it simply reduces total N intake (Ferguson et al., 1998). Furthermore, broiler litter N was reduced more than 16% when dietary CP was reduced by 2%, while maintaining similar levels of dietary amino acids (Applegate et al. 2008).

2.7 Role of proteases enzymes on the nitrogen and environmental condition

In order to meet the increasing demand for meat and egg supplies, the poultry industry has made adjustments. The poultry sector has been growing at more than 5 percent annually, over the past three decades, and its part in world meat production increased from 15 percent three decades ago to 30 percent in 2006 (FAO, 2006). This growth has been associated with intensifying and concentrative of poultry operations. The demand of lower production costs and enhance supply promote more efficient operations, by growing to more large, more specialized, and more integrated facilities by improving in animal genetics use, proper nutrition, and latest technologies of production. Animals that are reared in intensive production systems consume a large amount of protein and other nitrogen-containing substances in their diets. The changing of dietary nitrogen to animal products is relatively inefficient, with 50 to 80 percent of the nitrogen is excreted (Gerber et al., 2015). Nitrogen is excreted in two form of compounds- organic and inorganic compounds. Four main forms of Nitrogen emitted from manure take: ammonia (NH3+), di-nitrogen (N_2), nitrous oxide (N_2O) and nitrate (NO_3) (Gerber et al., 2015). The production of nitrogen excreted from intensive livestock and poultry operation is a serious environment concern. Besides polluting the air and water, nitrogen in poultry fecal matter or litter is converted to volatile ammonia through microbial fermentation which affect the health of birds and farm workers (Hassan et al., 2011). Nitrogen pollution is one of the major risk to the quality of soil and water. These risks relate to high levels of nitrates that can be leached to the groundwater table or to surface water causing eutrophication. In its nitrate form, nitrogen can easily be leached below the rooting zone and into groundwater. Poultry manure contributes to the structural nutrient overload in these areas. Moreover, the manure may be applied to crops or fish ponds in

excess or in addition to chemical fertilizers or fish feed, resulting in an over-supply of nutrients. Such saturated systems will release a huge amount of nutrients into the environment. Excessive levels of nitrogen in the environment lead to negative effects (De Vries et al., 2003). Increased levels of nitrogen in the environment may have several adverse effects such as- reduced plant species diversity in the ecosystems, eutrophication of surface waters, groundwater pollution due to nitrate leaching, and global warming by nitrous, nitrogen oxide, and ammonia (N2O, NOx, and NH3) emissions (Gerber et al., 2015).

One of the major air pollutant of present time is Atmospheric ammonia (NH3) due to its role in regional and global-scale negative effects when deposited into ecosystems. Ammonia is a gas which is soluble and reactive (Sutton and Fowler, 1995). Which indicates that it dissolves, for example in water, and that it will form ammonia-containing compounds by reacting with other compounds to. The ammonia concentrations in the air are greatest in intensive livestock farming areas where there is Agricultural land receiving large inputs of nitrogen from manures normally acts as a source of ammonia. A net source of ammonia is little deposition of intensively managed ammonia gas to farmland (Sutton and Fowler, 1995). Atmospheric ammonia can be absorbed by water, land and vegetation known as dry deposition. Rain or snow can remove ammonia from the atmosphere by wet deposition. Consequences of ammonia deposition include; acidification of soil and water, eutrophication due to nitrogen enrichment with consequent species loss, damage of vegetation , and increases in emissions of the greenhouse gases such as nitrous oxide (Gerber *et al*., 2015). Nitrogen excretion from farm animals is indicator of an unfriendly environmental footprint. So, the advantage of

using protease enzymes may not only be feed efficiency and utilization improvement by the animal to reduce production cost, but also to decrease the total content of nitrogen being excreted in the manure (Kamel *et. al,* 2015).It shows that for optimum environmental performance of broilers, one of the more promising nutritional strategies is the use of a protease in feed. It can be used alone or combined with other dietary alterations or changes in poultry production (Smith, 2015). Hassan *et al*., (2011) found that the addition of protease in broiler diet decreased the N excretion by 8.33, 7.60, and 7.97% in starting, growing, and finishing periods, respectively. the combination of protease, xylanase, amylase, and phytase is found to br useful in improving the digestibility of lipid, amino acids, energy, Ca, and P, dry mater, M, N, of maize/soybean meal-based diets for broiler chickens (Cowieson *et al*., 2006). Also, Ghazi *et al*.,(2010) have found that the protease increased apparent nitrogen (N) digestibility and apparent N retention across the whole digestive tract in broilers. On the other hand for chicks fed low-protein diets, nitrogen was found lower. So, no markable impactt of protease enzyme supplementation was observed (Yamazaki *et al*., 2002).

2.8 Role of microbial enzymes on protease inhibitors:

Protease inhibitors are small protein molecules that can interfere with the action of the proteolytic enzymes causing break down of protein into amino acid components.Protease Inhibitors are found from many legumes, including soybeans, heat can destroy these inhibitors, that is why whole soybeans must be roasted before adding in poultry diets (Jacob, 2015, the heat treatment must inactivate the antinutritional substances as well as transform the raw protein into a more bird-available digested form) for the maximum conversion of the proteins of soybeans and other legumes into products with good

nutritional quality (Rackis et al., 2014). Protease inhibitors are the factors which can limit protein digestibility and growth performance (Jacob, 2015).

2.9 Impact of exogenous proteases on the growth responses of broiler chicken:

Numerous exogenous enzymes are commercially available and consistently using as supplement of pro-nutrient for enhancing the productivity of poultry. Different types of enzymes say amylolytic, proteolytic and lipolytic enzymes act on the specific substrates through after supplementation in the diet. The main function of enzymes is to enhance feed digestibility and thereby the growth performance of the animal. Though numerous experiment has been done with the enzymes focusing broiler productivity, the findings are different and showed inconclusive results in most of the cases. However, several studies have shown that supplementation of protease improved body weight, feed consumption and feed conversion efficiency in broilers (Angel *et al.* 2011; Freitas *et al.* 2011; Cowieson *et al.* 2017; Mahmood *et al.* 2017a,b). The increased feed consumption of broilers fed on supplemented diets could be probably due to rapid growth rate of broiler chickens and therefore, more nutrients are required to ensure their faster growth. Cowieson, 2010 and Zanella *et al*,.1999 reported that exogenous enzymes can increase the feeding value of soy and other grain products, when fed to broilers, leading to increased growth performance and efficiency. In another studies conducted by Ghazi *et al.* (2002) and Kaczmarek *et al.* (2014) reported no improvement or poorer growth performance in broilers fed diets supplemented with proteases. The authors attributed their observation to the possible negative effects of exogenous protease supplementation on secretion of the endogenous proteolytic enzymes.

Ghazi et al. (1997 and 2010) reported on the use of different mono-component proteases. In one study soybean meal was pretreated with 2 mono-component proteases and fed to broilers. One of the enzymes resulted in no change in performance, whereas the other protease had a positive effect on both BWG and FE. In a separate study, Ghazi reported that the use of one mono-component protease resulted in increased BWG and FI, but that FE was either negatively affected or not affected at all, depending on the protease concentration used. Persia et al. working with turkeys and using a multienzyme complex containing protease, reported no differences in BWG but improvements in FE from 9 to 15 wk of age. Yan *et al.* (2012) have reported that the effect of protease to the diet of broiler chickens during the starter phase is greater compared to that during other growth phases, suggesting that young animals might be more sensitive to supplementary protease, in agreement with the findings of this current study. It has been reported that adding protease to diet has a positive influence on the digestibility of amino acids (Angel *et al.,* 2011; Liu *et al.,* 2013).

Protease enzymes have several benefits including reducing undigested proteins in the diet, enhancing amino acid availability, reducing protein needs in the diet, maintaining weight gain and feed efficiency, reducing proteolytic fermentation, and decreasing biogenic amines and bacterial toxins (Buttin et.al, 2016). Therefore, protease enzyme has gained interest for many poultry companies and nutrition supplementation companies for use as an important supplement digestive enzyme in broiler diets. Proteases have also been found to affect mucus layer thickness in the GIT, apparently alleviating the effect of a coccidial infection, resulting in higher weights (Peek et al., 2009). the effects of proteases cannot be limited to hydrolysis of dietary proteins only. Interactions between

the digestions of other nutrients in the feed matrix are possible, as well as changes in the microbial communities due to modifications in the availability of easily accessible proteins in different parts of intestinal lumen (Morita et al., 1998; Scott et al., 2013). Additionally, mild interactions with the intestinal mucosa such as an increment in the thickness of the mucus layer in the intestinal lining of young chickens (Peek et al., 2009) have been reported, and ascribed potentially beneficial effects in conditions of coccidiosis challenges, which have not been fully demonstrated.

2.10. Effect of exogenous proteases on the meat, carcass characteristics and gastro-intestinal development of broiler chicken:

The sources or types of enzymes used in the feed formulation for poultry not only affect the quality of the formulated diets but also influence their optimum growth, carcass yield parts, and other internal organ development of the broilers. Different researchers have been reported both positive and negative responses regarding body growth and the internal organs development of broilers fed on enzymes or non-enzymes supplemented diets. Researchers claimed different opinions on the effects of protease on carcass. Protease has a positive impact on meat yields specifically breast meat and carcass quality of broiler. Freitas *et al.* (2011) reported that supplementation of protease to diets containing varied levels of CP had no effect on dressing percent and percent of deboned breast meat. However, Ajayi (2015) reported that percentage of dressed weight and breast meat yield of birds on low protein diet with protease was significantly increased when compared to the birds on same diet without protease. This difference might be due to different diet and level of enzyme used. Yadav and Sah (2005) showed that dietary reduction of crude protein increased the average dressing percent of broilers over those

fed basal diet which was statistically not significant. Further also reported that increased protease supplementation of reduced crude protein diets had no significant effect on the dressing percent of broiler.

2.11 Effect of exogenous protease on the gut health and intestinal morphology of broiler chicken:

Broiler chickens are non-ruminant, and therefore rely on enzymes from their own organ for nutrient digestion. Only a small amount of digestion takes place in ceaca by the microbial activity. Apart from this, the poultry industry relies on many microbial enzymes to enhance digestive function and improve bird productivity. Amongst multiple factors, the diet can play an important role in initiation of enzyme secretion and activities. Type of feeds or proteins used in the diet formulation of poultry might affect digestive enzyme activities. Broiler diets prepared exclusively with all-vegetable ingredients may increase dietary fibre considerably in association with phytic acid. More than 5.0 % crude fibre in the practical diet of broiler is not desirable at all, because chicken is a non-ruminant animal and so high fibrous feed materials depress their digestibility and thus reduce their performance.

The effects of protease supplementation on microbial ecology have not been studied by using NGS (Next Generation Sequence) techniques. However, different scenarios of consequences of protease supplementation on the microbiota can be deduced from the literature. In a study which used qPCR methodology to target specific microbial groups, protease found to be increase the presence of *Lactobacillus* spp but decrease the presence of *Clostridium perfringens* in the ileum (Giannenas et al., 2017). Another study pointed

that, protease supplemented in combination with α-amylase and glucoamylase increased the relative abundance of *Bifidobacterium, Staphylococcus, Bacteroides*, and *Megamonas* (Yin et al., 2018), which are considered to be beneficial bacteria. There is also a possibility that protease supplements alter the microbiota composition by modifying the substrates that the microorganisms access. For instance, higher availability of Amino acid was shown to be either beneficial or harmful to the growth of certain microorganisms (Dahiya et al., 2007). Other metabolites like short chain fatty acids, amines, and AA derivatives were also shown to having an impact on the microorganisms (Hemarajata and Versalovic, 2013). Therefore, effects of protease supplementation on pc digestibility might partly be explained by a shift in the microbial composition. To our knowledge, such a relationship has not been investigated to date. (*Kamel et al., 2015; Yuan at al., 2008*) indicates the protease causes improved crypt depth, villus width, VH:CD ratio and surface area of broiler chicken.

The advantages of using protease enzymes in poultry diets include not only improved bird performance and feed conversion, but also reduced environmental problems due to reduced output of excreta. Protease enzymes have several benefits including reducing undigested proteins in the diet, enhancing amino acid availability, reducing protein needs in the diet, maintaining weight gain and feed efficiency, reducing proteolytic fermentation, and decreasing biogenic amines and bacterial toxins (Buttin *et.al,* 2016). Therefore, protease enzyme has gained interest for many poultry companies and nutrition supplementation companies for use as an important supplement digestive enzyme in broiler diets. Proteases have also been found to affect mucus layer thickness in the GIT, apparently alleviating the effect of a coccidial infection, resulting in higher weights (Peek

et al., 2009). the effects of proteases cannot be limited to hydrolysis of dietary proteins only. Interactions between the digestions of other nutrients in the feed matrix are possible, as well as changes in the microbial communities due to modifications in the availability of easily accessible proteins in different parts of intestinal lumen (Morita *et al.*, 1998; Scott *et al.*, 2013). Additionally, mild interactions with the intestinal mucosa such as an increment in the thickness of the mucus layer in the intestinal lining of young chickens (Peek et al., 2009) have been reported, and ascribed potentially beneficial effects in conditions of coccidiosis challenges, which have not been fully demonstrated.

2.12 Effect of exogenous protease on the blood metabolites of broiler chickens:

The serum blood metabolites such as total protein, glucose, albumin, uric acid, creatinine and triglycerides etc. of broiler chicken might be affected by dietary supplementation of exogenous protease enzymes. Because protease enzymes could perform multiple functions in the body. It helps in protein metabolism, protein accretion, increase amino acid availability, reduce nitrogen waste, decrease environmental pollution and so on. The protease e enzyme is also produced in an insufficient amount in the body and should be provided from external sources. It acts on protein anti-nutrients found in some feed ingredients such as soybean, thus making the dietary protein more available. The role of protease enzymes on blood profile in the poultry is still unclear or contradictory. Different researchers demonstrated both positive and negative findings regarding this.

Law *et al*.(2019) reported that diet had no effect on serum glucose (GLU) and total protein (TP) of broiler fed protease supplemented diet .This may be due to the strict regulation of carbohydrate metabolism in the same birds to maintain the blood GLU level

(Hada *et al.* 2013). Corzo *et al*. (2005) and Hernández *et al*. (2012) fed broiler chickens with low-CP diets (3–4% in CP) and observed no change in serum TP. Corzo *et al*. (2009) and (Ahmadi et al. 2015) commented that TP will only be affected when diets ingested by the animals are deficient in AA. Thus, it appears that meeting the AA requirement could be more important than the CP *per se*. However, feeding low-CP diets, as demonstrated in the current experiment lead to elevation of liver lipogenesis and thus TG. Similarly, Swennen et al. (2006) and Dehghani-Tafti and Jahanian (2016) reported that irrespective of energy density, birds grown on low-CP diets had higher TG. It appears that the TG level is associated with calorie/protein and the excessive energy intake above the requirement level resulted in higher TG and therefore higher abdominal fat deposition (Rosebrough and Steele 1985; Sterling et al. 2002; Malheiros et al. 2003; Swennen et al. 2007). Abdominal fat is an unfavourable trait in carcase quality and reduces its acceptability by the consumers. The increase in abdominal fat deposition is a major disadvantage of feeding low-CP diet (Sklan and Plavnik 2002). However, the percentage of breast meat yield was not affected by diet in the present experiment. Similar results have been reported previously by van Nguyen and Bunchasak (2005) and Infante-Rodríguez et al. (2016).

2.13 Effect of exogenous protease on the survivability of broiler:

Numerous studies have shown no adverse effects of enzyme supplementation in broiler diets on body weight, mortality, health, feed intake, FCR, nutrient digestibility, meat quality and production costs. However, there is still a large amount of uncertainty regarding the use of enzymes (Doscovic *et al*, 2013). Khan *et al*. (2006) observed maximum mortality in control group compared to those of enzymes treated groups.

Freitas *et al*. (2011) reported in his experiment using Protease enzyme that there was no effect by treatment on mortality. Hashemi, and Davoodi, (2010) reported that enzymes improve the weight of immune organelles such as burse and spleen which can promote the immune situation and efficacy of health and livability. Sohail and Roland, (1999) stated that phytase supplementation significantly increased body weight, bone mineral content, bone density, and livability of broilers. Several studies demonstrated that enzyme could stimulate the natural immune response in poultry which could stimulate the similar livability of broiler chickens regardless of treatment. The immune system of birds is complex and is composed of several cells and soluble factors that must work together to produce a protective immune response.

2.14 Effect of exogenous protease on the cost-effectiveness or profitability of broiler chicken:

The farm's profitability depends on not only single factor. Mainly feed cost or total production cost along with multiple factors affect farm's profitability. For this reason, today's broiler industries are vibrant, taking dynamic polices for cutting production cost and increasing farm's profitability. Supplementation of enzymes in diet reduces nutrient need and increases profitability.

Broilers fed diet supplemented with 0.075 percent protease had the highest increase in income over feed cost reported by Yadav and Sah (2005) in a study. The results indicated that the reduction of crude protein decreased income over feed cost. Protease supplementation of reduced crude protein diets increased the income over feed and chick cost to the level of basal diet. The decreased in income over feed and chicks cost with reduction of crude protein of the diet could be attributed to the increased mean

cumulative feed consumption over basal diet fed. Enzymes treated groups generated more profit than that of control group reported by Khan *et al*. (2006) . Ward and Deer (2012) concluded that protease is the next new frontier in enzymes for poultry feeds. At today's ingredient prices, $3.00 per ton or more can be saved in starter feeds with this new protease. Enzyme supplementation caused higher body weight gain in periods of 7-21 days of age and 22-42 days of age which increase the profitability. These results agree with those of Carvajal *et al*.(2010) who reported that protease supplementations in broiler diets improved body weight, body weight gain and feed conversion ratio during starter and grower periods. By this way, relative economical efficiency (REE) is increased. The improvements in REE are due to reduced total feed costs for starter and grower diets and the high body weight gain. It is agreed with Mateo and Carandang (2006) who found that the feed cost per kg gain was significantly low in corn-sorghum and sorghum based diets. Moreover, Medugu *et al*. (2010) reported that replacements of maize by sorghum in broiler diets reduced the feed cost.

Enzyme supplementation typically costs around US$2.0 per tonne of feed. However, enzyme supplementation can reduce the feed costs of broilers by up to US$4.0 -11.0 per tonne, for layers up to US$9.8 per tonne, and the feed cost per 1000 kg of eggs produced can be reduced by US$?21.2 per tonne. In a study on the effect of the use of enzymes on feed costs, a blend of amylase, protease, and phytase was used in corn-based broiler feeds at 1 g/kg. The results show that these enzymes reduced the amounts of nutrients required in the diet, while maintaining live-weight gain and feed conversion at the same level as birds fed a standard diet (145 Kcal/kg reduction in ME, 4% reduction in amino acids, 0.10% reduction in total phosphorus, and 0.12% reduction in calcium), with a resulting

feed cost saving of around US$ 11.00 per tonne. The addition of exogenous enzymes also appears to reduce feed costs by replacing expensive feed materials with cheaper ones. For example, it was found that adding phytase enzyme to rapeseed meal – a cheap protein source – was just as nutritious for broiler chicks as the widely-used soybean meal (rapeseed meal is about US$ 130 a tonne cheaper than soybean according to a 2014 estimate). In addition, phytase releases phosphorus from the phytate molecule in the GI tract and makes it bioavailable to the birds, thus reducing the cost of inorganic phosphorus supplementation that is needed for the development and maintenance of their skeletal system. It also minimises the amount of phytate-bound phosphorus that is excreted and hence prevents negative impacts on the environment. While common phytase is derived from various species of fungi, new generation products are derived from bacterial sources. The new-generation phytase is around 45% more effective in increasing body weight gain and around 70% more effective in improving feed conversion. These improvements are due to the superior ability of the new-generation phytase to liberate more phosphorus from the dietary phytate, in addition to its beneficial effect in reducing the anti-nutritive properties of phytate. Economically, the use of the new-generation phytase can result in a saving of about US$ 4.0-6.0 per tonne of low-quality feed.

2.15 Importance of the study

The study is undertaken to determine the usefulness of including protease enzyme in poultry diets, by assessing growth performance, carcass yield, viability, blood metabolites, gut morphology and profitability of broiler fed plant-sourced diet. The results from the research study might act as guidelines for the poultry farmers, poultry

researchers to formulate efficient diet formulation which could help poultry integrators to increase the broiler meat production with more efficiently, and thus could supply premium quality meat to the consumer across the globe.

2.16 Conclusion

In conclusion, the use of a protease not only has the potential to increase the profitability of a broiler enterprise, but can also have a positive impact on the environment and help improve litter quality and associated incidence of economically important welfare parameters. Choosing a protease which has been selected, and developed, with the right characteristics for use in feed production is, however, key if these benefits are to be realized. Supplementation of exogenous enzyme in protein vegetable diet on broiler chicken might be economical, and it could reduce excreta nitrogen and thus environmental pollution. Further, it might enhance nutrient availability of bird and thus increase the productivity of poultry.

Chapter-III

Materials and Methods

3.1 Statement of the experiment

The experiment was carried out at the Department of Dairy and Poultry Sciences, Chattogram Veterinary and Animal Sciences University (CVASU) to ascertain the efficacy of protease enzyme on the growth performance, carcass yield traits, gastro-intestinal development, gut morphology, blood metabolites and profitability of broiler chickens. Biological trial was performed at the Poultry Research Shed of CVASU campus, during September-October, 2019. Laboratory analyses were performed in the Physiology and biochemistry lab, PRTC, Histology lab of CVASU, Khulshi, Chattogram.

3.2. Enzyme composition and activity

Commercial mono-component protease (a proteolytic exogenous enzymes) was used in this study, and the enzyme was derived from the DSM (Ronozyme ® ProAct; DSM Nutritional Products Ltd, Switzerland). The exogenous enzymes were included in the test diets at a rate of 500 and 300 ppm to provide a minimum of 300000 units/kg and 15000 PROT unit/kg of feed, respectively, as per the recommendations of the manufacturing companies. Both enzymes were alkaline serine endopeptidase proteases derived from *Bacillus lichenformis*.

3.3 Preparation of the experimental shed

The experimental poultry shed was prepared first by swiping and removing of dust dirt by broom. The battery cage was also washed and cleaned by whisk. Both shed and battery

cages was then washed and cleaned properly with tap water containing detergent. The shed and cages was left for air drying for 3 days. After that, ceiling, wall and floor along with battery cages were treated with disinfectant with FAM 30R (5ml/1L water) via sprayer and again left for drying for 1 week. The cage divided into 12 pens of equal size to accommodate broiler chicken. The individual tube feeder, drinker and each pen were marked properly by sticker (bearing cage no. and treatment) before allowing chicks entry into the cages. Chicks were brooded with an electric bulb (60 watt) set at the roof of each pen by hanging condition. The floor space provided for each bird was 0.5 sq. ft in the cage. The floor of each pan was covered with medium thick paper to reduce leg injury and to maintain warm temperature within each pen. All equipment was cleaned and disinfected accordingly outside the shed.

3.4 Collection of day-old broiler chicks and experimental design

A total of 112 (Cobb 500) day-old broiler chicks of either sex was purchased from the renowned hatchery (M M Aga Farm Ltd) on a pre-order basis to run the experimental trial from d1 to 33 days. The chicks were weighed on receipt (46.34±0.27g) and then randomly assigned into four dietary treatment groups, these are- Maize-wheat based diet without enzyme (**MS**-), Maize-wheat based diet with enzyme (MS+), Maize-wheat-soya based diet without enzyme (MWS-) and Maize-wheat-soya based diet with enzyme (MWS+), where each treatment was replicated 4 times with 7 birds per replicate in a 2 × 2 factorial design. The layout of the experimental trial was demonstrated below in Table 1.

Table1: Layout of the experiment

Treatment	No. of birds per Replicate				No. of birds per treatment
	R_1	R_2	R_3	R_4	
MS-	7	7	7	7	28
MS+	7	7	7	7	28
MWS-	7	7	7	7	28
MWS+	7	7	7	7	28
Total	28	28	28	28	Grand Total=112

[Treatment MS--and MWS- refer to basal diets maize-soya based and maize-wheat-soya based without supplementation of any enzymes, whereas treatment MS+ and MSW+ denote Maize-soya based and maize-wheat-soya based diets supplemented with enzymes (protease)].

Fig 2: purchased Day 1 chick

Fig 3: Transportation of chick

Fig 4: Weighing of micronutrient ingredients

Fig 5: Mixing of feed

Fig 6: Mixing of feed ingredients

Fig 7: packaging of test diets with marking

Fig 8: Prepared brooding pen

Fig 9: Spreading pre-starter feed on

Fig 10: Floor space for 7 birds in

Fig 11: Floor space for 7 birds

Fig 12: Feeding and watering of chick

Fig 13: Diluted vaccine

Fig 14: Vaccination via eye drop

Fig 15: Measuring feed weight for feed intake record

Fig 16: giving Diet from marked specific packet to specific pen.

Fig 17: Weighing of birds

Fig 18: Post-mortem of birds

Fig 19: Dissection of Bird

3.5 Collection of the experimental feed and feedstuffs

Broiler pre-starter (crumble) diet (Nourish^{TM}) was collected from the local market and used to feed the birds up to 2 weeks of age. After that, finisher or test diets were prepared manually and feed the birds from 15-33 days. The macro-feed ingredients (maize, wheat, soybean meal, til oil cake, palm oil, and limestone) required for the feed formulation were purchased from the local market of Pahartali and Rajakhali Bazar, Chattogram. Each macro-ingredient was purchased based on thorough selection by visual observation like organoleptic test (color, odor, moisture etc.). The micro-nutrients were procured from another local market (Hazari lane, Terry Bazar, Chattogram). Particularly, test ingredient (protease) was collected from a pharmaceuticals company named Eon Group Bangladesh Co. Limited. The proximate composition and reporting values of Nutritive composition of ready-made pre-starter diet (Nourish^{TM}) were shown in Table 2

Table 2: Nutrient composition of ready-made starter diet

Nutrient components (%)	Proximate values of ready-made (Nourish feed)	Reporting values of ready-made (Nourish feed)
ME (kcal/kg)	3356.68	2950
Moisture	9.95	9.98
DM	90.05	90.02
CP	21.20	21
CF	4.77	5.0
EE	6.98	5.00
Ash	5.23	6.0
Ca	1.00	1.00
Total P	0.58	0.45
Lysine	-	1.30
Methionine	-	0.55

3.6 Formulation of test diet

Four experimental diets (MS-, MS+, MWS- and MWS+) were formulated with maize, wheat and vegetable oil as the main energy sources, along with soybean meal and protein concentrate as the main protein sources (Table 3). The diets were formulated exclusively with the ingredients of plant origin with or without addition of microbial enzymes (proteases). Two basal diets (MS- and MWS-) were formulated with soybean meal, protein concentrate along with basal grains including other feedstuffs as shown in Table 3. These were fed as such (MS- and MWS-) or supplemented with microbial enzymes (RONOZYME® ProAct –protease, DSM Pty Ltd., Switzerland) to create diets MS+ and MWS+. Protease is a mono-component proteolytic enzyme supplemented to the basal diet at the rate of 0.03g and 0.05g/kg, respectively. All the test diets were iso-caloric and iso-nitrogenous in nature, and supplemented with or without exogenous cocktail enzymes. Bird was fed ready-made starter broiler diet for the first 14 days and then finisher or formulated diet in mash form was used as feed to nourish the broilers entire trial period from the rest of the trial period (d 15 to 33 days) , as shown in Tables (2, 3).

3.7 Feed grinding, mixing and preparing the diet

First of all, the macro ingredients were collected from local market in ground form having a desirable particle size. The micro-ingredients were also weighed by electric balance one by one and then put in a small bucket for each diet and mixed properly by turning layer by layer. After that, the weighed macro-ingredients were spread on the wide plastic paper kept on floor of house and mixed thoroughly by the help of shovel. After that, the micro-nutrients were mixed on feed mixture equally. Vegetable oil (Palm) was added at half of the required amount by sprinkling over the feed mixture and then mixed

thoroughly by both hand and shovel. Remaining half amount of vegetable oil was finally sprinkled over feed mixture and again mixed thoroughly by both hand and shovel. A thorough mixing was done manually with shovel after weighing all ingredients as per the requirement of individual diet. Finally, the mixed diets were stored in the bags having marking sign and later used for feeding the bird as mash feed. Same procedures were followed for the preparation of all diets.

Table 3: Ingredient and nutrient composition of test diet for broiler from d14-33d

Ingredients (%)	Treatment			
	MS-	MS+	MWS-	MWS+
Maize	58.40	58.40	58.00	58.00
Wheat	0.00	0.00	2.00	2.00
Soybean meal	32.00	32.00	29.50	29.50
Protein concentrate	2.10	2.10	3.74	3.74
Palm oil	4.20	4.20	3.60	3.60
DCP	0.60	0.56	0.36	0.36
Limestone	1.68	1.68	1.50	1.50
Table salt	0.25	0.25	0.25	0.25
Choline chloride	0.04	0.04	0.04	0.04
Vitamin mineral - premix	0.25	0.25	0.25	0.25
L-lysine	0.18	0.18	0.16	0.16
DL-methione	0.25	0.26	0.25	0.25
Enzymes (protease)	**0.00**	**0.03**	**0.00**	**0.05**
Toxin binder	0.05	0.05	0.05	0.05
Sand	0.00	0.00	0.3	0.25
Total	100.0	100.0	100.0	100.0
	Nutrient (%)-calculated			
ME (Kcal/kg)	3118	3118	3118	3118
CP	21.00	21.00	21.00	21.00
Ca	1.10	1.09	1.10	1.10
P	0.67	0.67	0.64	0.64
EE	3.48	3.48	3.5	3.51
CF	3.49	3.49	3.43	3.43
Proximate values (%)				
DM	88.50	89.00	88.00	87.50
CP	20.50	20.68	20.50	20.80
CF	5.80	5.50	5.20	5.10
EE	6.23	5.89	5.50	5.40
Ash	5.20	4.10	5.60	5.00
NFE	55.1	58.2	56.7	55.7

[MS--and MWS- refer to Maize-soya based and Maize wheat soya based basal diets without supplementation of any enzymes, whereas MS+ and MSW+ denote diets supplemented with enzymes (protease). Enzyme was added in the diet as per the recommendation of the manufacturer company

3.8 Management

The following management procedures were pursued during the whole experimental period and tried to maintain the uniformity (similar feeding, lighting, environmental condition) in the management practices as much as possible.

3.8.1 Brooding

For the first two days, the birds were provided with a temperature of 33 ^{o}C. The temperature then was gradually reduced by 1 or 2 ^{o}C every 1 or 2 days until the chicks were 19 days old atwhich point the temperature was maintained at 24^{o} C for the rest of the trial. Each pen was furnished with a feeder and drinker. Feeders were cleaned before supplying diets, and drinkers were washed weekly to maintain hygienic conditions. Excreta trays were cleaned after three weeks, or earlier if they were filled with excreta material.

3.8.2 Floor space

Bird was raised in battery cages by dividing 16 pens of equally sizes. Each pen was labeled properly with different treatment groups before entering chicks into the cage. Dry and clean newspaper was placed on the floor of each pens as bedding materials prior to allowing chicks in the room, and the paper was replaced with new one as or when it get too dirt during the whole brooding period. Birds were reared in battery cages. Each pen (4.4 sq. ft.) was allotted for 7 birds. Therefore, floor space for each bird was 0.63 sq. ft.

3.8.3 Feeder and drinker space

Each pen was furnished with a feeder and one drinker. One feeder (60 cm × 8 cm × 5 cm) and one round drinker with a capacity of 2.5 litres were provided for each pen. The feeder and drinker were placed in such a way so that the birds were able to eat and drink conveniently.

3.8.4 Feeding and watering

Feed and drinking water were supplied *ad-libitum* to the birds throughout the experimental period. Pre-starter feed was supplied to birds from d 1-14 days once a day in the tube feeder in the early morning as an adjustment period. Paper along with tube feeder and drinkers were used for feeding and watering the chicks during the early stages soon after coming from the hatchery. The finisher mash diets were given to the experimental birds from d 15-33 days two times daily, where once in the morning at 6 AM and another in the afternoon at 6 PM. Fresh, clean and cool drinking water was supplied the birds three times a day *i.e.* at 6 AM, 12 AM, and 6 PM.

3.8.5 Lighting:

The birds were exposed to a continuous lighting (23 h: 1h) in each 24 hrs of photoperiod.

3.8. 6 Immunization of birds

Birds were vaccinated against Ranikhet (New Castle Disease), and Gumboro disease according to the schedule mentioned in Table 4. Ranikhet live vaccine (Cevac New L^R) and Gumboro live vaccine (Cevac Gumbo L^R) were procured from local veterinary medicine Dispensary. Vaccines were collected in ice contained air tight flask and

individual vaccine was collected at the vaccination date. 25 ml distilled water was added to vaccine vial via syringe to make 500 dose diluted live vaccine. Vaccine was administered to individual birds via eye drop method within 2 hours of collection. Individual vaccines were administered at the evening time of respective vaccination date.

Table 4: Vaccination schedule

Age (Days)	Name and type of the vaccine	Name of disease	Route of administration
5	Cevac New L^R, Live	Newcastle disease	One drop in one eye
12	Cevac Gumbo L^R, Live	Gumboro	One drop in one eye
17	Cevac New L^R, Live	Gumboro	One drop in one eye
22	Cevac Gumbo L^R, Live	Newcastle disease	One drop in one eye

3.8. 7 Sanitation and hygienic management

Adequate and proper hygiene and sanitary measures were adopted and followed throughout the experimental period. Proper cleaning and disinfection of all equipment were done prior to the beginning of the trial. Potassium permanganate ($KMnO_4$) solution (1.5%) was prepared and kept into a plastic bottle fitted with a sprayer at its opening mouth. It was kept at the entry point of poultry shed and used as disinfectant before entry into poultry shed. Hands and feet were also properly disinfected with 70% alcohol before entry into the shed,

3. 9 Data and sample collection

Both pre-starter feed sample and test diets sample were collected prior to supplying birds with test diets for the assessment of the nutritive value of each diet. Body weight, feed intake and remaining feeds were recorded in record sheet in weekly basis to calculate body weight gain and feed conversion ratio (FCR). Blood samples of two birds from each replicate cage were collected on day 30 to assess blood metabolites. Besides, two healthy birds were also selected randomly from each replicates and then slaughtered by halal method to collect intentional tissue (small Intestine), visceral organs (heart, pancreas, liver, spleen, proventiculus and gizzard), breast, thigh, drumstick, wings, neck, head and shanks sample weights. Meat yield traits like dressing percentage, breast weight, thigh weight, drumstick weight, shank weight, giblet weight were also recorded at the end of the trial period. Individual weight of gastrointestinal organs (liver, pancreas, bursa of fabricius, heart, small intestine, proventiculus, gizzard, spleen) was also recorded to ascertain the gastrointestinal organ development of the birds. Cost benefit analysis was calculated at the end of trial period.

3.9.1 Method of broiler processing:

At the end of trial period, two broilers were selected randomly from each replicate cages and killed humanely to assess the carcass yield traits, gastro-intestinal development and gut morphology. Feed and water were withdrawn from the pens for 3 hours before slaughtering to facilitate proper bleeding and skinning and then selected broilers were slaughtered by halal method. After slaughter, birds were processed by removing the feather, skin, head, shank, viscera, oil gland, heart, kidneys, liver, lungs and small and large intestine of the carcasses. Heart and liver were removed from the gastro-intestinal

tract by cutting and traction gently to let them loose. Gall-bladder was removed from liver. Gizzard and proventiculus was separated from gastro-intestinal tract by cutting it loose in front of the duodenum and behind the last end of oesophagous. Spleen was also collected by gentle cut and traction from liver parenchyma. Pancreas was collected from loop of duodenum by gentle cut and traction by scissors. Small intestine was collected by making two cut where one at the proventiculus and gizzard junction and another cut in front of the blunt sac of cecum.

3.9 2 Record keeping: The following parameters were recorded during the entire experimental period.

Mortality: Mortality was also recorded if death occurred.

Body weight:

Live weight of broiler was taken replication wise for each treatment at each weekend. Average live weight of the broilers was also recorded at the beginning of the experiment and at the end of each weekend.

Feed intake: Feed intake was calculated by deducting the left over from the total amount of feed supplied to birds at each weekend.

3.10 Calculation of data

3.10.1 Weight gain

The weight gain was calculated by deducting the initial body weight from the final weight.

3.10.2 Feed conversion ratio (FCR)

The amount of feed needed for per unit of production is called feed conversion ratio. It was calculated by using the following formula.

$$\text{FCR} = \frac{\text{Feed intake}}{\text{Body weight gain}}$$

3.10.3 Mortality and livability

Mortality of birds was calculated number of dead birds throughout the experimental period divided by the total number of birds housed at the start of experiment. Livability was calculated from mortality of birds per replicate cage.

The percentage of mortality was calculated by this formula.

$$\text{Mortality (\%)} = \frac{\text{Number of birds died}}{\text{Total No. of birds housed}} \times 100$$

3.10.4 Dressing percentage

The dressing percentage of birds was calculated as follows: Dressing (%) = $\frac{\text{Dressed Weight}}{\text{Body Weight}} \times 100$

Slaughtering data' such as body weight, blood loss, feather loss, abdominal fat, shank weight, heart weight, gizzard weight, heart weight, small intestine, pancreas, bursa etc., were expressed in percentage.

3.11 Sample processing and Analyses:

3.11.1 Feed sample:

Eight feed samples were collected from ready-made and formulated test diets prior to feeding the birds. The samples were processed by grinding with the help of mortar and pestle and then mixed thoroughly for lab analyses. About 500gm of each diets of finisher as well as starter diet were taken and sent to the Animal Nutrition Lab for proximate analysis. Each analysis was done three times for each sample to minimize technical errors. The samples were tested for proximate analysis having dry matter (DM %), moisture %, crude protein (CP %), crude fiber (CF %), ether extract (EE%) and ash using standard laboratory procedures (AOAC, 2007). Dry matter estimation was done by oven dry method. Crude protein estimation was accomplished by Kjeldahl Method. Ether Extract estimation was done by Soxhlet apparatus. Ash was measured by igniting the pre-ashing sample on a Muffle furnace at a temperature of 600°C for four to six hours. Additionally, Calcium (Ca %) and phosphorus (P %) was determined by atomic absorption and spectrophotometry, respectively (AOAC, 2007).

3.12 Evaluation of parameters:

3.12.1 Serum biochemical parameters and analyses

On day 30, one bird was selected randomly from each replicate cage, weighed and killed humanely to collect blood sample for the analysist of blood metabolites such as total protein (TP), glucose, albumin (Alb), uric acid (UA), creatinine and triglycerides (TG). The blood samples were then centrifuged at 3000 g at 4^{o}C for 15 minutes to obtain the serum, and these serum samples were collected in clean-plastic vials, and immediately frozen at -80^{o}C, until further chemical analyses were done in the lab. Serum total protein,

albumin, triglyceride, glucose, uric acid and creatinine were determined using standard kits (Randox Laboratories Ltd., UK) and automatic analyzer (Humalyzer300, Merck®, Germany) as per the instructions given by the manufacturers' company.

3.12.2 Gut morphology of broiler

Bird from each replicate was taken randomly and sacrificed after 12 hours of fasting and samples of ileum were taken to measure intestinal tissue morphology on day30. The specimens were fixed in 10% formalin after which they were dehydrated in 100% ethanol. The specimens were then cleared with xylene and embedded in paraffin. A microtome was used to make 5mm cuts that were mounted on glass slides and stained using the H and E (Haematoxyline and Eosin) method. Five readings each of villus height and crypt depth were taken per specimen. This was done with a light microscope (Olympus). Villus height was measured from the apical to the basal region which corresponded to the superior portion of the crypts. Crypts were measured from the basis until the region of transition between the crypt and the villus.

3.13 Production cost

Cost of production was calculated considering the expense on chick, feed, medicine, labor, etc. Chick cost was calculated from the purchasing cost. Feed cost was considered from sale price of the feed marketed through dealers. Market price of all feeds was shown in Appendix (Table 8).

3.14 Statistical analyses

All data were subjected to analyze by the GLM procedure of Minitab statistical software (Minitab, 2000). The significance of differences between means was tested using the Duncan multiple-range test. Statistical significance was considered at $P \leq 0.05$.

Chapter-IV

RESULTS

The experiment was carried out to find out the effect of vegetable based diets supplemented with exogenous mono-enzyme proteases on the gross responses (body weight, feed intake, FCR), carcass yield traits (dressing percentage, drumstick weight, thigh weight, breast weight, giblet weight, shank weight, neck weight, back weight), intestinal tissue morphology, livability, blood metabolites and profitability of broiler chickens. The results obtained from the study are stated below in this chapter.

4.1 The gross responses and livability of broiler chickens fed vegetable based diets supplemented with exogenous enzymes

4.1.1 Body weight

The results of body weight (BW) of broiler chickens fed vegetable based diet supplemented with exogenous mono-enzyme were shown below in Table 5. The data showed that diet and interaction (Diet × Enzyme) had no effect ($P>0.05$) on the BW of broiler chickens except for enzyme. Increased ($P<0.01$) BW was found in the broilers fed diet supplemented with enzyme (MS+, MWS+) compared to others diets (MS-, MWS-) with no enzyme during d 14-21 d 21-28 d, and d 28--33, respectively. Highest live weight (1853.0g/ bird) was recorded at MS+ diet with enzyme supplementation and the lowest live weight (1590g/ bird) was recorded at MWS- diet without enzyme supplementation from d 28-33 days.

Table 5: Body weight (BW) of broiler fed enzyme supplemented diets

Diets	Enzymes	BW of bird on different ages (days)-1-33			
		D14	D21	D28	D33
MS	-	453.20	976.20[b]	1335.50[b]	1722.00[b]
	+	502.86	1021.40[a]	1474.33[a]	1853.00[a]
MWS	-	454.60	943.22[b]	1308.56[b]	1590.0[b]
	+	508.00	1013.22[a]	1491.00[a]	1847.10[a]
SEM		6.264	8.718	13.423	19.342
Level of significance					
Diet (A)		0.205	0.183	0.536	0.113
Enzyme (B)		0.992	**0.01**	**0.01**	**0.01**
A × B		0.824	0.857	0.657	0.141

[MS--and MWS- refer to basal diets without supplementation of enzymes, whereas MS+ and MSW+ denote diets supplemented with enzymes (protease).). [a,b] Means bearing uncommon superscript in the column differed significantly at the level given above

4.1.2 Feed intake

Feed intake (FI) of broiler chickens indicates that diet and interaction had no significant (P>0.05) effect on broiler chickens during the trial period, as shown in Table 6. The FI was increased in the enzyme diets (MS+, MWS+) compared to non-enzyme diets (MS-, MWS-). The highest FI (3151.20 g/b) was observed in the broilers fed vegetable based diet supplemented with enzyme (MWS+) and lowest FI (2960 g/b) being in the non-supplemented diet (MWS-) at 33 d.

Table 6: Feed intake (FI) of broiler fed enzyme supplemented diets

Diets	Enzymes	FI of bird on different ages from d1-33 days				
		D14-21	**D21-28**	**D28-33d**	**D14-33**	**D1-33 days**
MS	-	781.48[b]	957.80[b]	760.00[b]	2605.40[b]	3055.40[b]
	+	858.71[a]	986.8[a]	968.0[a]	2707.3[a]	3157.40[a]
MWS	-	763.62[b]	904.30[b]	776.00[b]	2509.80[b]	2960.00[b]
	+	940.71[a]	1024.30[a]	842.0[a]	2741.20[a]	3191.20[a]
SEM		10.229	9.931	12.615	23.192	22.401
Level of significance						
Diet (A)		0.156	0.658	0.061	0.511	0.511
Enzyme (B)		0.04	**0.05**	**0.02**	**0.01**	**0.01**
A × B		0.01	0.01	0.01	0.187	0.189

[**MS--and MWS- refer to basal diets without supplementation of enzymes, whereas MS+ and MSW+ denote diets supplemented with enzymes (protease).). [a,b] Means bearing uncommon superscript in the column differed significantly at the level given above**

4.1.3 Feed Conversion Ratio (FCR)

The results of FCR shown in Table 7 denote that interaction between diet and enzyme had no significant (P>0.05) effect on the FCR of broiler chickens from d 14-21, d 21-28 and d 28--33, respectively. Improved (P<0.01) FCR was found in the enzyme-supplemented diets (MS+, MWS+) compared to those birds fed diets without enzyme during d 14-33.

Table 7: Feed conversion ratio (FCR) of broiler fed enzyme supplemented diets

Diets	Enzymes	FCR of bird on different ages from d1-33 days				
		D14-21	**D21-28**	**D28-33d**	**D14-33**	**D1-33 days**
MS	-	1.91	2.75[a]	2.57	2.17[a]	2.65[a]
	+	1.56	2.14[b]	1.97	2.03[b]	2.45[b]
MWS	-	1.86	2.52[a]	2.67	2.29[a]	2.82[a]
	+	1.72	2.10[b]	2.20	2.03[b]	2.44[b]
SEM		0.053	0.078	0.196	0.028	0.036
Level of significance						
Diet (A)		0.602	0.418	0.259	0.332	0.301
Enzyme (B)		0.355	**0.01**	**0**.561	**0.01**	**0.04**
A × B		0.052	0.519	0.065	0.320	0.257

[MS--and MWS- refer to basal diets without supplementation of enzymes, whereas MS+ and MSW+ denote diets supplemented with enzymes (protease). . [a,b] Means bearing uncommon superscript in the column differed significantly at the level given above

4.2 Livability:

The livability (%) of broilers was not influenced (P>0.05) by diet, enzyme and its interaction, as shown below in Figure 1. It implies that the diet, supplemented diet and their interaction had a similar effect on the growth, development and mortality of broilers. The livability of broiler chickens was not affected by the supplementation of enzyme in the diet in this current study

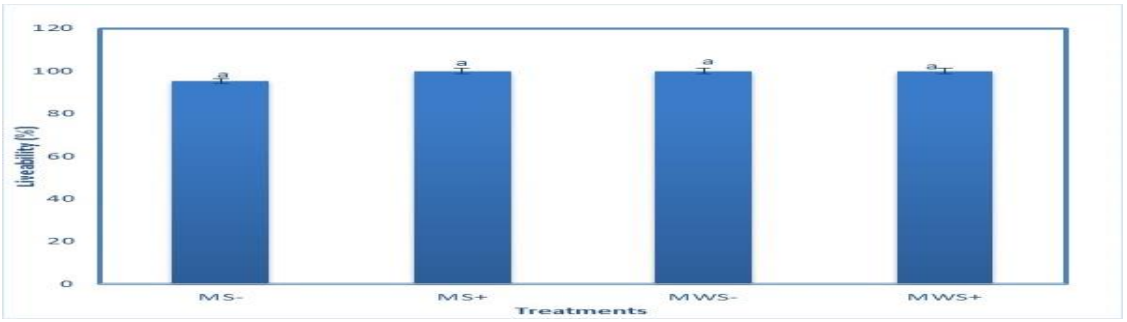

Figure: Livability (%) of broilers fed enzyme supplemented diets on day 33; Bar with similar letter has no significant differences (P>0.05) between treatments

4.3. Meat yield traits of broiler chickens

The results of meat yield characters *i.e* dressing yield, drumstick weight, thigh weight, breast weight, and abdominal fat weights of broilers chickens fed vegetable diets are shown below in Table 8. The data show that diet had no significant effect (P>0.05) on these parameters measured herein this study. Enzyme has influenced (P<0.05) the dressing %, thigh weight, breast weight, and abdominal fat weights of broilers. Interaction (Diet × enzyme) had a significant effect (P<0.05) on the breast weight only. Breast weight tended to be significant (P<0.06) by diet as well.

Table 8: Meat yield traits (g/b) of broiler fed enzyme supplemented diets on 33days

Diets	**Enzyme**					
		Dressing %	Breast weight	Drumstick weight	Thigh weight	Abdominal fat weight
MS	-	63.60b	436.75^{b}	138.80	156.43^{b}	19.47^{b}
	+	66.16a	553.69^{a}	155.30	187.61^{a}	27.08^{a}
MWS	-	64.49b	484.40^{b}	175.00	149.85^{b}	21.38^{b}
	+	71.96a	489.78^{a}	188.44	192.63^{a}	29.33^{a}
SEM		0.385	5.951	7.496	4.115	0.906
Level of significance						
Diet (A)		0,06	0.403	0.112	0.924	0.298
Enzyme (B)		**0.02**	**0.01**	**0.716**	**0.05**	**0.02**
A × B		0.141	0.01	0.510	0.531	0.848

[MS--and MWS- refer to basal diets without supplementation of enzymes, whereas MS+ and MSW+ denote diets supplemented with enzymes (protease).). [a,b] Means bearing uncommon superscript in the column differed significantly at the level given above

4.4 Visceral organ weights (g/b) of broiler chickens fed enzymatic diet

The data show that diet and interaction had no significant effect (P>0.05) on the visceral organ weights (Table 9) of broiler chickens except for enzyme. Liver and pancreas weights were increased (P<0.05) by supplementation of enzyme in broiler diet.

Table 9: Visceral organ weight (g/b) of broiler fed enzyme diets on 33days

Diets	Enzyme				
		Gizzard-proventriculus weight	Liver weight	Heart weight	Pancreas weight
MS	-	68.33	39.84[b]	8.57	2.93[b]
	+	66.34	51.26[a]	9.34	3.40[a]
MWS	-	76.63	36;78[b]	11.05	2.96[b]
	+	79.17	47.72[a]	11.51	3.57[a]
SEM		2.171	1.952	0.419	0.086
Level of significance					
Diet (A)		0.153	0.446	0.056	0.593
Enzyme (B)		**0.58**	**0.05**	**0.56**	**0.05**
A × B		0.826	0.953	0.86	0.706

[MS--and MWS- refer to basal diets without supplementation of enzymes, whereas MS+ and MSW+ denote diets supplemented with enzymes (protease).). [a,b] Means bearing uncommon superscript in the column differed significantly at the level given above

4.5 Blood serum metabolites of broiler chickens fed enzymatic diet

The results of blood metabolites *i.e* albumin, triglycerides (TG), total protein, glucose (Glu) and creatinine and uric acid of broilers chickens fed vegetable diets are shown

below in Table 10. The data showed that the interaction between diet and enzyme had no significant effect (P>0.05) on the blood profiles of broiler chicken except for TG, which is influenced (P<0.05) by dietary treatment only. Only Glu level was increased (P<0.01) by enzyme diet. Apart from this, TG (P=0.087) and uric acid (P=0.09) were also slightly influenced by enzyme supplemented diet.

Table 10: Blood metabolites of broiler fed enzyme supplemented diets on 33days

Diets	Enzyme	Blood serum metabolites of birds on 33 days					
		Albumin (g/L)	Glucose (g/L)	Triglyceride (mg/dL)	Creatinine (mg/dL)	Total protein (g/dL)	Uric acid (mg/dL)
MS	-	15.75	232.10[b]	86.52[a]	0.48	1.63	4.35
	+	18.90	262.70[a]	91.15[a]	0.50	1.70	3.56
MWS	-	16.30	237.74b	76.55[b]	0.51	1.65	2.35
	+	1.60	268.23[a]	81.96[a]	0.55	1.82	7.10
SEM		0.619	2.842	1.143	0.051	0.055	0.551
Level of significance							
Diet (A)		0.475	0.382	0.013	0.736	0.534	0.432
Enzyme (B)		0.26	**0.01**	**0.087**	**0.910**	**0.321**	**0.09**
A × B		0.286	0.99	0.84	0.736	0.674	0.065

[MS--and MWS- refer to basal diets without supplementation of enzymes, whereas MS+ and MSW+ denote diets supplemented with enzymes (protease). Enzyme was added in the diet as per the recommendation of the manufacturer company). [a,b] Means bearing uncommon superscript in the column differed significantly at the level given above]

4.6 Ileal intestinal morphology of broiler chickens fed protease supplemented diet

The results of intestinal morphometric measurements *i.e* villus height (VH), crypt depth (CD), villus width (VW), VH: CD ratio, and surface area of broilers chickens fed

vegetable diets are shown below in Table 11. The data showed that interaction between diet and enzyme had no significant effect (P>0.05) on the intestinal tissues of broiler chicken. The morphology of intestinal tissues (VH, CD, VW, VH: CD ratio and surface area) were increased (P<0.05) by enzyme diet.

Table 11: Intestinal morphometric measurements of broiler fed enzyme diets on 33d

Diets	Enzymes	Ileal morphology of broiler				
		Villus height (µm)	Crypt depth (µm)	Villus width (µm)	VH:CD ratio	Surface area (mm^2)
MS	-	607.50^{b}	113.50^{b}	215.00^{b}	4.50^{b}	0.165^{b}
	+	623.50^{a}	138.00^{a}	243.50^{a}	5.35^{a}	0.280^{a}
MWS	-	614.00^{b}	109.50^{b}	$214,00^{b}$	4.63^{b}	0.140^{b}
	+	615.00^{a}	132.50^{a}	241.60^{a}	5.62^{a}	0.260^{a}
SEM		0.587	1.520	1.139	0.058	0.008
Level of significance						
Diet (A)		0.442	O,176	0.612	0.164	0.198
Enzyme (B)		**0.03**	**0.01**	**0.01**	**0.01**	**0.02**
A × B		0,09	0.759	0.758	0.579	1.00

[MS--and MWS- refer to basal diets without supplementation of enzymes, whereas MS+ and MSW+ denote diets supplemented with enzymes (protease). ^{a,b} Means bearing uncommon superscript in the column differed significantly at the level given above]

4.7 Economic analyses and cost effectiveness

The data on cost benefit analyses of broiler were presented in the Table 12. Significantly higher (P<0.05) total production cost was found in the birds fed non-supplemented or basal diets (MS-, MWS-). Total cost of production (Tk/Kg live broiler) was significantly (P<0.05) less for the birds fed enzyme- supplemented diets (MS+, MWS+). Higher (P<0.01) profit margin was obtained for the supplemented dietary group.

Table 12: Cost-benefit analyses of broiler fed enzyme diets on the last day of trial

Diets	Enzymes	Feed cost (TK / kg live bird)	Total production cost (TK /kg live bird)	Market price (TK/kg live bird)	Profit (Tk /kg live bird)	Cost: Benefit ratio
MS	-	67.00[a]	117.59[a]	130.00	12.41[b]	9.48[a]
	+	63.00[b]	112.29[b]	130.00	17.71[a]	6.34[b]
MWS	-	66.35[a]	118.08[a]	130.00	11.92[b]	9.90[a]
	+	63.40[b]	111.85[b]	130.00	18.15[a]	6.16[b]
SEM		0.0937	0.0314	130.00	0.031	0.0241
Level of significances						
Diet (A)		0.49	O,712	-	0.710	0.06
Enzyme (B)		**0.03**	**0.05**	**-**	**0.01**	**0.01**
A × B		0.06	0.067	-	0.05	0.05

[MS--and MWS- refer to basal diets without supplementation of enzymes, whereas MS+ and MSW+ denote diets supplemented with enzymes (protease). [a,b] Means bearing uncommon superscript in the column differed significantly at the level given]

Chapter V
Discussion

5.1 The gross responses of broiler chickens fed protease supplemented diet

The most important criteria for evaluating the growth performances of broilers are feed intake (FI), body weight (BW) and feed conversion ratio (FCR). These criteria *i.e* FI, BW and FCR are considered as the key performance indicators (KPI) for assessing the broiler's potentiality in regard to growth responses. The results from our research study demonstrated that the addition of protease enzymes in the diet significantly ameliorated the average BW, FI and FCR of broilers. The improved growth performances of broilers might be a result of increased feed intake and better feed efficiency of the broiler chicken, as observed in our current study. The increased feed consumption of broilers fed on supplemented diets could be probably due to rapid growth rate of broiler chickens and therefore, more nutrients are required to ensure their faster growth. Moreover, it might be a resultant of supplementation of protease enzyme in the broiler diet. It can be assumed that protease enzymes can enhance digestibility of feed protein, which could make more available necessary amino acids in the diets for increasing full growth potential of the broiler chicken. The improvement in growth performance of broiler could be probably due to the increment of protein and amino acid digestibility through after enzyme (protease) application in the diets. It has been reported that protease inclusion in the diet has a positive influence on the digestibility of amino acids (Angel *et al.,* 2011; Liu *et al.,* 2013). Lazaro *et al.* (2004) reported that digest a viscosity might be reduced by exogenous enzymes, which could facilitate the improved contact between endogenous enzymes and nutrients, thereby improve the digestibility of the nutrients. However, our

results are in agreement with the report of previous investigators conducted in a several studies (Ghazi *et al,* 1997, Angel *et al.,* 2011; Freitas *et al.,* 2011; Yan *et al,* 2012, Cowieson *et al.,* 2017, Mahmood *et al.,* 2017a,b). Carvajal *et al*. (2010) reported that protease supplementations in broiler diets improved body weight, body weight gain and feed conversion ratio during starter and grower periods.

5.2 Livability of Broiler Chicken:

The livability (%) of broilers was not affected by supplemental enzymes in the diet. The findings of current study agrees with **the result of** Freitas *et al.(*2011) which reported that, using protease enzyme in the diet had no effect on the mortality of birds. Similar result was obtained by another researcher when broiler fed diet supplemented with multi-enzymes–containing protease (Abu-Tayyeb *et al.,* 2019). *Hashemi and Davoodi* (2010) reported that enzymes improve the weight of immune organelles such as burse and spleen which can promote the immune situation and efficacy of health and livability. Several studies demonstrated that enzyme could stimulate the natural immune system of poultry (Lohakare *et al.,* 2005).

5.3 Meat yield characters and visceral organ weight of broiler chicken

The carcass yield traits of broiler were affected by enzyme supplemented diets in this study. This might be due to improved growth responses of broilers fed on enzyme supplemented diet. The breast weight, dressing %, abdominal fat content and thigh weight were found to be improved by enzyme supplemented diets. The results obtained coincided with the findings of previous researcher (Ajayi, 2015). The higher abdominal fat deposition might be due to probably the

excessive energy or feed intake above the requirement level resulted in higher triglyceride (TG), as TG is associated with higher calorie/protein (Rosebrough and Steele, 1985; Sterling *et al*,. 2002; Malheiros *et al.,* 2003; Swennen *et al.,* 2007). Studies have indicated that an increase in dietary protein content could result in increased carcass protein content and decreased carcass fat content (Bedford & Summers, 1985). In addition, the visceral organ weights, particularly liver and pancreas of broiler were also improved by supplemented diet in this study. The increased visceral organ weight might be a result of heavier body weight of broiler fed on enzyme diet, as is seen in this study.

5.4 Blood metabolites of broiler fed enzyme supplemented diets

Dietary supplementation of exogenous protease enzymes could affect the blood metabolites say total protein, glucose, albumin, uric acid, creatinine and triglycerides (TG) etc.. Because enzymes could play as catalyst for multi-functional role in the body mechanism. The present study indicates an improvement in plasma glucose level of broiler fed protease supplemented diet.The reason for this probably due to increased metabolism of carbohydrate caused by supplemental enzyme in the diet. A report stated that protein and energy levels in the diets did not affect carbohydrate metabolism (Swennen *et al.,* 2007, Hada *et al.,* 2013). So it can be assumed that due to the strict regulation of carbohydrate metabolism in the same birds to maintain the blood glucose level (Hada *et al.,* 2013). Apart from this, TG and uric acid were found to be slightly influenced by enzyme supplemented diet. Our results agree with the findings of Ndazigaruye et al. (2019), which reported that serum total protein, albumin, TG, CHO, creatinine, HDL-cholesterol, GOT, and GPT levels did not change in broiler fed diets with protease . Our results are not in harmony with the report of previous investigator

(Allouche *et al.*,2015), who found that plasma protein concentrations were enhanced by dietary protease supplementations. These findings might be attributed to the reduction of plasma triglycerides, total cholesterol and LDL- cholesterol (Wang *et al*,. 2011; Saleh *et al*., 2018). The discrepancy between two experiments might be due to many factors such as the nutrient composition of the diets (sufficient/insufficient protein), enzyme used (source of enzymes, doses), and raw protein sources used which could alter some conditions of the digestive tract such as the pH. In addition, dietary protease tended to increase TG and uric acid (UA), especially when added to the vegetable diet. It is often reported that serum UA concentrations can be used as an indicator of amino acid utilization in broilers fed amino acid-sufficient and amino acid-deficient diets. Further, increased TG in the blood profile might be a result of increased feed intake and calorie-protein of the birds (Malheiros *et al.,* 2003; Swennen *et al.,* 2007).

5.5 Intestinal morphometric measurements of broiler fed enzyme diets

The results of ileal tissue morphometric measurements demonstrated that villus height (VH), crypt depth (CD), villus width, VH: CD ratio, and surface area (SA) of broilers were significantly increased by enzymatic diet, but no dietary and interactive effects were found over this intestinal tissues. It implies that dietary exogenous enzymes improved the gut morphology of broiler chickens (Kamel *et al.,* 2015; Yuan *at al.,* 2008). The increased feed utilization, absorption and assimilation of nutrients by exogenous enzymes might result in better ileal tissue growth, proliferation and development of the broiler. Villus height is very inherently related with the absorption, utilization and assimilation of food nutrients (*Choct et al., 2006)*. The crypt is the villi factory, and deep crypts denote quick tissue growth and a high demand for new tissue. These results agreed with the

findings of Yan et al. (2011) who studied the effect of dietary protein level and protease supplementation on gut health. Enzyme -protease was found to be associated with increased growth efficiency in the gut and reduced systemic inflammation as demonstrated by improved crypt villus ratio.

5.6 Economic analyses and cost-effectiveness of broiler fed enzyme diet

The profit of broiler chicken relies on multiple factors, which include feed, diseases, management, supplement, chick, market price, selling method, production cost, feed cost, and so on. Supplementation of enzymes in diet reduces nutrient need and increases profitability. Today's broiler industry is very sophisticated and adopting different innovative policy to sell their finished products in the market in diversified forms, to earn more profit. However, it is obvious from our current study that production cost was lower and profit margin being higher in the enzyme-supplemented dietary group of broiler than the control. On the contrary, low profit and increased production cost were observed in the birds fed basal diet, as is evinced from our current study. Our result agrees with the previous researchers (Yadav and Sah, 2005, Khan *et al*, 2006, Ward and Deer, 2012, Abu-Tayyeb, 2018). Broilers fed diet supplemented with 0.075 percent protease observed more income over feed cost by Yadav and Sah (2005) in a study. Khan *et al*. (2006) obtained more profit on enzyme supplemented diet Today's ingredient prices, $3.00 per ton or more can be saved in starter feeds with this new protease enzymes stated by Ward and Deer (2012).

Chapter- VI

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, the results of this study suggest that broiler chickens fed plant- based diets supplemented with exogenous enzyme proteases had a positive responses over feed intake, body weight, carcass yield, gastro-intestinal organ, gut morphology and profitability. Supplemented diet also improved blood glucose level of broiler without affecting other blood metabolites measured in this study. So the study suggests that broiler fed on this supplemented diet could be reared profitably as the production cost incurs lower than the un-supplemented diets. From the results, it could be assumed that application of supplemental feed on the broilers chickens, might enhance broiler production and cut cost significantly. Supplementation of enzyme in plant based diet for broiler chicken might be economical or profitable to rear under the farming condition. Further, it might enhance nutrient availability of bird and thus increase the productivity of broiler chickens. Data on plant based diet with addition of exogenous enzyme proteases not enough, and it might warrant further study to elucidate the present findings for the broiler production profitably.

In this study , broilers were reared in cage system , which might be shifted to floor system for conducting commercial production. Some abnormalities such as breast blister and leg problem might arise from rearing broilers in cages, and it might downgrade the carcass quality of broiler chcikens. Due to financial constraints and technical limitations, nutrients digestibility, residual effect, enzyme efficacy were not analyzed. These parameters could have vital effect on enriching the present data. The study could explore

new horizon for investigating those parameters with larger sample size and variable temporal pattern as future study. These results suggest that supplementation of exogenous enzymes in broiler diet could improve the benefit of using such feed additives for increasing efficiency of nutrient utilization. Further research study focusing protease enzyme could be undertaken in order to reduce excreta nitrogen emission, environmental pollution and to reduce production cost of broiler.

Chapter-VII

REFERENCES

Abu-Tayyeb, M. I. Jahan, M.A. Hossain, M.M. Hossain, N. Akter and B.K. Nath, (2019). The Responses of Exogenous Enzymes (Multi-Enzymes) on the Productivity and Stage of Production of Broilers Fed Vegetable-sourced Diets. *International Journal of Poultry Science, 18: 515-522.*

Ajayi, Helen. (2015). Effect of protease supplementation on performance and carcass weights of broiler chickens fed low protein diets. Nigerian Journal of Agriculture, Food and Environment, *8: 109-112.*

Angel, C.R., Saylor, w., Vieira, S.L., and Ward, N. (2011). "Effects of a monocomponent. protease on performance and protein utilization in 7- to 22-day-old broiler chickens". Metabolism and Nutrition. Department of Animal and Avian Sciences, University of Maryland.

Angel, R., W. Powers, S. Zamzow, and T. Applegate. (2006). "Dietary modifications to reduce nitrogen consumption and excretion in broilers". *Poultry Science 85(Suppl. 1):25.*

Applegate, T., Angel, R. (2008). "Protein and Amino Acid Requirements for Poultry". NRCS, USDA.

Bailey, C., Cantor, A. (2016). "POULTRY NUTRITION". Poultry Science Manual, 6th Edition. 13 pages. Texas A&M Poultry Science Department. Bedford, M., and Walk C., (2014). "Enzymes and their effect on amino acid nutrition". AB Vista Feed Ingredients, Marlborough, Wilts UK. Bregendahl, K., Sell L., and Zimmerman R., (2002). "Effect of

low protein diets on performance and body composition of broiler chicks". Poultry Science. 81:1156 –1167.

Barekatain, M. R., Antipatis, C., Walkden-Brown S.W., Iji, P. A., and Choct, M. (2013). "Evaluation of high dietary inclusion of distillers dried grains with solubles andsupplementation of protease and xylanase in the diets of broiler chickens under necrotic enteritis challenge." School of Environmental and Rural Science, University of New England, Armidale, NSW, Australia. *Poultry Science 92 :1579–1594*.

Bregendahl, K., J. L. Sell, and D. R. Zimmerman. "Effect of low-protein diets on growth performance and body composition of broiler chicks." *Poultry science* 81.8 (2002): 1156-1167.

Buttin, P., Yan, F., Dinbner, J., Knight, C.D., Vazquez-Anon, M., Odetallah, N., Carter, S. (2016). "Effect of dietary protein and protease supplementation on performance and gut health of broiler chicks" NOVUS.

Campbell, G. L., and M. R. Bedford. "Enzyme applications for monogastric feeds: A review." *Canadian journal of animal science* 72.3 (1992): 449-466.

Cao, Li, et al. "Reduced lesions in chickens with Clostridium perfringens-induced necrotic enteritis by Lactobacillus fermentum 1.2029." *Poultry science* 91.12 (2012): 3065-3071.

Choct, M. (2006). Enzymes for the feed industry: Past, present and future. *Worlds Poultry Science. J. 2006, 62, 5–16. [CrossRef]*

Cowieson, A. J., and Bedford, M. R. (2009). The effect of phytase and carbohydrase on ileal amino acid digestibility in mono-gastric diets: complementary mode of action?*World's Poultry Science Journal, 65*, 609-624.

Cowieson, A. J., Singhi, D. N., and Adeola, O. (2006). "Prediction of ingredient quality and the effect of a combination of xylanase, amylase, protease and phytase in the diets of broiler chicks. 2. Energy and nutrient utilization". British Poultry Science Volume 47, Number 4, pp. 490—500. Campbell G.L., Bedford M.R. (1991). "Enzyme applications for monogastic feeds". Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Dale, Nick (2009). "A Few Basic Points about Protein". The Poultry Site. De Vries, W., Kros, J., Oenema, O. & de Klein, J. (2003). "Uncertainties in the fate of nitrogen II: a quantitative assessment of the uncertainties in major nitrogen fluxes in the Netherlands". Nutrient Cycling in Agroecosystems, 66(1): 71–102.

De Vries, Sjerp, et al. "Natural environments—healthy environments? An exploratory analysis of the relationship between greenspace and health." *Environment and planning A* 35.10 (2003): 1717-1731.

Dozier, W.A., III, M.T. Kidd, and A. Corzo. (2008). "Amino acid responses of broilers". J. Appl. Poultry. Res. 17:157-167.

FAO. (2006). "World agriculture: towards 2030/2050 interim report". Rome

Faridi, A., A. Gitoee, and J. France. "A meta-analysis of the effects of nonphytate phosphorus on broiler performance and tibia ash concentration." *Poultry Science* 94.11 (2015): 2753-2762.

Ferguson, N. S., et al. "The effect of dietary crude protein on growth, ammonia concentration, and litter composition in broilers." *Poultry Science* 77.10 (1998): 1481-1487.

Ferguson, N.S., R.S. Gates, J.L. Taraba, A.H. Cantor, A.J. Pescatore, M.L. Straw, M.J.Ford, and D.J. Burnham, (1998). "The effect of dietary protein and phosphorus on ammonia concentration and litter composition in broilers". Poultry Science 77:1085-1093.

Feye, K. M., et al. "Influential factors on the composition of the conventionally raised broiler gastrointestinal microbiomes." *Poultry science* 99.2 (2020): 653-659.

Forler, Daniel, et al. "An efficient protein complex purification method for functional proteomics in higher eukaryotes." *Nature biotechnology* 21.1 (2003): 89-92

Freitas, D. M., Vieira, S.L., Angel, C.R., Favero, A., Maiorka, A., (2011). "Performance and nutrient utilization of broilers fed diets Supplemented with a novel mono component protease". J. Appl. Poult. Res. 20, 347–352.

Fru-Nji, F., Kluenter, A.M., Fischer, M., Pontoppidan, K., (2011). "A feed serine protease improves broiler Performance and energy digestibility". The Journal of Poultry Science, vol. 48, No. 10, 239–246.

Geraert, P. A., J. C. F. Padilha, and Solange Guillaumin. "Metabolic and endocrine changes induced by chronic heatexposure in broiler chickens: biological and endocrinological variables." *British Journal of Nutrition* 75.2 (1996): 205-216.

Gerber, P., Opio, C., Steinfeld, H. (2015). “Poultry production and the environment – a Review”. Poultry in the 21st Century. Animal Production and Health Division, Food and Agriculture Organization of the United Nations.

Ghazi, S., Rooke, J.A., Galbraith H. & Bedford, M.R. (2010b). “The potential for the improvement of the nutritive value of soya-bean meal by different proteases in broiler chicks and broiler cockerels”. British Poultry Science Journal. Pp. 70-77.

Giannenas, Ilias, et al. "Effects of protease addition and replacement of soybean meal by corn gluten meal on the growth of broilers and on the environmental performances of a broiler production system in greece." *PloS one* 12.1 (2017): e0169511.

Gitoee, A., Janmohammadi, H., Taghizadeg, A., Rafat, S.A. (2015). “Effects of multi-enzyme on performance and carcass characteristics of broiler chickens fed corn-soybean meal basal diets with different metabolizable energy levels”.Journal of Applied Animal Research, 2015 Vol. 43, No. 3, 295–302.

Group, E. (2013). “The Health Benefits of Protease”. Global Health Center.

Hassan, A., Abd-Elsamee, O., El-Sherbiny, E., Samy, A., and Mohamed, A. (2011). “Effect of Protein Level and Avizyme Supplementation on Performance, Carcass Characteristic, and Nitrogen Excretion of Broiler Chicks” American-Eurasian J. Agric.& Environ. Sci., 10(4):551-560,2011

Infante-Rodríguez, F., et al. "Effect of diets with different energy concentrations on growth performance, carcass characteristics and meat chemical composition of broiler chickens in dry tropics." *SpringerPlus* 5.1 (2016): 1-6.

Jacob, J. (2015). “Antinutritional Factors in Feed Ingredients”. University of Kentucky. eXtension. extension.org.

Kalmendal, R., and R. Tauson. "Effects of a xylanase and protease, individually or in combination, and an ionophore coccidiostat on performance, nutrient utilization, and intestinal morphology in broiler chickens fed a wheat-soybean meal-based diet." *Poultry Science* 91.6 (2012): 1387-1393.

Kamel, N.F., Naela, Ragaa M., El-Banna, R.A., Mohamed, F.F. (2015). “Effects of a Monocomponent Protease on Protease on Performance Parameters and Protein Digestibility in Broiler Chickens.” Department of Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Cairo University, Egypt. Agriculture and Agricultural Science Procedia 6 (2015) 216 – 225. www.sciencedirect.com. Elsevier B.V.

Khan, S. H., R. Sardar, and B. Siddique. "Influence of enzymes on performance of broilers fed sunflower-corn based diets." *Pak. Vet. J* 26.3 (2006): 109-114.

Khattak, F. M., et al. "Enzymes in poultry nutrition." *J. Anim. Pl. Sci* 16.1-2 (2006): 1-7.

Kocher, A., Choct, M., Ross, G., Broz, J., Chung, T., (2003). “Effects of enzyme combinations on apparentmetabolizable energy of corn-soybean meal- based diets in broilers”. *School of Rural Science and Agriculture, University of New England, Armidale, NSW 2351, Australia In: pp. 275-283.

Lazaro, R. M., Latorre, A., Medel,P., Garcia,M.,and Mateos, G. G. (2004). Feeding regimen and enzyme supplementation to rye-based diets for broiler. *Poultry Science, 83,* 152-160.

LECO Corporation. www.leco.com. Mótyán J., Tóth F., and Tőzsér J., (2013). “Research Applications of Proteolytic Enzymes in Molecular Biology”. US National Library of Medicine National Institutes of Health. Biomolecules Journal 2013 Dec; 3(4): 923- 942.

Lin, H., et al. "Impaired development of broiler chickens by stress mimicked by corticosterone exposure." *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 143.3 (2006): 400-405.

Liu, S., Selle, P., Court, S., Cowieson, A., (2013). “Protease supplementation of sorghum based diets enhances amino acid digestibility coefficients in four small intestinal sites and accelerates their rates of digestion”. Animal Feed Science and Technology. In. LECO CN628 Manual. Version 1.3x, Part Number 200-747 (2014).

Lohakare, J.D., M.H. Ryu, T.W. Hahn, J.K. Lee and B.J. Chae, (2005). Effects of supplemental ascorbic acid on the performance and immunity of commercial broilers. J. Applied Poult. Res., 14: 10-19.

Madrid, J., Catalá-Gregori, P., García, V., Hernández, F. (2010). “Effect of a Multienzyme complex in wheat-soybean meal diet on digestibility of broiler chickens under different rearing conditions.” Italian Journal of Animal Science 2010; volume 9: e.

Marsman, G. J., et al. "The effect of thermal processing and enzyme treatments of soybean meal on growth performance, ileal nutrient digestibilities, and chyme characteristics in broiler chicks." *Poultry science* 76.6 (1997): 864-872.

Martínez-Ballesta, M. C., et al. "Minerals in plant food: effect of agricultural practices and role in human health. A review." *Agronomy for sustainable development* 30.2 (2010): 295-309.

Medugu, Clement Ijaduwa, et al. "Carcass and blood components of broiler chickens fed sorghum or millet as replacement for maize in the semi arid zone of Nigeria." *Agriculture and Biology Journal of North America* 1.3 (2010): 326-329.

Mohamed, M. A., et al. "Growth performance and histological changes in ileum and immune related organs of broilers fed organic acids or antibiotic growth promoter." *Int. J. Poult. Sci* 13.10 (2014): 602-610.

Mótyán, János András, Ferenc Tóth, and József Tőzsér. "Research applications of proteolytic enzymes in molecular biology." *Biomolecules* 3.4 (2013): 923-942.

Oxenboll, K.M., Pontoppidan, K., and Fru-Nji, F. (2011). “Use of a Protease in Poultry Feed Offers Promising Environmental Benefits”. International Journal of Poultry Science 10 (11): 842-848.

Peek, H. W., et al. "Dietary protease can alleviate negative effects of a coccidiosis infection on production performance in broiler chickens." *Animal feed science and technology* 150.1-2 (2009): 151-159.

Pope, T., L.N. Loupe, P.B. Pillai, and J.L. Emmert. (2004). “Growth performance and nitrogen excretion of broilers using a phase feeding approach from twenty-one to sixty-three days of age”. Poult. Sci. 83:676-682.

Rackis, J., Gumbmann, R. (2014). “Protease Inhibitors: Physiological Properties and Nutritional Significance” Northern Regional Research Center Agricultural Research Science and Education Administration. USDA.

Rogers, k. (2015). “Proteolytic enzyme”. Encyclopædia Britannica.

Romero, L. F., et al. "Comparative effects of dietary carbohydrases without or with protease on the ileal digestibility of energy and amino acids and AMEn in young broilers." *Animal feed science and technology* 181.1-4 (2013): 35-44.

Rosebrough, R. W., and N. C. Steele. "Energy and protein relationships in the broiler: 1. Effect of protein levels and feeding regimens on growth, body composition, and in vitro lipogenesis of broiler chicks." *Poultry Science* 64.1 (1985): 119-126.

Sanz, María Luz, et al. "In vitro investigation into the potential prebiotic activity of honey oligosaccharides." *Journal of agricultural and food chemistry* 53.8 (2005): 2914-2921.

SAS User’s Guide. 2007. Version 9.2 ed. SAS Inst. Inc., Cary, NC.

Selim, N. A., et al. "Effect of inclusion inorganic, organic or nano selenium forms in broiler diets on: 1-growth performance, carcass and meat characteristics." *International Journal of Poultry Science* 14.3 (2015): 135.

Sergeant, Martin J., et al. "Extensive microbial and functional diversity within the chicken cecal microbiome." *PloS one* 9.3 (2014): e91941.

Simbaya, J., et al. "The effects of protease and carbohydrase supplementation on the nutritive value of canola meal for poultry: In vitro and in vivo studies." *Animal Feed Science and Technology* 61.1-4 (1996): 219-234.

Smith, A., (2015). “Protease reduces environmental impact of broiler production”. Feed Enzymes EMEA Animal Nutrition & Health DSM. Poultry world.

Smits, Coen HM, and Geoffrey Annison. "Non-starch plant polysaccharides in broiler nutrition–towards a physiologically valid approach to their determination." *World's poultry science journal* 52.2 (1996): 203-221.

Sohail, S. S., and D. A. Roland Sr. "Influence of supplemental phytase on performance of broilers four to six weeks of age." *Poultry Science* 78.4 (1999): 550-555.

Spiehs, M. (2005). “Nutritional and Feeding Strategies to Minimize Nutrient Losses in Livestock Manure”. The College of Agriculture, Food and Environmental Science, University of Minnesota.

Sutton, M. & Fowler, D. (1995). “Atmospheric deposition of nitrogen compounds to heathlands”. Aarhus Geoscience, 4, 61–71.

Swennen, Katrien, Christophe M. Courtin, and Jan A. Delcour. "Non-digestible oligosaccharides with prebiotic properties." *Critical reviews in food science and nutrition* 46.6 (2006): 459-471.

Temim, S., et al. "Muscle protein turnover in broiler chickens: effects of high ambient temperatures and dietary protein intake." *Reproduction, Nutrition, Development* 2.38 (1998): 190.

Van Nguyen, Tuan, and Chaiyapoom Bunchasak. "Effects of dietary protein and energy on growth performance and carcass characteristics of Betong chicken at early growth stage." *Growth* 27.6 (2005): 1172.

Villanueva-Lopez, D. A., et al. "Effect of Dietary Frying Fat, Vegetable Oil and Calcium Soaps Of Palm Oil on the Productive Behavior and Carcass Yield of Broiler Chickens." *Brazilian Journal of Poultry Science* 22.4 (2020).

Yadav, J. L., and R. A. Sah. "Supplementation of corn-soybean based broiler's diets with different levels of acid protease." *Journal of the Institute of Agriculture and Animal Science* 26 (2005): 65-70.

Yamazaki, M. Murakami, H., and Nakashima, K. (2002). "Effect of Enzyme Supplementation on Performance and Nitrogen Excretion of Broiler Chick Fed Low-Protein Diet Based on Corn and Soybean Meal". Bulletin Nat. Ins. Lives Grassland Sci. 2:9-13.

Yan, F., Garribay, L., Arce, J., Lopez-Coello, C., Camacho, D., Disbennet, P., Vazquez-Anon, M., Manangi, M., Odetallah, N.H., Carter, S., (2012). "Effects of protease supplementation on broiler performance and in vitro protein digestibility". Australian Poultry Science Symposium, 134-137.

Yu, Jie, et al. "Dietary protease improves growth performance and nutrient digestibility in weaned piglets fed diets with different levels of soybean meal." *Livestock Science* 241 (2020): 104179.

Yun, J. H., et al. "Comparative efficacy of plant and animal protein sources on the growth performance, nutrient digestibility, morphology and caecal microbiology of early-

weaned pigs." *Asian-Australasian journal of animal sciences* 18.9 (2005): 1285-1293.

Zanella, I., N.K. Sakomura, F.G. Silversides, A. Fiqueirdo and M. Pack, 1999. Effect of enzyme supplementation of broiler diets based on corn and soybeans. Poult. Sci., 78: 561-568

Bio-data

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