Effect of inclusion of enzymatically pre-treated Sunflower Meal and Wheat Middlings in Broiler chicken diets

By

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Declaration

This thesis is my original work, and it has never been submitted for any degree at any other university.

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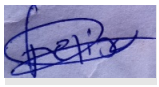
Signature:  Date: 02 June 2022

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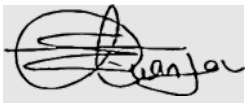
Signature  Date: 02 June 2022

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Dedication

I dedicate this work to my mother, Alice Njeri, my brother Edwin Mwaniki and my late brother Anthony Gachira for their prayers and love during my study.

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Abstract

The study investigated 1) the effect of *in vitro* pre-treatment of sunflower meal (SM) and wheat middlings (WM) using fiber degrading enzymes (FDE) without or with protease on mono-sugars release, organic acid concentration (SCFA), organic matter solubilisation, protein solubilization and apparent disappearance (AD) of neutral detergent fiber (NDF). Each feedstuff was mixed with 1% of FDE without or with protease in a ratio of 1:4 wt/wt for feedstuff:water, incubated for 24h and 48h at 40˚C, and freeze-dried. 2) Impact of the inclusion of pre-treated sunflower (TSM) and wheat middling (TWM) in a maize-soybean meal (MSBM) ration on growth performance, tibia attributes, apparent retention (AR) of components and caecal sugars and short-chain fatty acids production. A total of 288 Ross-708 d old male broiler chicks were placed in cages (6 birds/cage) based on body weight (BW) and allocated to 6 diets in a completely randomized design giving 8 replicates per diet. The diets were: I) PC, a MSBM, positive control (PC), II) NC, PC plus untreated sunflower meal (USM) and wheat middling (UWM), and III) 4 test diets in which USM and UWM were replaced with TSM and TWM at 25% (N25), 50% (N50), 75% (N75) and 100% (N100). For pre-treatment, each feedstuff was mixed with 1% of FDE in a ratio of 1:2 wt/wt for feedstuff:water, incubated for 24h at 40˚C, and oven-dried. All diets had titanium dioxide (0.3%) for AR determination. The body weight (BW), feed intake (FI), and mortality were recorded. Excreta was collected and frozen (-20°C). The data were subjected to PROC GLIMMIX of SAS 9.4, and LS means were separated using the Tukey test ($P<0.05$).

The analyses of freeze-dried samples showed improved in-vitro solubilisation of organic matter, increased mono-sugars, organic acids concentration, protein solubilization, and AD of NDF in both SM and WM. Analyses of oven-dried samples showed that pre-treatment increased soluble protein (SP) and decreased neutral detergent protein (NDF-P) and NDF in TWM. Compared to

USM, TSM had less SP while NDF-P and NDF were greater. The overall BW and BW gain (BWG) of NC were lesser than PC throughout the study ($P \leq 0.001$). The N75 and N100 BW and BW gain were less than PC ($P \leq 0.001$) throughout the trial. Overall feed intake was not different during the starter, finisher, and overall period ($P \geq 0.217$) relative to PC and NC. Also, feed conversion ratios were not different throughout the study ($P \geq 0.151$). The NC tibia attributes were not different from PC throughout the trial; test diets tibia length was lesser than PC ($P = 0.005$) and had a linear decrease response with higher inclusion levels ($P = 0.004$) on d42. The test diets did not affect the AR of CP ($P = 0 < 0.001$); there was decreased apparent NDF retention on d 21 and d 42 ($P \leq 0.001$) and apparent gross energy retention on d 21 and d 42 ($P \leq 0.001$) compared to PC. The NC and TD did not affect caecal sugars and SCFA production throughout the feeding period. In conclusion, pre-treating increased *in vitro* digestibility of sunflower meal and wheat middling, but their pre-treatment did not improve broiler chicken performance and AR of components.

Keywords: **fiber degrading enzymes; broiler; performance; component retention**

Chapter One: Introduction

1.1 Background

The poultry industry is an essential component of the food sector. Their products form an essential source of protein in the diets, especially for people in developing countries, and as such, their feed conversion efficiency is paramount (Mottet & Tempio, 2017; Sell-Kubiak *et al*., 2017). The production of broilers has seen a tremendous increase since the 1980s due to the awareness of the nutritional attributes of poultry meat compared to other meat protein sources (Horn & Süt, 2014).

In developing countries, Kenya included, the poultry industry is being confronted by various challenges, including the high cost of maize and soybean used as conventional feed ingredients (Alagawany *et al*., 2015). Maize and soybean are also consumed as human food in these countries (Mottet & Tempio, 2017). Due to the lack of quality feed ingredients at affordable prices, poultry production in these countries is low compared with developed countries

(Ravindran, 2013). This has prompted an urgent search for affordable and nutritious feeds. Since feed accounts for approximately 70% of total poultry production cost, researchers have given great attention to using agro-industrial by-products as alternative feed resources (Alagawany *et al*., 2018; Alagawany & Attia, 2015). The use of by-products has been intensified by the shortage of conventional raw materials such as maize, which is now also used to produce biofuels (Zhang & Lis, 2020), and soybean price keeps on fluctuating due to fuel market volatility, thus becoming unreliable (Mutisya *et al*., 2021).

In Kenya, the common poultry feed ingredients are maize and maize milling by-products (maize germ), soybean meal, and fish meal (‘omena’) (Abro *et al*., 2020). Most feed ingredients are selected based on the availability, nutrients they can provide, the presence or absence of anti-nutritional factors, palatability or their effect on voluntary feed intake and cost (Raza *et al*., 2019).

Apart from whole cereals, their milling by-products such as wheat middling's (also known as pollard), wheat bran, and rice bran are used in developing countries to lower the cost of production by acting as a source of energy (Bhattacharjee & Dey,2012; Supriyati *et al*., 2015; Osunbami, 2021). By-products such as sunflower seed cake, rapeseed meal, canola meal have also attracted attention as potential targets for replacing soybean meal in broiler diets (Biesek *et al*., 2020; Mbukwane *et al*., 2022).

The main limitation of using the agricultural by-products is the presence of anti-nutritional factors: cellulose, hemicellulose, and non-starch-polysaccharides (NSP), for which poultry lacks endogenous enzymes that can hydrolyze them (Raza *et al*., 2019). This has led to the development of exogenous enzymes that can hydrolyze these NSP, thus allowing nutrients to the bird (Fafiolu *et al*., 2015). The use of enzymes has enabled the poultry industry to save billions of shillings and, at the same time, reduce environmental pollution from unutilized nutrients in poultry faecal wastes (Dosković *et al*., 2013). Numerous studies have shown that poultry performance and feed conversion ratio improve when diets containing high NSP such as sorghum, barley, and wheat or triticale are supplemented with exogenous enzymes. Saleh *et al*. (2020) showed that the performance of birds feeding on olive seed cake was improved when an exogenous enzyme was added to it.

Enzymes have also been used in poultry diets to improve broiler performance and gut health (Kiarie *et al*., 2013). Exogenous enzymes help fight coccidiosis through short-chain fatty acids production in the intestines and overcome coccidia's adverse effects on bones (Kiarie & Mills, 2019). Although the use of enzymes in poultry feeds has become a norm, there are physiological limitations such as pH and feed gut transit time (less than 90 minutes in proventriculus/gizzard (Ravindran, 2013) that affects their response (Rodrigues & Choct, 2018). These limits affect even

highly digestible ingredients containing starch as only about 90% is digested; it would still be unrealistic to expect 100% nutrient digestion after directly supplementation enzymes (Ravindran, 2013). Researchers are now attempting to use feeds pretreated with enzymes to overcome these limitations (Rahimi *et al*., 2020). Due to the reduced retention time and the increased use of byproducts high in crude fiber, there is a limited time for enzyme-substrate interaction when exogenous enzymes are directly mixed (premixing) with poultry feeds (Ravindran, 2013). Pre-treatment with FDE of these ingredients high in crude fiber outside the bird GI system would provide a controlled environment which is optimum for fiber degrading enzymes to hydrolyse the crude fiber (Rahimi *et al*., 2020).

1.2 Problem statement

For quality broiler meat that is affordable to be produced there is a need for a nutritionally balanced diet that is cost-effectively formulated as feeds account for greater than 60% of total variables of broiler production (Sonkar *et al*., 2020). However, the poultry industry faces a feed shortage due to overreliance on maize and soybean meal which are only produced by a few countries (Wahyono & Utami, 2018). In Kenya, maize, fish meal, and soybean meal are the primary energy and protein sources used in poultry feeds and are mostly imported (Abro *et al.*, 2020; Gakuya *et al*., 2014). Byproducts such as sunflower meal and wheat middlings, which are relatively cheap, are increasingly being used to partially replace soybean and maize, respectively, thus reducing the cost of broiler production (Gerzilov & Petrov, 2022; Abudabos, 2016; Mbukwane *et al*., 2022). Sunflower meal has particularly been pointed out as the soybean alternative in regions that don't produce enough (Ditta & King, 2017a).

With the evolution of intensive feeding, the role of the gizzard and crop has been ignored as they are now more of transit organs, which affects feed retention time in the bird and enzyme activity (Kiarie & Mills, 2019). Due to the reduced retention time and the increased use of byproducts high

in crude fiber, there is a limited time for enzyme-substrate interaction when exogenous enzymes are directly mixed (premixing) with poultry feeds (Ravindran, 2013). Most enzymes have an optimum pH of between 4.0-6.0, and in the chicken gut, the pH varies a lot (gizzard 2.0-4.0 and small intestine pH between 6.5-7.5) (Svihus, 2011; Ndou *et al*., 2015). Some exogenous enzymes, such as phytase, are only considered effective in the crop (where the pH is optimum) (Sommerfeld *et al*., 2018). Hence more research is needed on a combination of feeding strategies, exogenous enzymes, and the structure of feeds to overcome the gut limitations (Kiarie & Mills, 2019; Rodrigues & Choct, 2018). Pretreatment of meals with heat, 0.5% HCl and enzymes is an effective method of ensuring nutrients are available to chickens more than conventional methods of enzyme application (pre-mixing) alone (Rahimi *et al*., 2020). Still, more studies on pretreatments are required to provide practical ways of decreasing antinutrients in feeds (Rahimi *et al*., 2020).

The above shows that there is a need to enhance the use of non-conventional animal feed ingredients to improve their nutritional quality, ensure stability in the price and supply of mixed feeds. This would further alleviate hunger, malnutrition, and poverty as there would be affordable, high-quality protein from broilers. Agricultural byproducts such as wheat pollard, rice bran, and sunflower seed cake readily available in Kenya would be suitable byproducts whose nutritional value can be improved through enzymatic pretreatment.

1.2 Objectives

1.2.1 Broad objective

To evaluate the effect of enzymatic pre-treatment of high crude fiber feedstuffs on nutrient digestibility and performance of broiler chickens.

1.2.2 Specific objectives

1. To evaluate the effect of *in vitro* pre-treatment of sunflower meal (SM) and wheat middlings (WM) on mono-sugars, organic acids and short-chain fatty acid concentration, protein solubilisation and apparent disappearance of neutral detergent fiber.

2. To evaluate the effect of inclusion of enzymes pre-treated SM and WM in a maize-soybean meal diet on growth performance, breast muscle weight, and tibia attributes of broiler chicken.

3. To evaluate the effect of inclusion of enzymes pre-treated SM and WM in a maize-soybean meal diet on apparent retention of components, cecal sugars, and short-chain fatty acids production in broiler chicken.

1.3 Hypothesis (HO)

1. Pre-treating sunflower meal (SM) and wheat middlings (WM) has no effect on mono sugars, organic acids, and short-chain fatty acids concentration in the supernatant, protein solubilization apparent disappearance of neutral detergent fiber.

2. Inclusion of enzymes pre-treated SM and WM in a maize-soybean meal diet has no effect on growth performance, breast muscle weight, and tibia attributes of broiler chicken.

3. Inclusion of enzymes pre-treated SM and WM in a maize-soybean meal diet has no effect on apparent retention of components, cecal sugars, and short-chain fatty acids production in broiler chicken.

1.4 Justification

Maize and soybean meal are the commonly used conventional poultry feed ingredients due to their superior nutritional quality. These traditional ingredients are expensive due to competition between humans and animals for the same ingredients (te Pas *et al*., 2021; Woyengo *et al*., 2014), and maize is currently being used to produce biofuels (Woyengo *et al*., 2014). Due to the unavailability of conventional ingredients at a relatively low price, there is a need to seek other alternatives (Laudadio *et al*., 2014). Most developing countries have started focusing on using agricultural by-products, which are abundant, as alternatives to conventional feedstuffs (Alshelmani *et al*., 2021). However, these by-products are rich in non-starch polysaccharides (NSP) such as mannans, arabinose, and xylans which suppresses the bird's performance (Aftab & Bedford, 2018), because of increased gut viscosity (Singh & Kim, 2021a). The poultry industry has recognized the value of exogenous enzymes in improving the growth performance and efficiency of feed utilization in the non-conventional feedstuffs (Adeola & Cowieson, 2011; Alagawany *et al*., 2018; Cozannet *et al*., 2017).

There is a short retention time of feeds in the poultry digestive system, and therefore the results of enzymes directly pre-mixed with feeds can vary (Sacranie *et al*., 2017). The use of exogenous enzymes in pre-treatment has the potential to solve these problems (Denstadli *et al*., 2007; Matshogo *et al*., 2021). Boroojeni *et al*. (2017) conducted an experiment showing that enzymatic pretreatment of peas drastically improved standardized ileal digestibility of nutrients in turkey.

Chapter Two: Literature Review

2.1 The poultry industry in Kenya

Kenya is considered one of the few countries in Africa with a well-developed animal feed industry, but the industry is not self-sufficient (Kenya Markets Trust, n.d.).The poultry industry is divided into indigenous and commercial systems and contributes about 30% of the agriculture GDP and 8% of the country's total GDP (FAO, 2017). The estimated poultry population is 42 million (*EB-2020*.), 82% indigenous chicken, 9% layers, and 7.4% broilers (FAO, 2018).

Due to the rapidly growing population and uneven economic development of the Kenyan people, the requirement for formal jobs has not been met (KIPPRA.,2016). The high annual urbanization (4%) has led to poverty and food insecurity in Kenyan urban areas (*National-Food-and-Nutrition-Security-Policy-2012*.). This has led people to venture into the informal sector, including urban poultry farming (Omondi *et al., 2017*). Most Kenyan farmers have faced various challenges, such as the high cost of feeds, poultry diseases, and lack of information on market trends (Extension Service, 2022). The country has a well-established feed manufacturing industry, but it remains far from being self-sufficient (Onono *et al*., 2018). Kenya is also among the sub-Saharan states seeking alternative poultry feed ingredients (Nakimbugwe *et al*., 2021).

2.2 Use of feed additives in poultry

2.2.1 Enzymes

The increased cost of animal feeds has been the main challenge facing developing countries, and as such cheaper non-conventional feedstuffs should be used (Alagawany *et al*., 2018). The main problem with most non-conventional feed is the high non-starch polysaccharides (NSP) (Alagawany *et al*., 2018). Non-starch polysaccharides are a group of starch with unique physio-

chemical properties whose size, shape, and solubility in water inside poultry gut vary a lot (Santos *et al*., 2007). The price volatility of poultry feed ingredients has increased due to the use of conventional feedstuffs such as maize in the production of biofuels (Raza *et al*., 2019). When formulating poultry feeds, the most limiting factor is the ability of the bird to utilize various ingredients components such as fiber (Zarghi, 2018). High fiber ingredients such as milling byproducts, ethanol industry byproducts, and oilseed meals are incorporated into poultry diets to partially substitute maize, wheat, and soybean meal (Teymouri *et al*., 2018). As a result, the quality of poultry feeds has become incredibly variable.

These byproducts have high levels of both fibrous carbohydrates and NSP (Raza *et al*., 2019). Broiler chickens cannot digest these fibrous feeds, and this causes an increase in intestinal viscosity, thus slowing down nutrient movements and absorption, which negatively affects their health (Raza *et al*., 2019). High fiber diets also cause an increase in subclinical pathogens that leads to the development of other pathogenic infections such as necrotic enteritis (Kaldhusdal, 2000).

Most of the enzymes used by the poultry industry are non-starch polysaccharides digesting (NSPases) that break down NSP in highly viscous ingredients such as wheat and triticale and phytases that target complex plant phytate (Ravindran & Son, 2011). These enzymes have improved digestibility in poultry diets with high non-starch polysaccharides with positive results on nutrient digestibility and availability (Saleh *et al*., 2018; Yan *et al*., 2017). Enzymes do not only improve performance by increasing the digestibility of ingredients but also through other mechanisms such as influencing gut microflora and altering gut morphology (Aftab & Bedford, 2018). Research has shown that when NSPases are used to cleave NSP, some of the oligosaccharides produced can act as prebiotics in the chicken gut (Masey O'Neill *et al*., 2014).

Oligosaccharides are formed during cell wall cleavage by enzymes, and when they reach the cecum, they support the proliferation of healthy microorganisms (Zarghi, 2018). Currently, enzymes have shown contradictory results when used in monogastric animal feeds, which has limited their wide acceptance (Ravindran & Son, 2011).

The use of exogenous enzymes in poultry feeds is not a new concept, and there are many studies conducted regarding this topic (Bedford, 2000; Pettersson & Aman, 1989; Rho *et al*., 2020). The current debate is the mechanism by which enzymes promote performance (Bedford, 2018).

2.3 Use of wheat and its by-products in poultry diets

Agricultural by-products have increasingly been used to reduce the cost of chicken production. However, their inclusion also increases dietary fiber, which acts as an antinutritional factor (Singh & Kim, 2021). The by-products contain non-starch polysaccharides that are not utilized by the chicken due to the presence of β-linkages, leading to reduced nutrient absorption and increased gut infection as they increase intestinal viscosity (Raza *et al*., 2019). Exogenous enzymes have been successfully used to improve the utilization of this dietary fiber by reducing these antinutritional factors (Singh & Kim, 2021). These enzymes have encountered various limitations in broiler chicken (Ravindran, 2013), limiting understanding of how these exogenous enzymes work inside the bird(Bautil *et al*., 2021). Direct supplementation of enzymes has reported mixed results, with some researchers showing improved broilers' performance (Agboola *et al*.,2015; Odu *et al*., 2015; Kiarie *et al*., 2017), while others have reported no improvement (Mohammed *et al*., 2017; Olgun *et al*., 2018; Walters *et al*., 2018).

It has been shown that wheat cell wall contains non-starch polysaccharides, which have an antinutritional effect in the gut of pigs and poultry, thus reducing its inclusion in poultry feeds (Choct, 2006). In broilers, the wheat's nutritional value is determined by its apparent metabolizable

energy (AME) (González-Ortiz *et al*., 2019). This AME is calculated as the energy required for growth and maintenance (Mateos *et al*., 2015).

According to Woyengo *et al*., (2014), agro-industrial byproducts such as wheat middlings have become more common for non-ruminant animals. According to Loy & Lundy (2019), when these byproducts are compared with cereal grains, they have high fat and crude protein. However, their fiber content is higher, negatively affecting digestibility and feed efficiency (Rosenfelder *et al*., 2013). Cellulose, arabinoxylans, and mixed linkage β-glucans are the primary byproduct fibers (Jaworski *et al*., 2015).

The gut of poultry and pig is colonized by diverse microbiota that ferments different substrates that are availed (Brestenský *et al*., 2017). Monogastric cannot utilize cereal byproducts due to the high content of insoluble fiber. Also, they have an insoluble portion which is usually hydrophobic, crystalline, and resistant to fermentation (Knudsen, 2014). Pre-treatment of co-products by soaking them in water and exogenous enzymes improves feed efficiency in monogastric (Hurst *et al*., 2008).

2.3.1 Non-starch polysaccharides in wheat milling byproducts

The main wheat milling byproducts used in poultry diets are wheat bran and wheat middlings (pollard). Wheat middlings does not compete with human as a food source and, therefore, has the potential to reduce the cost of feeds (Ahmadi & Karimov, 2010). Wheat middlings contains 16% CP, 10.5% ADF and 2,540 Kcal ME/kg (Ahmadi & Amini, 2014; Casas *et al*., 2018). The level of bran, germ, characteristics of wheat, and milling process affect the composition of wheat middlings (Rosenfelder *et al*., 2013).

According to Ahmadi & Karimov (2010) and Abudabos (2011), wheat milling byproducts can replace 30-40% of maize in poultry diets. These wheat milling byproducts have high insoluble Non-starch Polysaccharides (NSP) (Choct *et al*., 1996). In wheat, the NSPs are arabinoxylan, mannose, arabinogalactan, and β-glucan (Li *et al*., 2020). Non-starch polysaccharides send satiety signals when they mix with water and swell to form chime in the gut, thus reducing feed intake (Leo *et al*., 2012). Cozannet *et al*. (2017) showed that NSP affects intestinal microorganisms' composition and increases intestinal viscosity. In addition, they reduce the motility of the intestines and can block nutrients' absorption (Cozannet *et al*., 2017), thus reducing the performance and growth of the animal. Enzymes (carbohydrases) can be added to wheat by-products in poultry diets to minimize the effects of NSP (Abudabos, 2011). According to Gallardo *et al*. (2018), the inclusion of carbohydrases in broiler diets increases nutrient retention, also reduces digesta viscosity (Amerah *et al*., 2015), and improves the accessibility of endogenous enzymes to nutrients (Woyengo & Nyachoti, 2011).

Due to the adverse effects of high fiber diets on broiler chicken gut health and production performance (Hamedi *et al*., 2011), coupled with the physiological limitation of poultry gut on the effectiveness of exogenous enzymes supplemented in their diets (Ravindran, 2013), there is increasing research on pre-treating high fiber ingredients before feeding them to poultry. Some of the pre-treatment methods with available data include the use of probiotics (Yeh *et al*., 2018), organic acids such as citric acid (Zangaro & Woyengo, 2022), physiochemical such as low moisture anhydrous ammonia (Mahmud & Rosentrater, 2021; Yoo *et al*., 2011) and exogenous enzymes (Rho *et al*., 2017). Most of these methods have faced common challenges that have prevented them from being applied commercially. These challenges include unfavourable

processing methods, the high moisture content in the final product, and others producing a lot of waste (Mahmud & Rosentrater, 2021).

Fermentation has been shown to improve the nutritional value of agricultural by-products such as wheat bran by breaking down cellulose and increasing the level of acid soluble protein (Teng *et al*., 2017; Yeh *et al*., 2018) and removing toxic substances in feeds (Xu *et al*., 2012). While there are opportunities to develop highly efficacious feed enzymes to improve animal nutrient utilization and performance, there will always be physiological limitations by gut pH and nutrient retention time at specific gut sections (Adeola & Cowieson, 2011; Ravindran, 2013). Even when enzymes are supplemented in diets, the digestibility is not 100%; for example, the digestibility of phosphorous in swine without exogenous enzymes supplementation is 40% - 50 %, while with supplementation, the digestibility is increased to about 60%-80% (Lei *et al*., 2013; Selle & Ravindran, 2008). There is a need to develop dietary strategies to overcome these limitations, such as using high inclusion levels of enzymes/or creating enzymes that are designed to be released at specific gut sites (Kiarie *et al*., 2016). The use of exogenous enzymes to pre-treat products of low digestibility in the monogastric gut might be one of the strategies to be explored.

2.5 Use of sunflower seed cake in poultry diets

Due to the high cost of conventional protein sources such as Soybean meal (SBM), there has been an increased need to seek alternative sources for poultry feeds and other monogastric in developing countries (Oliveira *et al*., 2012). Researchers have taken up this challenge by using agricultural by-products as cheap alternatives (Alagawany & Attia, 2015). Sunflower seed cake (SFC) has high-fat content and can be used as a source of energy in poultry diets (Oliveira *et al*., 2012), but its use is limited by a high content of NSP, mainly cellulose and lignin (Attia *et al*., 2003). These

limitations can be overcome by supplementation with fiber digesting enzymes (Berwanger *et al*., 2017). However, supplementation of SFC with enzymes at the broiler starter phase does not increase nutrient utilization, decreasing performance. At the same time, it can only be included at 10% without affecting performance in the finisher phase (Berwanger *et al*., 2017). In Kenya, sunflower is grown in various regions such as in the Western, Meru and Rift Valley region (Bidco Kenya and wakulima-hub, anon, Okoko *et al*., 2008). The quality of the sunflower meal sold for animal feeds in Kenya is; 33-34 % CP, 9.2-9.3 crude fat, and 37.3-37.4 crude fiber (Sakwa *et al*,. 2019).

Most of the NSPs in the SFC hull, including arabinoxylans, xyloglucans or cellulose are insoluble in water (Ditta & King, 2017). At the same time, soluble NSPs (pectin and arabinans) are associated with the embryo and cotyledon (Düsterhöft *et al*., 1992). The insoluble fraction increases the movement of digesta from small to large intestines, thus reducing feed transit time and the activity of digestive enzymes, reducing nutrients digestibility (Bedford & Classen, 1992). Although physiological effects caused by enzymes on the gut have not been well characterized (Kiarie *et al*., 2013), there are several studies describing the effects of carbohydrates on the gastrointestinal barrier, intestine mass, nutrients transporters expression, and immunity (Agyekum *et al*., 2012: Agyekum *et al*., 2015). Feed enzymes have an antimicrobial effect and modulate gut microbiota (Kiarie *et al*., 2013).

2.6 Enzymatic pre-treatment/pre-treating of feeds

The use of enzymes to pre-treat/steep ingredients started way back in the 1950s when chickens were fed barley soaked in water before feeding, and they showed improved performance (Fry *et al*., 1957). Recent research has confirmed that soaking any feed ingredient in water (pre-treating) improves its nutritional value (de Lange & Zhu, 2012). Pre-treating time is critical as it alters the

microbial and chemical profile of the ingredients. The ideal broiler feed has a high lactic acid concentration and a low pH (Brooks *et al*., 2003). Researchers have reported that the perfect pre-treating time is 10-72h (Wiseman *et al*., 2017). Maize pre-treated with phytase leads to the release of all phytate-phosphorous (Niven *et al*., 2007), with only a tiny amount of the phytase needed to improve phosphorous retention in starter pigs fed maize-soybean based diet (Columbus *et al*., 2010).

It's essential to first understand the products of enzymatic hydrolysis of non-starch polysaccharides as some products, such as xylose and arabinose, if produced at a high level, will be detrimental to the bird's performance (Schutte, 1990). The use of fiber degrading enzymes (FDE) to pre-treat (steep) ingredients releases simple sugars such as xylose and arabinose that monogastrics such as pig are unable to utilize (de Lange *et al*., 2006), and therefore use of FDE need to be combined by slow fermentation during pre-treatment (Lange *et al*., 2006). These results triggered the use of exogenous enzymes in feeds (Jensen *et al*., 1957). The current understanding of the mode of action of enzymes in improving performance is through reducing gut viscosity, breaking plant cell walls, and production of prebiotics (Bedford, 2018).

Saenphoom *et al*. (2013) showed that enzymatic pre-treatment of palm kernel expeller with enzymes improved AME by hydrolyzing cellulose and hemicellulose. When food-processing by-products are soaked with FDE before feeding, their fiber is pre-digested (Rho *et al*., 2020). The poultry gut is small, and hence the digesta retention time is limited, which influences the type of bacteria that colonizes their gut (Pan & Yu, 2014). In poultry, fermentation of by-products occurs from crop to ceca, with most of it happening in ceca (Pan & Yu, 2014). The primary substrates for fermentation are NSP and resistant starch, with SCFA, methane, hydrogen, and carbon dioxide

(Mroz *et al*., 2006). The SCFA act as an antimicrobial by inhibiting the growth of pathogenic bacteria in the poultry gut (Ricke, 2003).

It has been suggested that feed pre-treatment with enzymes might increase the feed conversion ratio and body weight gain and increase the bioavailability of minerals such as phosphorus (Rahimi *et al*., 2020). However, more studies are required to understand the effect of pre-treatment of feeds with enzymes and also provide practical ways of decreasing feed antinutrients (Rahimi *et al*., 2020). The value of agro-processing byproducts can be improved when inoculated with an enzyme in a liquid diet (Wiseman *et al*., 2017). Of late, there is much interest in the pre-treatment of byproducts, especially in swine (Rho *et al*., 2020; Wiseman *et al*., 2017), where byproducts are pre-treated for a prolonged period and fiber degrading enzymes added.

Feeding rations containing coproducts with enzyme premixing have shown positive results in nutrient utilization and growth performance (Tsai *et al*., 2017), but there have also been contradicting results (Kerr & Shurson, 2013). Hence, more research is needed to achieve consistency in cereal byproducts utilization (Huntley & Patience, 2018). Exogenous enzymes are only activated in a liquid medium, and pre-treating feeds in the presence of exogenous enzymes bring about synergism (Svihus, 2010). Pre-treating feedstuffs promote hydrolysis of fibrous materials and their utilization (Jakobsen *et al*., 2015).

Rho *et al.* (2018) pretreated maize distillers dried grain with solubles (DDGS) by adding feed enzymes to fermented or non-fermented liquid feeds fed to pigs. There was an improvement in feed efficiency compared to control, although the mechanism by which this improvement was achieved was unclear to them (Rho *et al*., 2020). Their results showed that crude fiber digesting enzymes could be used to solubilize insoluble crude fibers that are then made available for fermentation in the hide gut (Rho *et al*., 2020). However, more research is needed to validate the

effect of pre-treatment byproducts with fiber degrading enzymes in solubilizing resistant fiber to fermentation by monogastric gut microbial (Rho *et al*., 2020). The short retention time of feeds in the poultry gut does not allow enough time for exogenous enzymes interaction, and the pH varies across the gut with the substrate in the gut (Ravindran, 2013). Enzymes prefer a pH of between 4 and 6 (Svihus, 2011), thus justifying the need to pre-treat. There is also minimum literature on the effects of feed pre-treatment in poultry.

2.7 Fiber and Chicken gut microflora

There are various ways of classifying fiber, such as crude fiber, acid detergent fiber, neutral detergent fiber, soluble and insoluble fiber, based on either analytical methods or their digestibility (Raza *et al*., 2019). The NSP constitutes β-fibers and has different degrees of solubility in water. The most abundant NSP in plant cell walls are cellulose, arabinoxylans (hemicellulose), and β-glucan (Bach Knudsen, 1997; Knudsen, 2014). Some fibers, such as oligosaccharides, have been shown to exert prebiotic effects by enhancing gut health. With most countries banning the use of antimicrobial in animals, prebiotics are now being used to promote the growth of the gut microbiome by acting as their substrate (Singh & Kim, 2021b). The hydrolysis and fermentation of these fibers in the chicken's intestine lead to the production of short-chain fatty acids (SCFA) such as propionic, butyric, and acetic acids which promotes gut health (Lin & Olukosi, 2021).

Fiber degrading enzymes (FDE) help to hydrolyze NSP whose products act as nutrients for beneficial gut microorganisms after fermentation (Bedford & Cowieson, 2012). Xylanase has been shown to enhance the release of xylooligosaccharides from xylan degradation (Morgan *et al*., 2017), which is an essential product to the host as it acts as a prebiotic, which promotes the growth of good bacterial for cecal fermentation in broilers (Samanta *et al*., 2015). There is evidence that gut microbiota influences nutrients utilization in chicken (Jha *et al*., 2019a). Dietary fiber, which

can be defined nutritionary as carbohydrates not utilized by endogenous enzymes (Jha & Mishra, 2021), utilization by pigs and poultry is determined by the extent of microbial fermentation in the large intestine (Perez Bonilla, 2013).

Intestinal microorganisms rapidly ferment soluble fiber as the net effect of these soluble fibers is to increase gut viscosity, digesta movement rate reduction, and feed intake reduction (Hetland *et al*., 2004). It is possible to manipulate intestinal microflora during a broiler chicken's pre-hatch and post-hatch life (Jha *et al*., 2019b). Fiber is fermented in the large intestine to produce short-chain fatty acids (SCFA), and these metabolites also affect the composition of intestinal microorganisms (Jha & Berrocoso, 2015). Galacto-oligosaccharides and fructo-oligosaccharides have been studied widely and proven to alter intestinal microbiota (Park *et al*., 2017; Slawinska *et al*., 2019).

Most enzymatic pre-treatment of ingredients has been conducted in pigs with mixed results. There is limited literature on the effect of this technology on broilers and poultry in general.

2.8 Short-chain fatty acids in poultry

When broilers are fed high fiber diets, the NSP may account for up to 10% of total nutrients in the diet (Nguyen *et al*., 2022). The soluble fraction of the NSP is the most important as it causes viscosity, thus affecting nutrients absorption and excreta moisture. At the same time, it’s the portion that provides the intestinal microbiota with fuel for proliferation (Nguyen *et al*., 2022). Poultry lacks endogenous enzymes to utilize NSP and is, therefore, less efficient in fiber utilization which is further complicated by their short gut and digesta transit time (Iji *et al*., 2001). Commensal microorganisms utilize non-starch polysaccharides and other indigestible starch in the large intestines (Rinttilä & Apajalahti, 2013b). When exogenous enzymes are used in broiler diets, they hydrolyze NSP into oligosaccharides such as xylose, mannose, arabinose, and glucose (Smeets *et*

al., 2014). The cecal microbiota then metabolizes oligosaccharides to produce SCFA, which acts as a prebiotic (Stanley *et al*., 2014).

Studies in humans have shown that SCFA provides between 5 and 15% of the energy requirements (Bergman, 1990). The NSPs increases gut viscosity, thus depressing growth performance (Jia *et al*., 2009). Small oligosaccharides are produced after hydrolysis of NSP, and they could act as prebiotics (Courtin *et al*., 2008). Products of hydrolysis are suggested to be the ones that are fermented by beneficial bacteria into SCFA (Lee *et al*., 2017) since the increase in SCFA is associated with the growth of beneficial bacteria (Ravangard *et al*., 2017). Supplementation of broiler diet with xylanase has affected SCFA production (Lee *et al*., 2017). The cecum is the section where fermentation of polysaccharides occurs and plays a significant role in poultry health and performance (Stanley *et al*., 2014). Polysaccharide fermentation generates short-chain fatty acids (SCFA) mainly in the form of acetate (Yeoman *et al*., 2012). The SCFA provides low pH that inhibits the growth of acid-sensitive pathogens, improves mineral absorption, and promotes the development of epithelial cells (Oakley *et al*., 2014).

When mice and broiler are fed dietary fiber which is fermented in the intestine into SCFA, mice colon and epithelial cells utilize SCFA as the primary energy source (Donohoe *et al*., 2011), while in broiler chicken, they are used during intestinal villi development (Panda *et al*., 2009).

Chapter Three. The effects of pre-treating wheat middlings and sunflower meal with enzymes on crude protein, apparent disappearance of crude fiber, and concentration of mono-sugars, and organic acids.

Abstract

An *in vitro* experiment was conducted to investigate the effects of pre-treating wheat middlings (WM) and sunflower meal (SM) with exogenous enzymes on solubilization of crude protein and fiber and mono-sugars and organic acids concentration in the supernatant. The WM was pre-treated with fibre degrading enzymes (FDE) (2×2 factorial) and SM with FDE with or without protease (FDEP) (2×3 factorial). For each feedstuff, four replicates of 50 grams of material were mixed with 1% of the enzyme (none for control), suspended in 200 mL of distilled water and placed in an incubator shaker set 40℃ and 200rpm. The WM enzyme activity was xylanase-3,000u/g, cellulase-45,000u/g and β-glucanase-40,000u/g, the SM enzymes activities were cellulase-45,000u/g, β-glucanase-40,000u/g, mannanase-12,000u/g, pectinase-250,000u/g and protease-1,000,000 u/g. Pre-treatment was either 24h or 48h, and samples were processed for apparent disappearance of neutral detergent fiber (NDF), concentration of mono-sugars and organic acids. Pre-treatment of WM increased crude protein, soluble protein (SP), and fat by 1.83%, 7.09%, and 1.93% at 24h, respectively, and by 2.63%, 9.99%, and 2.55% at 48h, respectively, compared with the control at 0h. The FDE also increased apparent disappearance (AD) of NDF by 9.41% at 24h and by 10.23% at 48h. The FDE treated WM had higher total sugars than the control (P <0.001), there was also a time effect on total sugar concentration, with 24h being greater than 48h (P =0.029). Pre-treatment of SM with FDE increased CP by 2%, SP by 6.11% at 24h, and CP and SP by 2.36% and 7.64%, respectively, at 48h. The FDE with protease (FDEP) increased both CP and SP by 1.07% and 5.79% respectively at 24h and increased both CP and SP by 2.2% and 8.36% respectively at 48h. The total organic acid and mono-sugars

concentration was greatest with enzyme treatments relative to the control ($P < 0.001$). In conclusion, pre-treatment of both SM and WM with exogenous enzymes increased CP and apparent disappearance of crude fiber, increased mono sugars and organic acids concentration.

Keywords: **pre-treatment, enzymes, solubilization, apparent disappearance, mono-sugars, organic acids**

3.0 Introduction

Agricultural by-products such as rapeseed and sunflower meals have increasingly been used to reduce the cost of chicken production, but they also increase dietary fiber, which acts as an antinutritional factor (Kithama *et al*., 2021; Lannuzel *et al*., 2022; Singh & Kim, 2021b). The by-products contain non-starch polysaccharides (NSP) that are not utilized by the chicken due to the presence of β-linkages leading to reduced nutrient absorption and increased gut infection as they increase intestinal viscosity (Raza *et al*., 2019). Exogenous enzymes have been successfully used to improve the utilization of this dietary fiber by reducing these antinutritional factors (Singh & Kim, 2021b). These enzymes have encountered various limitations in broiler chicken (Ravindran, 2013), resulting in limited understanding of how they work within the bird (Bautil *et al*., 2021). Direct supplementation (premixing) of enzymes has reported mixed results, with some researchers showing improved broilers' performance (Agboola *et al*.,2015; Odu *et al*., 2015; Kiarie *et al*., 2017), while others have reported no performance improvement (Mohammed *et al*., 2017; Olgun *et al*., 2018; Walters *et al*., 2018).

There are various ways of classifying fiber, such as crude fiber, acid detergent fiber, neutral detergent fiber, soluble and insoluble fiber, based on either analytical methods or their digestibility (Raza *et al*., 2019). The NSP constitute β-fibers and have different degrees of solubility in water. The most abundant plant cell walls are cellulose, arabinoxylans (hemicellulose), and β-glucan (Bach Knudsen, 1997; Knudsen, 2014). Pre-treatment of by-products with fiber degrading enzymes such as xylanase, cellulase, and β-glucanase has resulted in the increased apparent disappearance of dry matter (DM), neutral detergent fiber (NDF), trace elements, crude protein (CP), and increased concentration of mono sugars such as xylose and arabinose (Jaworski *et al*., 2015; Rho *et al*., 2020; Yu *et al*., 2018). The hydrolysis and fermentation of these fibers in the chicken's intestine lead to the production of short-chain fatty acids (SCFA) such as propionic,

butyric, and acetic acids which promote gut health (Lin & Olukosi, 2021). It is important to understand the concentration of mono sugars generated by enzymatic hydrolysis of NSP as some of them, such as xylose and arabinose, if produced at a high level, will be detrimental to the broiler chicken performance (Regassa *et al*., 2017a; Schutte, 1990).

Most cereal grains, such as wheat, contain NSP in arabinoxylans, while protein-rich plants (dicotyledons) have xyloglucan as the predominant NSP (Caffall & Mohnen, 2009). Wheat grain has specifically 7.3% arabinoxylans(Knudsen, 2014). The NSP in Sunflower meals consist of 7.7% arabinose, 13.0% xylose, and 11.5% uronic acid (Carré & Brillouet, 1986). The concentration of NSP is much greater in by-products than in the parent grain (de Vries *et al*., 2013; Pedersen *et al*., 2014). Insoluble fiber prevents endogenous enzymes from accessing nutrients, causing them to pass through the gut unutilized (Bedford *et al*., 2022). Sunflower meal (SM) has a high level of lignification which affects the solubility of its NSP, with most of them being insoluble; these NSP are also rigid such that even the exogenous enzymes are unable to degrade them (Knudsen, 2014).

Whereas many studies have investigated the effect of individual SM and WM inclusion in broiler chickens' diets premixed with exogenous enzymes, there haven't been any published work on the impact of the inclusion of both enzymatically pre-treated SM and WM in broiler diets ((Abudabos, 2016; Adedokun *et al*., 2015; Kocher *et al*., 2000; Mbukwane *et al*., 2022; Singh *et al*., 2017)). Therefore the current study investigated the effect of *in-vitro* pre-treatment of SM and WM on AD of NDF, CP solubilization, mono sugars, and organic acids concentration in the supernatant of treated materials, and the impact of including pre-treated SM and pre-treated WM in a maize-soybean meal-based diet by having a positive control (PC); maize-soybean meal-based diet, negative control (NC) with the inclusion of both untreated SM and WM and test diets where the untreated ingredients were replaced with pre-treated SM and WM and hypothesized that the

replacement would enhance components retention and organic acids concentration in the caecal digesta comparable to PC in broiler chickens.

3.1 Materials and Methods

Wheat middlings (WM) and sunflower meal (SM) *in vitro* solubilisation of fiber and crude protein, mono sugars, and organic acids concentration in the supernatant were investigated as follows; The untreated wheat middlings (UWM) were obtained from Floradale Feed Mill Limited (Floradale, ON, Canada) and pre-treated without further processing. Sunflower meal was obtained from Persall Fine Foods Co. (Waterford, ON, Canada), where oil had been extracted using the expeller cold pressing method and used without any further processing. The WM had two treatments each with four replicates, and the treatments were 1) control pre-treated without enzymes; 2) control + FDE ((xylanase/cellulase/β-glucanase). Sunflower meal (SM) had three treatments with four replicates each. The treatments were 1) control pre-treated without enzymes, 2) control + FDE (1% (0.5g) cellulase/glucanase/mannanase and 1% (0.5g) Pectinase liquid), 3) Control + FDE + 1% (0.5g) protease liquid. A 50g sample of UWM and USM for all individual treatments was weighed and transferred to a 500ml plastic container, and 200 ml of water was added to it (1:4 w/v for WM: water). The container was then tightly capped and placed inside an incubator shaker (Controlled Environment Incubator Shaker, New Brunswick Scientific, Enfield, CT), where the temperature was set at 40^{o}C and shaking at 200 rpm and after 15min, the enzymes were added at the inclusion rate of 1% (w/w) the sample weight in accordance with manufacturers specifications, and the container closed. The pre-treatment happened in a semi anaerobic condition as no additional oxygen was pumped in. Samples were taken at the end of incubation time (24h). One replicate was freeze-dried and sent to a commercial laboratory (SGS labs, Guelph, ON, Canada) for analyses. In contrast, the remaining three replicates per treatment were centrifuged for 15 minutes, and the

supernatant and pellet separated into different aluminum trays. A 1ml aliquot was taken from all treatments replicate supernatants and frozen (-20˚C) awaiting analyses for mono sugars and organic acids analyses. The remaining supernatant and pellet per replicate were freeze-dried for analyses.

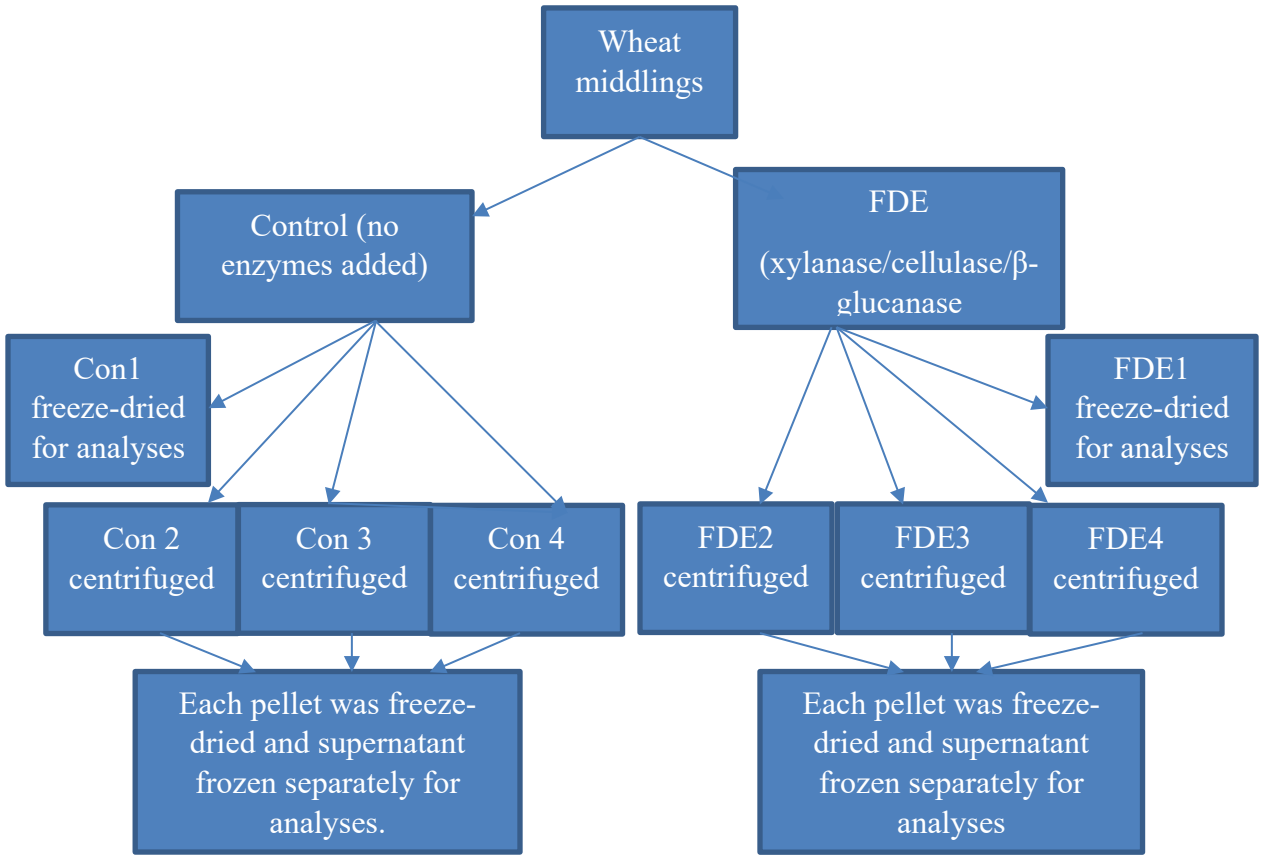

Figure 3.1 Wheat middlings pre-treatment flowchart

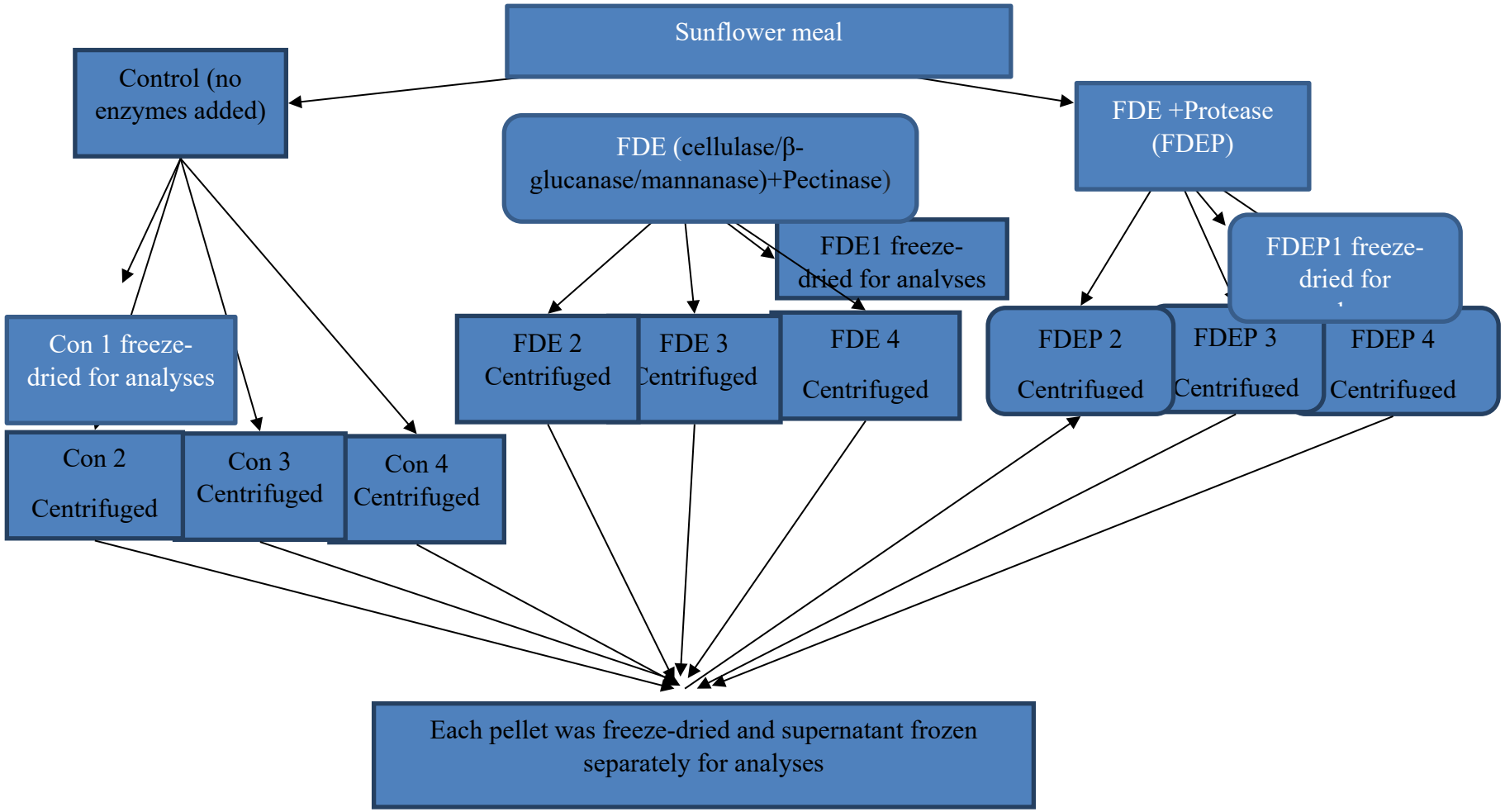

Figure 3.2 Sunflower meal pre-treatments flowchart

A similar set of above treatments and replicates were pre-treated for 48h instead of 24h, and the same laboratory analysis was done. The WM enzyme activity was xylanase-3,000u/g, cellulase-45,000u/g and β-glucanase-40,000u/g, the SM enzymes activity was cellulase-45,000u/g, β-glucanase-40,000u/g, mannanase-12,000u/g, pectinase-250,000u/g and protease-1,000,000 u/g (CBS Bio Platforms Inc, Calgary, Alberta, Canada).

3.1.1 Laboratory analyses

Only supernatant and pellet parts were analyzed at the University of Guelph, ON, Canada. The pellet was analysed for DM, NDF and nitrogen, while the supernatant was analysed for mono sugars, and organic acids concentration. The DM was analyzed using method 930.15 (AOAC, 2004), NDF was determined using an ankom 200 fiber analyzer (Ankom Technology, Macedon, NY) as described by (van Soest *et al*., 1991,) and nitrogen was determined using the LECO machine (LECO corporation, St. Joseph, MI) method 968.06 (AOAC International, 2005). The mono sugars, SCFA, and organic acids concentration were analyzed using HPLC after hydrolysis as described by Miron *et al*., (2001). The monosaccharide concentration, i.e., glucose, arabinose, and xylose, and organic acids, i.e., lactic acid, acetic, isobutyric, butyric, and propionate concentration in the supernatant of WM and SM, were analyzed using high-performance liquid chromatography (HPLC Agilent 1100 Series, Agilent Technologies; (Leung *et al*., 2018)). The samples were thawed and vortexed, and centrifuged for 15 minutes. Fat layer removed by sucking under vacuum. A 160 uL was diluted ×20 using 0.005N sulfuric acid, filtered using a 13 mililmetter syringe filter, and transferred to the HPLC vials. Separation of samples a 300 × 7.8-mm 8µ Rezex™ ROA-Organic Acid H+ (8%) column (Phenomenex, Torrance, CA). the HPLC settings were 60C for column temperature, 20ul as the injection volume; refractive index detector temperature was 35C, the 0.005N sulfuric acid mobile phase velocity was 0.5ml/min and a cycle

time of 45 minutes. Acetic acid, butyric, propionate, isobutyric, lactic, glucose, xylose, and arabinose retention time were 19.6, 28, 22.8, 25.5, 16.5, 12.39,13.22,13.65 minutes respectively. The following equation was used to calculate the apparent disappearance (AD) of components as described by (Rho *et al*., 2020).

$$AD\% = [\frac{(\text{concentration before pre-treatment}) - (concentration\ after\ pre-treatment)}{(\text{concecentration before pre-tratment})}] \times 100$$

3.1.2 Data analysis

The data was analyzed using proc GLM of SAS 9.4. The model had treatments, time, and their interactions as fixed factors and Tukey test was used to separate the significant means ($P<0.05$). The linear statistical model used is as shown in equation 1.

$$Y_{ijk} = \mu + b_i + c_j + bc_{ij} + \varepsilon_{ijk} \text{.....equation (1)}$$

Where:

Y_{ijk} = was observations recorded such xylose, arabinose…...,μ_i = overall population means, b_j= treatment effect,c=time effect, bc_{ij}=interaction effect between treatment and time, ε_{ijk} = random error effect associated with observations recorded.

3.3 Results and discussion

Analyzed chemical composition of untreated wheat middlings (UWM) and treated wheat middlings are shown in table 3.1. Treatment of UWM with just steeping it with water either at 24h or 48h led to improved compositional quality compared with a control. When soaked for 24h, soluble protein (SP) and fat increased by 3.8% and 1.36%, respectively, while 48h steeping increased both SP and fat by 4.71% and 1.53%, respectively. This is due to the activation of endogenous protease. These results agree with *et al*(Arte *et al*., (2015), who observed that water activates endogenous protease in wheat bran, leading to increased protein solubilization, especially

when the pH becomes acidic. The pH in the current study of steeped control dropped from 6.25 to 3.72 at 24 h and 3.57 at 48 h, as shown in table 3.2 The wheat middlings (WM) is a mixture of shorts, wheat bran, flour, germ, and other milling tails (Erickson *et al*., 1985). The FDE increased crude protein (22.25 vs 20.42) which can be due to increased microbial nitrogen, SP (16.16 vs 9.07), and fat by 1.83%, 7.09, and 1.93% at 24h, respectively, and by 2.63%, 9.99%, and 2.55% at 48h, respectively, compared with the control at 0h. The FDE also increased apparent disappearance (AD) of NDF by 9.41% at 24h and by 10.23% at 48h, and these results agree with (Moran *et al*., (2016a); Rho *et al*., (2020), who reported similar results whereby pre-treatment of UWM for either 24h or 48h with FDE led to the increased AD of NDF. The feed enzymes' mode of action is by breaking chemical bonds (especially the β-linkages that bird's endogenous enzymes are unable to hydrolyze), solubilization of NSP, and having an overall effect of encapsulating cell wall caged nutrients such as amino acids, starch, and minerals (Kiarie *et al*., 2021). The enzymes are also known to hydrolyze saturated fat (Munir & Maqsood, 2013), and this explains the high-fat content of FDE TWM in the current study. Lipids are generally attached to the surface of the plant cell wall or inside the starch granules (Jovanovich & Añón, 1999).

Table 3.1 Analysed chemical composition of wheat middlings pre-treated with or without fiber degrading enzymes on a dry matter basis.

Time[2]	**0h**	**24h**		**48h**	
Treatments[1]	Control (untreated)	Control[3]	FDE	Control[3]	FDE
CP%	20.42	20.76	22.25	21.50	23.05
Soluble protein%	9.07	12.87	16.16	13.78	19.06
Acid detergent protein%	0.57	0.65	0.61	0.74	0.66
Neutral detergent protein%	4.40	4.06	2.79	4.39	3.62
Acid detergent fiber%	13.64	13.54	12.14	14.48	11.89
Neutral detergent fibre%	40.65	40.77	31.24	42.34	30.42
Lignin%	3.68	5.78	6.62	4.72	6.11
Fat%	2.98	4.34	4.91	4.51	5.53
Starch%	18.86	17.14	17.56	16.70	18.74
Ash%	5.83	4.74	5.61	4.97	5.14
Ca%	0.13	0.12	0.12	0.12	0.14
P%	1.25	1.13	1.08	1.16	1.14
K%	1.35	1.22	1.2	1.27	1.22
Mg%	0.56	0.53	0.52	0.56	0.55
Na%	0.02	0.06	0.07	0.07	0.1
Cu (ppm)	11.29	15.11	15.02	14.61	13.68
Mn (ppm)	130.93	125.55	121.09	127.04	126.56
Zn (ppm)	107.55	103.18	100.4	103.58	102.58
Fe (ppm)	201.65	169.38	171.84	175.05	182.76

[1]FDE-fiber degrading enzymes (1% w/w of xylanase/cellulase/β-glucanase), Control- no enzymes added.
Enzyme activity was xylanase-3,00u/g, cellulase-45,000u/g, and β-glucanase-40,000u/g
[2]Time - 0h being the original sample, samples were steeped for either 24h or 48h
[3]Pre-treated by mixing with water only
50 g of wheat middlings was mixed with 200 mL of distilled water and 0.5 g of respective enzyme and incubated at 40 °C inside an incubator shaker (Controlled Environment Incubator Shaker, New Brunswick Scientific, Enfield, CT) with continuous agitation of 200rpm.

The pH and the apparent disappearance (AD) of DM, NDF, and CP in the pellet of wheat middlings pre-treated with fiber degrading enzymes (FDE) are presented in table 3.2. There was a treatment effect on AD of DM, NDF, and CP (P <0.001), while there was no time effect and treatment and time interaction effect on AD of DM, NDF, and CP (P ≥0.159) an indication of that pre-treating beyond 24h had no additional effect. The AD of DM was greater in FDE than in control without

enzymes, and this may be due to increased solubilization of CP, NDF, and other trace elements. These results agrees with Yu *et al*., (2018), who conducted an in-vitro study by pre-treating wheat middlings, wheat bran, maize, wheat, barley and soybean using either xylanase or β-glucanase and all treatments had increased disappearance of trace elements, xylan, β-glucan and phytic acid when compared to a control without enzymes treatment. The FDE have been reported to increase human and animal food/feed ingredients' protein, energy, and soluble mineral contents Habte-Tsion & Kumar, (2018).

In the current study, the AD of NDF in FDE was significantly higher than in control. FDE hydrolyzed cell walls and encapsulated nutrients by cleaving the β-linkages in NSP into small molecules, thus solubilizing trapped components (Yu *et al*., 2018). The endogenous plant enzymes could not hydrolyze the β-glycosidic bonds, and therefore, the control yielded less soluble matter. The AD of CP was greater in FDE than in control due to enzymes breaking down the cell wall to release trapped amino acids and other sources of nitrogen and agrees with Kouzounis *et al*., (2021), who showed that broiler chickens that were fed a wheat-based diet with added FDE added had increased digestibility of organic matter, crude protein, and starch. The control pH dropped from 6.250 at 0h to 3.717 at 24h and 3.570 at 48h, indicating the activation of endogenous microorganisms by water to produce lactic acid and other acids. There was no treatment effect on the pH of steeped control and FDE. There was no treatment and time interaction effect on the pH, and these results agree with Rho *et al*., (2020), who did not observe treatment effect when WM was pre-treated with or without FDE over time.

Table 3. 2 The pH of and apparent disappearance of NDF, CP, and DM of wheat middlings pre-treated with or without fiber degrading enzymes

Item (%)		**DM**	**NDF**	**CP**	**pH[1]**
Treatments[2]					
Con		35.38[b]	40.35[b]	47.42[b]	3.64
FDE		47.02[a]	60.41[a]	59.53[a]	3.60
SEM		0.87	1.44	0.87	-
Time[3]					
24h		40.62	50.13	52.52	3.68
48h		41.78	50.63	54.43	3.56
SEM		0.87	1.44	0.87	-
Treatment*Time					
C[2]	0h				6.25[a]
Con	24h	34.79b	40.36[b]	46.50[b]	3.72[b]
Con	48h	35.96b	40.34[b]	48.34[b]	3.57[b]
FDE	24h	46.44a	59.90[a]	58.54[a]	3.65[b]
FDE	48h	47.60a	60.91[a]	60.51[a]	3.56[b]
SEM		1.24	2.03	1.23	0.03
P-Value					
Treatment		<0.001	<0.001	<0.001	0.148
Time		0.373	0.813	0.159	0.001
Treatment*Time		1.000	0.807	0.959	0.312

[1]Measured before centrifuging the samples
[2]C- control at zero hours, Con- control, and FDE-fiber degrading enzymes (1% w/w of xylanase/cellulase/ β-glucanase). Enzyme activity were xylanase-3,00u/g, cellulase-45,000u/g and β-glucanase-40,000u/g
[3]Time- steeped for 24h or 48h
NDF-neutral detergent fiber, CP-crude protein, and DM-dry matter, after pre-treatment
Values within a column without a common superscript differ significantly by LS means at 5% probability.
SEM- standard error of means
50 g of wheat middlings was mixed with 200 mL of distilled water and 0.5 g of respective enzyme and incubated at 40 °C inside an incubator shaker (Controlled Environment Incubator Shaker, New Brunswick Scientific, Enfield, CT) with continuous agitation of 200rpm.

The mono sugars and organic acids concentration in the supernatant of TWM over time are shown in table 3.3. The composition of NSP of any grain and its by-product determines its energy value (Jaworski *et al*., 2015). The primary NSP in most cereal grains are insoluble cellulose, soluble arabinoxylan, and β-glucan (Marcotuli *et al*., 2020). The current study combined xylanase,

cellulase, and β-glucanase to hydrolyze the NSP, leading to increased xylose, arabinose and glucose in FDE TWM compared to the control (P <0.001), indicating that the FDE were effective in hydrolyzing NSP present in the treatment. The total sugars were higher in FDE than in control without enzymes (P <0.001). The xylanase function by cleaving the starch and xylan to release pentoses such as xylose and arabinose, cellulase hydrolyses cellulose into glucose, and β-glucanase breaks down β-glucan, cellulose, and starch leading to glucose production (Munir & Maqsood, 2013; Rajnish *et al*., 2021; Rastogi & Shrivastava, 2017).

In the current study, time had an effect on glucose concentration as the 24h concentration was more significant than the 48h (P =.004). This is because glucose is easily fermented, and with time, the substrate breakdown (starch) by enzymes might have declined, coinciding with an increment of SCFA and organic acids. (Blandino *et al*., (2003) reported that fermentation of cereals leads to carbohydrates decline. In the current study, there was no interaction between time and treatment on mono sugars release (P ≥0.163), showing that there is no beneficial effect of steeping beyond 24h, which agrees with (Rho *et al*., 2020), who reported similar observations when they steeped WM with FDE. The FDE treatment had higher total sugars than the control ((P <0.001), there was also a time effect on total sugar concentration, with 24h being greater than 48h (P =0.029), which was due to fermentation of the sugars into acids by microorganisms. There was no treatment and time interaction on total sugars (P =0.643). The higher concentration of total sugars in FDE than in control is due to the breakdown of hemicellulose (arabinoxylan) β-linkages by FDE used in current study into xylose and arabinose. The endogenous enzymes cannot cleave the arabinoxylans (Asmare, 2014), explaining the observed difference in total sugar concentration between the control and FDE.

It's important to note that not all mono sugars are beneficial to the animal. For example, xylose has been incriminated with depressing performance in broiler chickens and pigs (Agyekum *etet al*., 2016; Huntley & Patience, 2018). Regassa *et al*. (2017) showed that D-xylose reduces hepatic enzymes' utilization of nutrients as it interferences with genes required for lipids and glucose metabolism, thus reducing broiler chicken performance. The body has its mechanism for dealing with xylose when present in amounts less than 5% (Huntley & Patience, 2018). Most mono sugars are directly absorbed in the small intestines. Still, they would be more beneficial to the animal if they reach the distal ileum and ceca, where they are fermented into SCFA (Kiarie *et al*., 2008; Kim *et al*., 2022). The SCFA is beneficial to broiler gut health by acting as prebiotics. The functional oligosaccharides also act as antitumors (Wu *et al*., 2020). Carbohydrates yield xylose, fructose, and galactose/glucose which are considered functional oligosaccharides (Mussatto & Mancilha, 2007).

When any grain is soaked with water, endogenous enzymes, yeast, and bacteria are activated and start producing organic acids with time (Canibe & Jensen, 2012). In the current study, the steeping happened in a semi anaerobic condition as no extra oxygen was pumped in as the containers were tightly capped throughout the steeping time; therefore, once mono sugars were released, some of them got fermented into organic acids and SCFA. The hydrolysis and solubilization of mono sugars and oligosaccharides are associated with increased SCFA concentration (Broekaert *et al*., 2011a). In the current study, there was a time and treatment effect on lactic concentration ($P \leq 0.002$) with 48h and FDE being more concentrated, which is related to the activity of enzymes with time in hydrolyzing resistant starch (RS) and β-glucan, which is eventually converted into lactic acid by microorganisms present. The RS and β-glucan have been shown to promote the proliferation of lactobacillus, which are responsible for lactic acid production (Stack *et al*., 2010;

Tiwari *et al*., 2019). The lactic and propionic acid concentration was greater in FDE treated wheat middlings (TWM) than in control ($P \leq 0.007$), while acetic, iso-butyric, and butyric acids were not different ($P \geq 0.056$). There is a shift to amino acid fermentation when there is not enough energy source for fermentation, increasing iso-butyric production (Jha *et al*., 2019).

The current study had a time effect on iso-butyric production, rising at 48h. This means that pre-treatment should not exceed 24h to avoid amino acid deamination as in the current study. Protein is also used as a carbon source during oligosaccharides fermentation to produce SCFA (Zhao *et al*., 2018; Wang *et al*., 2019a), reducing nitrogen in the organic matter at 48h. Fermentation of protein leads to the proliferation of pathogenic bacteria (Jha & Berrocoso, 2016). The total organic acid concentrations were also greater in FDE relative to the control ($P \leq 0.001$). The high total sugars in FDE treatment may have contributed to the observed high amount of propionic and lactic acid in FDE treatment. The concentration of acetic, propionic, and butyric acid is determined by lignin content, the solubility of the fiber, the processing method, and the ratio between indigestible oligosaccharides and indigestible protein (Montagne *et al*., 2003; Morita *et al*., 2004). Since most mono sugars are absorbed in the intestines, the indigestible oligosaccharides and mono sugars are essential in promoting gut health after they are fermented (C. Zhao *et al*., 2017). Acetate and propionate are carried to the liver, where the former is utilized as a source of muscular energy while the latter is converted to glucose (Rérat *et al*., 1987). Colonocytes primarily use butyrate as a source of energy in many organisms, including humans (Roediger, 1982).

Table 3. 3 Effects of fiber degrading enzymes on mono sugars and organic acids production in supernatant of pre-treated wheat middlings over time

Item (µmol/kg)		**Xylose**	**Arabinose**	**Glucose**	**Lactic**	**Acetic**	**Propionic**	**Iso-butyric**	**Butyric**	**Total sugars**	**Total organic acids**
Treatments[1]											
Con		0.04[b]	0.01[b]	0.01[b]	0.38[b]	0.03	0.01[b]	0.003	0.01	0.06[b]	0.43[b]
FDE		0.17[a]	0.03[a]	0.04[a]	0.39[a]	0.04	0.03[a]	0.003	0.01	0.24[a]	0.46[a]
SEM		0.0003	0.0004	0.003	0.003	0.003	-	-	0.002	0.006	0.006
Time[2]											
24		0.11	0.02	0.03[a]	0.34[b]	0.04	0.01[b]	0.0025[b]	0.01	0.16[a]	0.39[b]
48		0.11	0.02	0.02[b]	0.43[a]	0.04	0.03[a]	0.003[a]	0.01	014[b]	0.50[a]
SEM		0.003	0.0004	0.003	0.003	0.003	-	-	0.002	0.006	0.006
Treatment*time											
Con	24h	0.05[b]	0.004[b]	0.02[cb]	0.33[c]	0.03	0.01[b]	0.0029[ba]	0.01	0.07[b]	0.38[c]
Con	48h	0.04[b]	0.01[b]	0.01[c]	0.42[a]	0.03	0.01[b]	0.0029[ba]	0.01	0.05[b]	0.47[b]
FDE	24h	0.17[a]	0.03[a]	0.05[a]	0.35[b]	0.04	0.02[b]	0.002[b]	0.01	0.25[a]	0.41[c]
FDE	48h	0.17[a]	0.03[a]	0.03[ba]	0.43[a]	0.04	0.05[a]	0.003[a]	0.01	0.23[a]	0.52[a]
SEM		0.004	0.0006	0.004	0.005	0.004	0.007	0.0002	0.002	0.008	0.008
p-value											
Treatment		<0.001	<0.001	<0.001	0.002	0.056	0.007	0.495	0.846	<0.001	<0.001
Time		0.254	0.436	0.004	<0.001	0.275	0.032	0.020	0.412	0.029	<0.001
Treatment*time		0.830	0.219	0.642	0.163	0.203	0.062	0.024	0.181	0.643	0.284

[1]*Con-control without enzymes, FDE-fiber degrading enzymes (1% w/w of xylanase/cellulase/ β-glucanase)*

Enzyme activity were xylanase-3,00u/g, cellulase-45,000u/g and β-glucanase-40,000u/g

[2]*Steeped for either 24h or 48h*

Values within a column without a common superscript differ significantly by LS means at 5% probability.

SEM- standard error of means

50 g of wheat middlings was mixed with 200 mL of distilled water and 0.5 g of respective enzyme and incubated at 40 °C with a continuous agitation (200rpm) inside an incubator shaker (Controlled Environment Incubator Shaker, New Brunswick Scientific, Enfield,CT

The analyzed composition of FDE treated sunflower meal (SM) without or with protease on a DM basis (as reported by the commercial lab, SGS labs, Guelph, ON, Canada) is shown in table 3.4. Pre-treating sunflower meals with water only did not improve any compositional quality of SM, and it increased NDF, ADF, and lignin. This increase was unexpected and no plausible explanation was found. But it shows that endogenous enzyme alone cannot improved theSM compositional quality as was the case with WM, whose soluble protein was increased by soaking with water only, as shown in Table 3.1 above. Current study agrees with the data reviewed by (Knudsen, 2014), which indicates that SM has a high level of lignification which affects the solubility of its NSP as most of them are insoluble; the NSP are also rigid such that even the exogenous enzymes are unable to degrade them. In current study, FDE without or with protease could only reduce NDF at 48h by 4.13% (27.24 vs 31.37) and 3.59%, (27.78 vs 31.37) indicating that the enzymes might have required more time or a higher inclusion rate to hydrolyze more of the fiber. When (Hassaan *et al*., (2018) fermented SM with either yeast or bacterial microorganisms, CP yield was maximum at 72h. Other researchers have reported that the inclusion of SM at 14-21% in turkey diets reduced small intestine weight and inhibited the cecal fermentation process (Juskiewicz *et al*., 2010). In the current study, relatively to the control, the FDE without protease increased CP by 2%, (33.52 vs 31.52%) SP by 6.11% (28.00 vs 21.89%) at 24h, and CP and SP by 2.36% (33.88 vs 31.52) and 7.64% (29.53 vs 21.89%), respectively, at 48h. The FDE with protease (FDEP) increased both CP and SP by 1.07% (32.59 vs 31.52%) and 5.79% (27.68 vs 21.89) respectively at 24h and at 48h both CP and SP were also increased by 2.2% (33.72 vs 31.52) and 8.36% (30.25 vs 21.89%) respectively. These results show that FDE used in current study effectively liberates fiber trapped nitrogen. The protease in current study was adequate and acted synergistically with FDE to increase CP liberation.

Table 3.4 Analyzed composition of sunflower meal treated with or without exogenous enzymes on a dry matter basis.

Time[2]	**0h**	**24h**			**48h**		
Treatments[1]	Control 0	Control 1	FDE[1]	FDEP[2]	Control 1	FDE[1]	FDEP[2]
CP%	31.52	31.54	33.52	32.59	29.08	33.88	33.72
Soluble protein%	21.89	15.26	28.00	27.68	16.96	29.53	30.25
Acid detergent protein%	0.86	1.58	1.19	1.05	1.22	0.98	0.91
Neutral detergent protein%	2.61	7.90	2.88	2.05	5.01	3.22	2.05
Acid detergent fiber%	20.85	34.73	25.45	25.40	25.55	20.22	20.82
Neutral detergent fibre%	31.37	47.97	34.79	34.63	34.78	27.24	27.78
Lignin%	7.83	17.98	21.03	12.09	16.78	15.53	16.67
Fat%	13.28	12.81	13.38	11.69	13.06	13.07	12.51
Starch%	1.06	0.64	0.77	0.71	0.64	0.78	0.89
Ash	6.54	6.05	2.38	4.85	4.97	6.19	3.34
CA%	0.22	0.27	0.26	0.27	0.26	0.28	0.29
P%	1.07	0.97	0.95	1	0.96	0.99	1
K%	1.56	1.47	1.49	1.53	1.44	1.54	1.55
Mg%	0.65	0.62	0.63	0.63	0.61	0.63	0.64
Na%	0.02	0.07	0.12	0.11	0.08	0.11	0.11
Cu (ppm)	32.76	32.9	33.9	31.57	33.53	32.2	33.7
Mn (ppm)	28.41	26.62	26.34	27.67	27.25	30.21	27.83
Zn (ppm)	84.14	80.36	78.03	81.42	82.44	80.86	84.49
Fe (ppm)	114.94	119.44	106.76	100.3	141.12	152.54	125.55

[1]FDE-fiber degrading enzymes (1% w/w of cellulase/β-glucanase/mannanase and pectinase), FDEP-FDE plus 1% w/w of protease, and Control- no enzymes added
Enzyme activity were cellulase-45,000u/g, β-glucanase-40,000u/g, mannanase-12,000u/g, pectinase-250,000u/g and protease-1,000,000 u/g
[2]Time- steeped for 24h or 48h
50 g of wheat middlings was mixed with 200 mL of distilled water and 0.5 g of respective enzyme and incubated at 40 °C inside an incubator shaker (Controlled Environment Incubator Shaker, New Brunswick Scientific, Enfield, CT) with continuous agitation of 200rp. The samples were then submitted to a commercial lab for analyses (SGS lab, Guelph, ON, Canada).

The pH and apparent disappearance of NDF, CP, and DM of sunflower meals pre-treated with fiber degrading enzymes (FDE) without or with protease are presented in table 3.5. The digestibility of organic matter (OM) can predict feed intake and metabolizable protein leading to better animal feed formulation (Beecher *et al*., 2015). In the current study, there was a treatment

effect and time effect on AD of DM (P <0.047), but there was no treatment and time interaction effect (P =0.589). The FDE and with protease (FDEP) had greater AD of DM than the control. The lignin content (7.83%) in the sunflower sample used in the current study can explain the DM disappearance differences between enzymes and control as sunflower lignin is resistant even to exogenous enzyme hydrolysis, as reviewed by (Knudsen, 2014). The NDF is made up of hemicellulose, cellulose, and lignin (Van Soest, 1994). In the current study, there was a treatment effect on AD of NDF (P <0.001), with FDE and FDEP being greater than the control, an indication of enzyme efficacy in solubilizing SM fiber, thus promoting AD since most SM fiber is insoluble and is found firmly attached to lignin (Ditta & King, 2017b). There was no time effect or treatment and time interaction effect on AD of NDF, showing that protease did not increase NDF solubilization nor pre-treating beyond 24h. There was a treatment effect on AD of CP with FDEP solubilizing more CP than FDE and control. Protease has been shown to increase protein solubilization when combined with FDE like xylanase in fibrous materials such as maize and wheat distillers' grains with soluble (DDGS) (Pedersen *et al*., 2015). In the current study, there was a time effect with 48h solubilizing more CP than 24h; there was also a treatment and time interaction effect on CP AD with 48h FDE and FDEP at both 24h and 48h solubilizing more CP, an indication that FDE without protease would require more than 24h to solubilize the same CP as FDEP at 24h.

The NSPs are more soluble in high gut pH (Malathi & Devegowda, 2001), and increased solubility of NSP leads to increased gut viscosity and thus low nutrients digestibility (Musigwa *et al*., 2021). Organic acids and SCFA influence the pH during the fermentation of oligosaccharides and monosugars (Broekaert *et al*., 2011b). The pH of the control dropped from 6.13 at 0h to 4.177 at 24h and 4.33 at 48h, an indication of fermentation by endogenous microbial enzymes in the SM.

There was a slight pH increase in the control at 48h, which agrees with other researchers who reported a slight rise in blank pH during fermentation of different fibrous substrates (Karimi *et al*., 2020), which is due to increased enzyme activity (Nawawi *et al*., 2017). The pH of FDE and FDEP was lower than the control (P <0.001), representing high fermentation products such as lactic and SCFA concentration. Time did not affect the pH (P =0.316), but there was time and treatment interaction on the pH, with control at 0 h being higher, followed by the control at 24h and 48h (*P* <0.001).

The effects of FDE with or without protease on mono sugars and organic acids concentration in the supernatant of pre-treated sunflower meal over time are shown in table 3.6. The SM is composed mostly of insoluble NSP (xylan and cellulase) and soluble NSP (pectin-derived compounds) (Ditta & King, 2017b). The xylose, arabinose, and glucose were the main sugars detected. Xylose was higher in FDE without protease (FDE) and FDEP relative to the control (*P* <0.001). Protease did not affect xylose and arabinose concentration as there was no difference between FDE and FDEP. Arabinose was not detected in control. There was no time effect on xylose concentration, but there was an interaction effect between treatment and time, with FDE having more concentration at 24h (*P* =0.015).

There was no time effect on arabinose concentration nor a time and treatment interaction effect (*P* = 0.630). The lack of arabinose and the little amount of xylose (compared to FDE and FDEP) in control is because most of the NSP in SM are insoluble as out of the total (27.6%) NSP, only about 4.5% are soluble (Ditta & King, 2017b; Irish & Balnave, 1993). The soluble NSP (pectin and arabinans) are associated with cotyledon and embryo (Düsterhöft *et al*., 1992).

Table 3. 5 The pH and apparent disappearance (%) of neutral detergent fiber, crude protein, and dry matter of sunflower meal pre-treated with fiber degrading enzymes without or with protease

Item (%)		DM	NDF	CP	pH[1]
Treatments[2]					
Con		20.53[b]	3.40[b]	32.42[c]	4.25[a]
FDE		33.98[a]	26.74[a]	40.97[b]	3.90[c]
FDEP		34.59[a]	25.85[a]	50.27[a]	3.92[b]
SEM		0.64	1.90	1.23	-
Time[3]					
24h		29.50	18.88	34.91[b]	4.01
48h		29.91	18.44	47.54[a]	3.97
SEM		0.52	1.55	1.00	-
Treatment*Time					
C	0h				6.13[a]
Con	24h	19.92[b]	5.31[b]	25.17[d]	4.18[bc]
Con	48h	21.15[b]	1.49[b]	39.68[bc]	4.33[b]
FDE	24h	32.76[a]	26.49[a]	32.80[dc]	3.81[d]
FDE	48h	35.21[a]	26.99[a]	49.15[a]	3.79[d]
FDEP	24h	35.81[a]	24.86[a]	46.76[ba]	4.03[c]
FDEP	48h	33.37[a]	26.85[a]	53.78[a]	3.80[d]
SEM		0.90	2.69	1.74	0.04
P-Value					
Treatment		<0.001	<0.001	<0.001	<0.001
Time		0.589	0.844	<0.001	0.316
Treatment*Time		0.047	0.549	0.046	<0.001

[1]Measured before centrifuging the samples

[2]C- control at zero hours, NDF-neutral detergent fiber, CP-crude protein, DM-dry matter, Con-control after pre-treatment, FDE- fiber degrading enzymes (cellulase/β-glucanase/mannanase and pectinase), and FDEP- FDE with protease

Enzyme activity were cellulase-45,000u/g, β-glucanase-40,000u/g, mannanase-12,000u/g, pectinase-250,000u/g and protease-1,000,000 u/g

[3]steeped either for 24h or 48h

Values within a column without a common superscript differ significantly by LS means at 5% probability.

SEM- standard error of means

50 g of wheat middlings was mixed with 200 mL of distilled water and 0.5 g of respective enzyme and incubated at 40 °C inside an incubator shaker (Controlled Environment Incubator Shaker, New Brunswick Scientific, Enfield, CT) with continuous agitation of 200rpm

In current study, glucose concentration was higher in enzymes treated SM relative to control (P <0.001), time also influenced its concentration, with 48h being greater than 24h (P <0.001). There was also an interaction between time and treatment with both FDE and FDEP concentrations being greater at the 48h (P <0.001). These results are due enzymes activity in breaking down the NSP to free up starch, which is then hydrolyzed into glucose. The data is in agreement with, Malathi & Devegowda, (2001), used FDE to pre-treat SM and observed an increase in glucose and mono sugars release compared to a control. Lactic acid concentration was higher in enzyme steeped SM than in control (P <0.001), resulting from more glucose released by enzymes and easily fermented into lactic acid. There was a time effect on lactic acid concentration with 48 h being greater than 24 h (P <0.001), and a time and treatment interaction effect on lactic acid concentration with FDEP having greater concentration than other treatments at 48h (P <0.001). The NSP in SM are hydrolyzed into mono and oligosaccharides which are fermented into SCFA (Hassaan *et al*., 2018; Kouzounis *let al*., 2021).

In current study, the concentration of acetic, propionic, and butyric acid was all higher in enzyme steeped treatments than in control (P <0.001) and is a result of enzymes hydrolyzing NSP which are eventually fermented into these SCFA (Karimi *let al*., 2020). Time did not influence acetic acid concentration, but there was a time and treatment interaction effect for acetic acid concentration, with FDE at 48h and FDEP at 24h and 48h being the highest (P =0.002). There was a time effect and time and treatment interaction effect on propionic acid concentration, with FDEP at 48 h being the greatest (P <0.001). This could be attributed to the rate of proliferation of Bacteroidetes, which are responsible for propionate production (Louis & Flint, 2017); FDEP might have supported the growth of Bacteroidetes more at 48h than other treatments. Propionic acid was not detected in the control due to a lack of arabinose which influences propionate concentration

(Chen *et alet al*., 2016). The propionic concentration can also be due to FDE releasing negligible amount of fermentable substrates such as glucose especially in sunflower meal which free glucose is negligible. For butyric acid concentration, there was a treatment effect (P <0.001) with FDE and FDEP being higher than the control, and a time effect (P =0.046) with 48h higher than 24h, but no treatment and time interaction (P =0.476). These results can be due to rapid drop of pH (from 6.13 at 0h to 4.25 for the control, 3.798 FDE and 3.918 for FDEP as shown in table 3.5) which prevented conversion of lactate to lactic acid, and it which is eventually converted to either butyric acid or acetic acid (Bourriaud *et al*., 2005; Twomey *et al*., 2003).

The total sugars concentration was greater in enzyme treatment relative to the control (P <0.001) due to hydrolyses of NSP by FDE and FDEP. There was no time effect and time and treatment interaction on total sugar concentration (P ≥0.100) which can be attributed to the rapid fermentation of these sugars and their preceding oligosaccharides into organic acids and SCFA (M. Wang *et al*., 2019). As discussed above, the total organic acid concentration was greatest with enzyme treatments relative to the control (P <0.001), which results from enzymes hydrolyzing NSP into sugars that are eventually converted to organic acids and SCFA. There was a time effect on total organic acids concentrations, with 48h being greater than 24h (P <0.001), there was also time and treatment interaction in total organic acids concentration, with FDEP concentration at 48h being the greatest (P <0.001). There was no treatment, time, or treatment and time interaction effect on iso-butyric acid concentration (P ≥0.145), which is an indication that the solubilized protein by FDE was not fermented since iso-butyric is one of the branched short-chain fatty acids (BCFA) normally recovered at the fecal level. They are formed as a result of protein fermentation (Rios-Covian *et alet al*., 2020).

Table 3. 6 Effects of fiber degrading enzymes with or without protease on mono sugars and organic acids concentration in the supernatant of pre-treated sunflower meal over time

Item (µmol/µL)		Xylose	Arabinose	Glucose	Lactic	Acetic	Propionic	Iso-butyric	Butyric	Total sugars	Total organic acids
Treatments[1]											
Con		0.01^{b}	-	0.003^{c}	0.25^{b}	0.02^{b}	-	0.004	0.007^{b}	0.02^{b}	0.28^{b}
FDE		0.04^{a}	0.02	0.02^{b}	0.38^{a}	0.04^{a}	0.01	0.004	0.020^{a}	0.09^{a}	0.45^{a}
FDEP		0.04^{a}	0.03	0.03^{a}	0.38^{a}	0.05^{a}	0.03	0.004	0.021^{a}	0.09^{a}	0.47^{a}
SEM		0.003	-	0.0003	0.007	0.003	-	0.0002	-	0.003	0.006
Time[2]											
24		0.03	0.02	0.014^{b}	0.31^{b}	0.03	-	0.004	0.015^{b}	0.07	0.37^{b}
48		0.03	-	0.020^{a}	0.37^{a}	0.04	0.02	0.004	0.017^{a}	0.06	0.44^{a}
SEM		0.003	-	0.0002	0.005	0.002	-	0.0002	-	0.002	0.005
Treatments*time											
Con	24	0.01^{c}	0.004^{b}	0.002^{d}	0.25^{d}	0.01^{d}	-	0.004	0.005^{b}	0.02^{b}	0.27^{d}
Con	48	0.01^{c}	-	0.004^{d}	0.24^{d}	0.02^{dc}	0.01^{c}	0.004	0.009^{b}	0.01^{b}	0.28^{d}
FDE	24	0.05^{a}	0.02^{a}	0.02^{c}	0.37^{b}	0.03^{bc}	0.01^{c}	0.004	0.018^{a}	0.09^{a}	0.42^{c}
FDE	48	0.03^{b}	0.02^{ab}	0.03^{a}	0.40^{b}	0.06^{a}	0.01^{c}	0.004	0.022^{a}	0.08^{a}	0.49^{b}
FDEP	24	0.03^{ba}	0.03^{a}	0.02^{b}	0.30^{c}	0.05^{a}	0.027^{b}	0.004	0.021^{a}	0.09^{a}	0.41^{c}
FDEP	48	0.04^{ba}	0.03^{a}	0.03^{a}	0.45^{a}	0.04^{ba}	0.033^{a}	0.004	0.021^{a}	0.09^{a}	0.54^{a}
SEM		0.004	0.004	0.0004	0.01	0.004	0.0006	0.0003	0.001	0.004	0.009
P-values											
Treatments		<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.668	<0.001	<0.001	<0.001
Time		0.145	0.475	<0.001	<0.001	0.059	<0.001	0.145	0.046	0.404	<0.001
Treatments*time		0.015	0.630	<0.001	<0.001	0.002	<0.001	0.528	0.476	0.100	<0.001

[1]Con-control without enzymes, FDE- fiber degrading enzymes (cellulase/ β-glucanase/mannanase and pectinase), and FDEP- FDE with protease Enzyme activity were cellulase-45,000u/g, β-glucanase-40,000u/g, mannanase-12,000u/g, pectinase-250,000u/g and protease-1,000,000 u/g

[2]steeped either for 24h or 48h

Values within a column without a common superscript differ significantly by LS means at 5% probability.

SEM- standard error of means

50 g of wheat middlings was mixed with 200 mL of distilled water and 0.5 g of respective enzyme and incubated at 40 °C inside an incubator shaker (Controlled Environment Incubator Shaker, New Brunswick Scientific, Enfield, CT) with continuous agitation of 200rp

3.4.0 Conclusions

1. Pre-treatment of WM with FDE increased CP solubilisation and apparent disappearance (AD) of crude fiber. The FDE also increased both mono sugars and organic acids concentrations.

2. Pre-treatment of SM with FDE increased CP solubilisation and AD of crude fiber. Addition of protease to FDE increased CP solubilization while it had no effect on AD of crude fibre. The FDE also increased both mono sugars and organic acids concentrations. Additional of protease to FDE increased both concentration of mono sugars and organic acids.

3.4.1 Recommendations

There is need to investigate why pre-treatment of sunflower meal with water only increased NDF relative to untreated one.

Chapter Four: The effect of inclusion of sunflower meal and wheat middlings pretreated with fiber degrading enzymes in maize-soybean meal diets on growth performance, organ weights, and tibia attributes in broiler chicken

Abstract

Pre-treating fibrous feedstuffs with fiber degrading enzymes (FDE) may improve utilization in poultry. The study investigated the impact of the inclusion of pretreated sunflower (TSM) and wheat middling (TWM) in a maize-soybean meal (CSBM) on growth performance and apparent retention (AR) of components. A total of 288 Ross-708 d old male broiler chicks were placed in cages (6 birds/cage) based on body weight (BW) and allocated to 6 diets in a completely randomized design giving 8 replicates per diet. The diets were: 1) PC, a CSBM, positive control (PC), 2) NC, PC plus untreated sunflower meal (USM) and wheat middling (UWM), and 3) 4 test diets in which USM and UWM were replaced with TSM and TWM at 25% (N25), 50% (N50), 75% (N75) and 100% (N100). For pre-treatment, each feedstuff was mixed with 1% of FDE in a ratio of 1:2 wt/wt for feedstuff: water, incubated for 24 hours at 40˚C, and oven-dried at 60-65˚C for 72 hrs before feed preparation. Diets were formulated for two phases (starter, d 0-21) and finisher (d 22-42). The negative control and test diets were formulated to be low nutrient-dense by having a lower AMEn and crude protein compared with PC. The data were analysed using PROC GLIMMIX of SAS 9.4 and Lsmeans separated using the Tukey test ($P<0.05$). Pre-treatment increased soluble protein (SP) by 5.08% and decreased both neutral detergent protein (NDF-P) and neutral detergent fiber (NDF) by 1.33% and 10.5%, respectively, in wheat middlings. Compared to USM, TSM had 3.44% less SP while both NDF-P and NDF were greater by 1.63% and 3.8%, respectively. The BW and BW gain (BWG) of NC were less than PC throughout the study ($P \leq 0.001$). The N75 and N100 BW and BW gain were less than PC ($P \leq 0.001$) throughoutthe

trial. Feed intake was not different during the starter, finisher, and overall period (P ≥0.217) relative to PC and NC. Feed conversion ratios were not different throughout the study (P ≥0.151), except for N75 during the starter phase (P = 0.005) when compared with PC and NC. The NC tibia attributes were not different from PC throughout the trial, also test diets did not affect tibia attributes on d21, but tibia length was lower for... than PC (P = 0.005) and had a linear decrease response with higher inclusion levels (P = 0.004) on d42. In conclusion, although pre-treatment altered fiber and fiber bound protein in test feedstuffs, there was no improvement in growth performance and component retention in broiler chickens.

Keywords: **pre-treatment, broiler, enzymes, performance improvement**

4.0 Introduction

The future growth of the poultry industry will continue to be challenged by health (Hafez & Attia, 2020) and feed cost (Makkar, 2018). At the same time, demand for poultry products will continue to increase especially in developing countries which are expected to have a bigger proportion of the expected world population of 9.1 billion by year 2050 (Alshelmani *et al*., 2021; Makkar, 2018). The cost of feed continues to be of concern as it account for 70-75% of the total cost of poultry production (Mbukwane *et al*., 2022). The shortage of conventional ingredients and their increased cost remains a constant constrain (Adeola & Cowieson, 2011). To alleviate this shortage and lower feed costs, animal nutritionists are continuously seeking for alternative feed resources.

Agricultural byproducts such as sunflower seed meal and wheat processing byproducts are available in abundance and have been explored as the alternative source of protein and energy to replace a portion of soybean meal and maize respectively (Alshelmani *et al*., 2021). The main challenge with the use of non-conventional feedstuffs is the presence of anti-nutritional factors such as non-starch polysaccharides (NSP) (Mbukwane *et al*., 2022). Exogenous feed enzymes such as phytase, carbohydrase and proteases have been widely researched and used as additives to degrade antinutritional factors and other substances that are either under-utilised or not utilised through endogenous enzymes (Adeola & Cowieson, 2011; Godoy *et al*., 2018; Kiarie *et al*., 2016).

Most of available data has focused on studies on the inclusion of a single high fiber ingredient but with the increasing shortage of conventional ingredients, poultry nutritionists, especially in developing countries, will find themselves being forced to include more that one agricultural by-product in feed formulations. The novelty of current study was that two high fiber treated ingredients were included to replace untreated of the same in practical broiler chicken diets, by formulating a negative control (NC) diet with higher neutral and acid detergent fiber compared

with a positive control (PC) and hypothesized that pre-treatment would restore the performance of our test diets to that or better than PC.

4.1 Materials and Methods

The experiment was carried out at the University of Guelph Arkell poultry research station with the approval of University of Guelph animal ethics committee. Broiler chicken care complied with Canadian Code of Practice for the Care and Use of Animals for Scientific Purposes (CCAC, 2009).

4.1.1 Ingredients and pre-treatment

4.1.2. Wheat middlings and sunflower meal

Wheat middlings (UWM) were obtained from Floradale Feed Mill Limited (Floradale, ON, Canada) and sunflower meal (USM) from Persall Fine Foods Co. (Waterford, ON, Canada).

Wheat middlings (TWM) and sunflower meal (TSM) were pre-treated separately by mixing with 1% (of its weight) enzyme complex made of xylanase/cellulase/glucanase for UWM and cellulase/glucanase/mannanase and pectinase for USM, this was then mixed with tap water in the ratio of 1:2 (Water:UWM/USM). Enzyme inclusion rate was based on manufacturer's recommendation. The wet mixture was then incubated for 24 hours at 40°C in the incubator shaker (Controlled Environment Incubator Shaker, New Brunswick Scientific, Enfield, CT) without shaking but was hand mixed using a wooden spatula 3 times within the 24hrs. The pre-treated product (TWM and TSM) was then oven dried at 60-65°C for 3 days. This procedure was repeated until enough material was obtained for experimental diets. Enzymes mixture with target activity of 60,000u/g xylanase, 8,000 u/g glucanase and 38,000 u/g cellulase for UWM and 40,000 u/g glucanase, 45,000 u/g cellulase, 12,000 u/g mannanase and 25,000 u/g pectinase for USM were obtained from Canadian Bio-Systems Inc., Calgary, Canada. The pre-treating conditions were deemed optimum as reported by Rho *et al*., (2020).

4.1.3 Diets formulation

Six dietary treatments were formulated to meet or exceed NRC nutrients requirement for broiler chicken (NRC, 1994). Birds were fed in two phases: starter (0-21 days) and finisher phase (day 21-42). The ingredients nutrients specification were obtained from the INRA feed tables (INRA CIRAD AFZ, 2022). The diets were: Trt 1) PC, a maize-soybean positive control, Trt 2) NC (negative control), PC plus inclusion of untreated sunflower meal (USM) and wheat middling (UWM), and Trt 3-6) 4 test diets (TD) in which USM and UWM were replaced with pretreated sunflower (TSM) and wheat middlings (TWM) at 25% (NC25), 50% (NC50), 75% (NC75) and 100% (N100) as shown in ***Table 4.1***. To account for the nutrients that would be released by pre-treatment (based on assumption both treated and untreated ingredients had similar nutrients composition), the NC and TD were formulated to be low nutrients dense by having a lower AMEn. The Negative control (NC) and test diets metabolizable energy was reduced by 80 Kcal/kg and 150 Kcal/kg in starter and finisher phases, respectively against that of PC. This reduction was done by hypothesizing that pre-treatment would unlock fiber bound nutrients in USM and UWM that would enable the birds utilize them, thus restoring performance to that of PC with NC expected to perform poorly. The diets were formulated based on equal total standardized ileal digestible AA. To meet phosphorous requirements all diets were supplemented with phytase (750 FTU/kg) obtained from CBS Bio platform inc. Alberta, Calgary, Canada.

4.1.4 Birds, housing, and experimental design

A total number of 288 Ross-708-day-old male broiler chicks were purchased from a local hatchery (Maple Leaf Foods, New Hamburg, ON, Canada) weighed and placed in cages (6 birds per cage). The trial was conducted in two phases: starter (0-21 days) and finisher phase (day 21-42). The cages were housed in a temperature and light regulated room. Temperature was maintained at 32°C

for the first 7 days and then reduced gradually by 3°C on weekly basis to 24°C for the first 3 weeks, then maintained at this temperature until the end of experiment while lighting was maintained for 17h the entire trial period. The six diets were allocated to cages in a completely randomized design to give 8 replicates per diet. Birds had free access to feed and water throughout the trial.

4.1.5 Measurements of growth performance and sampling

Feed intake and body weight of the chicken was determined on day 21 and 42 to calculate body weight gain (BWG) and feed conversion ratio (FCR). Mortality was recorded as it occurred and used to calculate corrected FCR. One bird per cage was randomly selected and sacrificed by cervical dislocation on day 21 and 42 for organs weights, cecal content and tibia samples collection.

4.1.6 Sample processing and laboratory analyses

The samples (ingredients and mixed feed) were ground using coffee grinder (CBG5 Smart Grind; Applica Consumer Products, Inc., Shelton, CT) before analysis. Gross energy (GE), AA and fat were determined at the University of Guelph while dry matter (DM), starch, crude protein, neutral detergent protein, soluble crude protein, neutral detergent fiber, acid detergent fiber, ash, calcium and phosphorous were determined in a commercial lab (SGS Canada Inc, Agricultural Services AGRI-FOOD LABORATORIES, Guelph, ON, Canada), Adiabatic bomb calorimeter was used to determine GE (IKA Calorimeter System C 6000; IKA Works, Wilmington, NC). Amino acids were analysed using ultra performance liquid chromatography (UPLC machine, Waters Corporation, Milford , CA) (Kim *et al*, 2022).

4.2.0 Calculations and statistical analyses

The cage was the experimental unit. The data was analyzed using PROC GLIMIXX of SAS 9.4 with diet as the fixed factor in the model.

There linear statistical model used is as shown in equation 2.

$$Y_{ij} = \mu + \alpha + \varepsilon_{ij} \text{.....equation (2)}$$

Where:

Y_{ij} = was observations recorded such BWG, BW, FI, FCR etc.

μ = overall population means,

α = treatment effect

ε_{ij} = random error effect associated with observations recorded

Least Square Means were separated using Tukey test, while pre-planned orthogonal and polynomial contrasts statements were used to compare the performance of NC against PC (PC vs NC) and response of diets containing test feedstuffs. The level of statistical significance was pre-set at $p \leq 0.05$.

Table 4.1. Composition of experimental diets[1], as fed basis

Ingredients, %	Cost/kg	Starter (day 0-21)							Finisher (day 21-42)					
		PC	NC	NC25	NC50	NC75	NC100		PC	NC	NC25	NC50	NC75	NC100
Maize	0.35	63.6	53.2	53.2	53.2	53.2	53.2		66.2	48.9	48.9	48.9	48.9	48.9
Soybean meal 46%	1.12	24.0	16.4	16.4	16.4	16.4	16.4		23.1	12.8	12.8	12.8	12.8	12.8
Wheat middlings	0.28	-	10.0	7.5	5.0	2.5	-		0.0	18.5	13.9	9.3	4.6	-
Treated wheat middlings	0.30	-	-	2.5	5.0	7.5	10.0		0.0	0.0	4.6	9.3	13.9	18.5
Soy oil	1.48	3.0	3.5	3.5	3.5	3.5	3.5		3.3	4.5	4.5	4.5	4.5	4.5
Sunflower meal	0.31	-	7.5	5.6	3.8	1.9	-		-	8.0	6.0	4.0	2.0	-
Treated sunflower meal	0.35	-	-	1.9	3.8	5.6	7.5		-	-	2.0	4.0	6.0	8.0
Fish meal	1.38	1.5	1.5	1.5	1.5	1.5	1.5		-	-	-	-	-	-
Pork meal	0.43	5.0	5.0	5.0	5.0	5.0	5.0		5.0	5.0	5.0	5.0	5.0	5.0
Phtase	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01
L-Lysine HCL	2.86	0.32	0.44	0.44	0.44	0.44	0.44		0.13	0.3	0.3	0.3	0.3	0.3
DL-Methionine	5.52	0.15	0.15	0.15	0.15	0.15	0.15		0.2	0.2	0.2	0.2	0.2	0.2
Threonine	2.95	0.00	0.02	0.02	0.02	0.02	0.02		-	-	-	-	-	-
Tryptophan	18.10	0.00	0.01	0.01	0.01	0.01	0.01		0.00	0.01	0.01	0.01	0.01	0.01
Limestone	0.06	0.46	0.43	0.43	0.43	0.43	0.43		0.37	0.33	0.33	0.33	0.33	0.33
Monocalcium phosphate	1.38	0.74	0.63	0.63	0.63	0.63	0.63		0.61	0.42	0.42	0.42	0.42	0.42
Sodium chloride	0.23	0.40	0.44	0.44	0.44	0.44	0.44		0.32	0.32	0.32	0.32	0.32	0.32
Vitamin and trace minerals	2.86	0.50	0.50	0.50	0.50	0.50	0.50		0.50	0.50	0.50	0.50	0.50	0.50
Titanium dioxide		0.30	0.30	0.30	0.30	0.30	0.30		0.30	0.30	0.30	0.30	0.30	0.30
TOTAL		**100**	**100**	**100**	**100**	**100**	**100**		**100**	**100**	**100**	**100**	**100**	**100**
Cost/tonne US$		623.4	564.3	565.6	567.2	568.1	569.4		601.4	522.7	524.6	526.8	528.4	530.3
Calculated nutrients														
AMEn, mcal/kg		2.96	2.88	2.88	2.88	2.88	2.88		2.99	2.84	2.84	2.84	2.84	2.84
CP, %		20	19.5	19.5	19.5	19.5	19.5		18.7	18.07	18.07	18.07	18.07	18.07
SID Lys, %		1.22	1.19	1.19	1.19	1.19	1.19		0.99	0.96	0.96	0.96	0.96	0.96
SID Met, %		0.45	0.40	0.40	0.40	0.40	0.40		0.48	0.46	0.46	0.46	0.46	0.46
SID Met + Cys, %		0.75	0.75	0.75	0.75	0.75	0.75		0.77	0.72	0.72	0.72	0.72	0.72
SID Thr, %		0.88	0.67	0.67	0.67	0.67	0.67		0.86	0.84	0.84	0.84	0.84	0.84
SID Trp, %		0.20	0.16	0.16	0.16	0.16	0.16		0.18	0.18	0.18	0.18	0.18	0.18
Ca, %		0.96	0.96	0.96	0.96	0.96	0.96		0.84	0.84	0.84	0.84	0.84	0.84
Available P, %		0.48	0.48	0.48	0.48	0.48	0.48		0.42	0.42	0.42	0.42	0.42	0.42
Na, %		0.207	0.16	0.16	0.16	0.16	0.16		0.16	0.16	0.16	0.16	0.16	0.16
Cl, %		0.285	0.16	0.16	0.16	0.16	0.16		0.21	0.23	0.23	0.23	0.23	0.23

[1]PC; maize-soybean based positive control; NC, PC plus untreated sunflower meal (USM) and untreated wheat middling's (UWM); N25, NC with USM & UWM replaced with 25% of treated sunflower meal (TSM) and 25% of treated wheat middling's (TWM), respectively; N50, NC with USM & UWM replaced with 50% of TSM and 50% of TWM, respectively; N75, NC with USM & UWM replaced with75% TSM and 75% TWM, respectively; N100, NC with USM & UWM replaced with 100% TSM and 100% of TWM, respectively.[2]Provided per kg of premix: vitamin A (retinol), 880 KIU; vitamin D_3 (cholecalciferol), 330 KIU; vitamin E, 4,000 IU; vitamin K_3 (menadione), 330 mg; vitamin B_1 (thiamin), 400 mg; vitamin B_2 (riboflavin), 800 mg; vitamin B_3 (niacin), 5,000 mg; vitamin B_5 (pantothenic acid), 1,500 mg; vitamin B_6 (pyridoxine), 300 mg; vitamin B_9 (folic acid), 100 mg; vitamin B_{12} (cyanocobalamin), 1200 mcg; biotin, 200 mcg; choline, 60,000 mg; Fe, 6000 mg; Cu, 1000 mg; I, 1 mg, Se, 30 mg.

4.3.0 Results and Discussion

4.3.1 Ingredients and diets

The ingredients analyses results are presented in Table 4.2. Pre-treated sunflower meal (TSM) had 3.96% more CP but 3.44% less soluble crude protein (SP), 3.8% more neutral detergent protein (NDF-P) and 1.63% more NDF compared to USM on as is basis. For TWM, CP was 1.71% more, 5.08% more soluble crude protein but 1.33 % less NDF-P, 10.5% less NDF and 1.99% less ADF compared with UWM on as is basis. Treatment diets analysis results are in ***Table 4.3***. Compared with PC, NC had 4.39% more NDF in starter diet and 7.53% more NDF in finisher diet.

Processing of ingredients at high temperature affects nutritive value of feedstuffs (Anderson-Hafermann *et al*., 1993; Lea & Hannan, 1949). Also, moisture content of any material being processed even at moderate temperature can affect the nutritional value of the feed stuff (Lea & Hannan, 1949). Nutrients affected by heat are products such as volatile acids, energy and protein (Zhang *et al*., 2022). Prolonged period of processing feeds at low temperature may have the same effect as processing the same at high temperature (Teodorowicz *et al*., 2018). In animal feed/ingredients, the effects of Maillard reaction on the final product are measured using reactive lysine (Boucher *et al*., 2009; Carpenter, 1960) or nutrients digestibility (Goering *et al*., 1972; Van Soest & Mason, 1991). Feed ingredients with advanced Maillard reactions have lysine that cannot be regenerated in the gut and cannot be utilized (Almeida, 2013) thus affecting performance.

Table 4.2 Analysed composition of enzyme treated and untreated ingredients, as fed basis

Item	sunflower meal		wheat middlings		Maize	Soybean meal	Fish meal	Pork meal
Ingredient	Untreated	Treated	Untreated	Treated				
Dry matter, %	90.7	92.4	87.8	87.6	86.1	88.1	92.3	93
Gross energy, kcal/kg	4550	4815	4126	4197	3913	4236	4088	4593
Starch	0.97	0.93	16.47	17.04	55.51	1.64	0.14	0.75
Crude fat, %	12.2	10.4	2.60	3.93	1.70	1.08	3.57	11.5
Crude protein, %	26.05	30.01	17.03	18.74	7.73	45.3	60.6	56.9
Neutral detergent protein, %	2.39	4.02	3.84	2.51	0.91	3.59	-	-
Soluble crude protein, %	20.07	16.63	7.92	13.0	1.40	8.45	-	-
Neutral detergent fiber, %	26.6	30.4	35.5	25.0	8.0	6.1	32.6	32.8
Acid detergent fiber, %	19.1	22.0	11.9	9.91	1.92	<0.10	-	-
Ash, %	6.00	4.40	5.09	5.10	1.05	5.59	24.1	18.8
Calcium, %	0.26	0.28	0.35	0.67	0.10	0.21	7.97	6.12
Phosphorous, %	0.94	1.09	1.09	1.23	0.31	0.79	4.28	3.10
Indispensable AA								
Arg	2.28	1.89	0.95	1.38	0.40	3.69	3.93	4.15
His	0.79	0.73	0.33	0.58	0.15	1.43	1.82	1.76
Ile	1.21	1.38	0.62	0.64	0.32	2.37	2.90	2.39
Leu	1.95	1.89	1.04	1.25	1.10	4.21	5.12	4.65
Lys	1.13	0.97	0.35	0.78	0.29	3.13	4.58	3.16
MET	0.72	0.26	0.28	0.97	0.17	0.76	1.93	1.24
Phe	1.46	1.34	0.66	0.89	0.46	2.97	3.00	3.07
Thr	1.09	0.98	0.60	0.65	0.33	1.96	2.73	2.20
TRP	0.41	7.06	0.21	0.58	0.08	0.82	1.35	13.68
Val	1.44	1.63	0.89	0.91	0.42	2.40	3.44	3.23
Dispensable AA								
Ala	1.34	1.57	0.97	1.04	0.66	2.30	4.22	4.61
Asp	2.88	2.95	1.45	1.60	0.76	6.30	6.53	4.91
CYS	0.56	0.58	0.39	0.44	0.18	0.75	0.63	0.56
Glu	5.69	5.75	3.61	3.68	1.72	9.83	8.59	7.83
Pro	1.40	1.58	1.33	1.38	0.78	2.89	3.39	5.30
Tyr	0.65	0.37	0.15	0.35	0.10	1.57	2.04	1.83
Ser	1.29	1.13	0.79	0.89	0.46	2.62	2.75	2.38
Total AA	26.29	32.07	14.61	18.02	8.38	50.00	58.96	66.94

Table 4.3. Analysed composition of experimental diets[1], as fed basis

	Starter							Finisher					
Item[1]	PC	NC	NC25	NC50	NC75	NC100		PC	NC	NC25	NC50	NC75	NC100
Dry matter, %	88.5	90.3	88.5	88.4	88.4	88.4		88.4	88.3	89.3	88.5	88.1	87.7
Gross energy, Mcal/kg	4.07	4.25	3.97	4.21	4.18	4.13		4.06	4.19	4.35	4.21	4.25	4.24
Poultry ME (Mcal/kg)	2.96	2.91	2.79	2.72	2.67	2.87		2.99	2.81	2.86	2.81	2.80	2.75
Starch, %	39.8	35.4	32.6	31.9	31.6	34.0		41.6	33.1	32.9	31.9	31.3	30.4
Crude protein, %	20.7	20.0	20.5	19.9	19.8	20.2		19.4	18.3	18.9	19.0	19.2	18.9
Crude fat, %	4.02	6.05	7.15	7.16	6.14	7.73		4.93	7.94	8.08	8.05	8.28	8.31
Neutral detergent fiber, %	7.41	11.8	12.2	11.5	11.8	11.3		7.37	14.9	14.8	14.8	12.1	13.1
Ash, %	5.38	5.75	5.82	5.84	5.87	5.86		4.65	5.22	5.03	5.29	5.21	5.40
Calcium, %	0.85	0.79	0.83	0.82	0.77	0.77		0.66	0.57	0.57	0.61	0.55	0.62
Phosphorous, %	0.68	0.73	0.75	0.74	0.72	0.76		0.56	0.68	0.70	0.70	0.68	0.71

[1]PC; maize-soybean based positive control; NC, PC plus untreated sunflower meal (USM) and untreated wheat middling's (UWM); N25, NC with USM & UWM replaced with 25% of treated sunflower meal (TSM) and 25% of treated wheat middling's (TWM), respectively; N50, NC with USM & UWM replaced with 50% of TSM and 50% of TWM, respectively; N75, NC with USM & UWM replaced with75% TSM and 75% TWM, respectively; N100, NC with USM & UWM replaced with 100% TSM and 100% of TWM, respectively

Enzymatic hydrolyses of cellulose and hemicellulose yields glucose from cellulose and several pentoses and hexoses from hemicellulose (Taherzadeh & Niklasson, 2004). Maillard reaction is a reaction between reducing sugar and amino acids such as lysine and the product of this process depends on processing temperature (Hofmann *et al*., 2004). In the current study, maillard reactions products were not tested for and therefore cannot authoritatively conclude whether it occurred although the treated materials had a lot of browning and great aroma emanating from them. However, oven drying of the pre-treated ingredients (though at moderate heat 60-65◦C) affected their nutritional quality negatively as evident by the TSM having 17.04% less SP and 40.54% more NDF-P relative to USM. For TWM, its high CP, more SP, less NDF-P, less NDF and less ADF compared with UWM in as is basis shows that pre-treatment improved the quality of WM. Although, current study did not measure maillard reaction, oven drying might have caused mallard reaction based on TSM having 3.8% more NDF relative to USM (table 4.2) and it has been reported that products of maillard reaction are picked as NDF during laboratory analysis of NDF (Oliveira *et al*., 2021).

3.2 Growth performance

The growth performance results are shown in Table 4.4. Compared with PC, the NC and test diets (TDs) significantly reduced the BW during the starter (P=<0.0001) and finisher (P≤0.0001) phases. The BWG was also lesser during the starter (P=<0.0001), finisher (p= <0.0001) and the overall performance (P= <0.0001). The TDs also showed a linear BW and BWG (P= <0.0001) response with increased inclusion rate of pre-treated ingredients during the starter (0-21), finisher (d21-42) and overall performance (d0-42). The BW of N75 and N100 were significantly lower than PC during the starter (p=0.0001) and finisher (P= <0.0001), their BWG were also significantly lower than PC during the starter (p= 0.0001), finisher (P= 0.0001) and their overall performance

(p= <0.0001). There was no FI and FCR difference (P>0.05) between PC and NC. Test diets FI and FCR were not significantly different from NC and PC with only N75 FCR being significantly (P=0.005) greater than PC during the starter phase.

Use of feed enzymes to pre-treat feeds and ingredients is gaining interest with inconsistent results. Svihus *et al*., (1997) fed broiler chicks with barley treated with a mixture of protease, xylanase and β-glucanase and reported improved BWG and FCR. In another trial, broilers were fed xylanase treated brewers spent grain and they showed an increased FI with no benefit on BWG and FCR (Denstadli *et al*., 2010). Saenphoom *et al*., (2013) conducted a trial where broilers were fed with enzymatically treated palm kernel expeller (PKE), dried at 75˚C and the performance of enzyme treated and enzyme supplemented PKE were lower compared with a positive control without PKE inclusion. In a fish experiment where a semi-solid mixture of sunflower-soybean meals and sunflower-rapeseed meals were pre-treated with a mixture of β-glucanase, hemicellulase and pectinase at 45˚C for 60 minutes and separately dried and then used to partially replace fish meal in rainbow trout diets, there was decreased growth and increased FCR compared with controls (Denstadli *et al*., 2010).

In another trial where broiler chicks were fed enzymatically fermented peas at 30% inclusion rate, there was reduced BWG and FI but a better FCR compared with a control (Boroojeni *et al*., 2017). The authors explained that the better FCR might be due to reduced feed intake, reduction of anti-nutritional factors (ANF) or due degradation effect of enzymes on complex nutrients. Similarly, Feng *et al*., (2020) showed that replacing maize partially with 5% of wheat bran fermented with xylanase producing microorganisms had no effect on finisher phase (d21-42) of broiler chicken but the diets led to increased intestinal microflora.

Table 4. 4. Growth performance of broiler chickens fed maize-soybean meal-based diets with pre-treated sunflower meal and wheat middlings

	Treatments[1]						SEM	Overall	Response of		Contrast
Item	PC	NC	N25	N50	N75	N100		*P*-value	Linear	Quadratic	PC vs. NC
Body weight (g/bird)											
d 0	42.7	42.8	42.6	43	42.4	43	0.17	0.178	0.553	0.301	0.391
d 21	814.3^{a}	774.6^{ba}	767.2^{ba}	768.3^{ba}	707.2^{b}	706.1^{b}	16.3	0.0001	<0.0001	0.296	<0.0001
d 42	2,609.1^{a}	2,462.0^{ba}	2,377.3^{bac}	2,393.8^{bac}	2,228.2^{bc}	2,161.2^{c}	61.6	<0.0001	<0.0001	0.359	<0.0001
Body weight gain (g/bird)											
d 0-21	771.6^{a}	731.7^{ba}	724.6^{bac}	725.4^{bac}	664.9^{bc}	663.1^{c}	16.2	0.0001	<0.0001	0.300	<0.0001
d 21-42	1,789.6^{a}	1,687.4^{a}	1,614.0^{ba}	1,625.4^{ba}	1,518.1^{bc}	1,449.0^{c}	46.7	0.0001	<0.0001	0.463	<0.0001
d 0-42	2,561.1^{a}	2,419.2^{a}	2,338.6^{ba}	2,350.8^{ba}	2,183.0^{bc}	2,112.1^{c}	52.8	<0.0001	<0.0001	0.334	<0.0001
Feed intake (g/bird)											
d 0-21	1,100.8	1,118.7	1,084.1	1,106.9	1,095.6	1,061.4	24.0	0.635	0.157	0.364	0.253
d 21-42	3,054.7	3,038.2	2,772.4	3067.3	2,872.3	2,709.3	128.5	0.217	0.036	0.528	0.064
d 0-42	4,155.4	4,156.9	4,174.2	3967.9	3,770.8	3,856.5	142.9	0.213	0.303	0.558	0.147
FCR											
d 0-21	1.43^{b}	1.54^{ba}	1.50^{ba}	1.52^{ba}	1.67^{a}	1.60^{ba}	0.04	0.005	0.081	0.101	0.005
d 21-42	1.71	1.80	1.73	1.89	1.94	1.94	0.10	0.409	0.303	0.486	0.116
d 0-42	1.62	1.72	1.72	1.73	1.72	1.81	0.05	0.151	0.039	0.48	0.181

SEM-standard error of means, BW-Body weight, BWG-Body weight gain, FI-feed intake, FCR-feed conversion ratio.

[a]Values within a row without a common superscript differ significantly by LS means at 5% probability.

[1]PC; maize-soybean based positive control; NC, PC plus untreated sunflower meal (USM) and untreated wheat middling's (UWM); N25, NC with USM & UWM replaced with 25% of treated sunflower meal (TSM) and 25% of treated wheat middling's (TWM), respectively; N50, NC with USM & UWM replaced with 50% of TSM and 50% of TWM, respectively; N75, NC with USM & UWM replaced with75% TSM and 75% TWM, respectively; N100, NC with USM & UWM replaced with 100% TSM and 100% of TWM, respectively.

In the current study, the BW of PC was greater than NC throughout the study (P≤0.001). The PC *vs* NC BWG was also greater throughout the study (P=<0.001). The N75 and N100 BW and BWG were lesser than PC (P<0.001) (Table 4.4). The test diets (TD) BW and BWG had a linear reduction with increased inclusion rate of pre-treated ingredients. The NC and TDs FI and FCR were not different (P>0.05) from PC despite the BW and BWG being lesser. The reduction in BW and BWG, especially at high inclusion rate of pre-treated ingredients, can partly be attributed to reduced soluble protein in TSM. While the laboratory analysis of TWM shows that it was superior to UWM, the performance of birds on test diets was less than that of NC, meaning that the TWM extra SP and energy (NDF was lesser than UWM) were unavailable/unutilised by the broiler chicken. This also shows that the current processing method had a negative effect on nutrients availability to the birds whereby starter, finisher phase and the overall (d0-42) growth performance response, linearly decreased with the increased inclusion rate of treated materials with N75 and N100 performing poorly.

Also, these diets were low in energy compared with PC and the expectation was for them to respond by increasing FI to compensate for the lower energy but there seems to be physiological limitation on the bird's gut system to the amount they could consume. The observed FI can also be due to increased solubilisation of fiber by FDE used which might have increased gut volume and reduced the gut emptying rate. This agrees with Tejeda & Kim, (2020) who observed similar FI between control and a diet containing 8% crude fiber despite the two being isonitrogenous and isocaloric. Diets with soluble fiber increases gut viscosity leading to reduced feed movement rate and reducing performance (Kiarie *et al*., 2014; Perera *et al*., 2019).

Dietary fiber (NSP), reduces BWG and increases FCR as there is a reduced interaction between endogenous enzymes and nutrients (Ayres *et al*., 2019; Graham & Åman, 1991), it also increases

intestinal viscosity, reduces feed gut transit rate and finally reduces FI and performance (Jiménez-Moreno *et al*., 2010). A study by Jiménez-Moreno *et al*.,(2010) showed that the high fiber in their diets was only reducing FI between d 1-12 of age and the birds later on recovered. This observation contrasts the current study where the chicks did not show any FI change from d1-21 and d21-42 and this discrepancy might show that the fiber in current diets had more water holding capacity and might have swelled thus decreasing the gut capacity and voluntary FI both in starter and finisher phases.

Although non-significant N75 and N100 consumed 9.29% (d21-42) and 10.82% (d0-42) less compared with NC respectively and this explain why their BWG was different ($P \leq 0.001$) from NC during these feeding phases, they also consumed 9.26% (d21-42) and 11.31% (d0-42) less compared with PC respectively and this might have contributed to the different BWG observed between these test diets and PC. The N75 starter FCR was significantly ($P=0.005$) greater than PC, which could not be explained. Another possibility that might have led to decreased performance in N75 and N100 is the high level of solubilized xylose, as TWM and TSM had 59.44 and 29.40 (µmol/ml) of xylose respectively. Xylose has been shown to reduce broilers chicken performance, as it is directly absorbed in the small intestines where it affects glucose and lipid utilization by the liver (Regassa *et al*., 2017b). The mono sugar would only be beneficial to the broiler chicken if fermented to organic acids in the ceca.

Most of pre-treatment studies have been done using microbiota ferment agricultural by-products and feeding the products in wet unprocessed form which have yielded mixed results. For example, Chiang *et al*. (2009) and Ashayerizadeh *et al*. (2018) showed that feeding broiler chicken fermented rapeseed meal increased BWG and reduced FCR. When Zhang *et al*. (2022) replaced maize with either 25g/kg or 50g/kg of wet *Lactobacillus acidophilus*, *Bacillus subtilis* and

Saccharomyces cerevisiae fermented wheat bran, there was no effect on performance compared with control. Fermentation increased CP (1.88%) while at the same time decreasing antinutritional factors and some toxic substances in feeds (Zhang *et al*., 2022). These studies show fermentation helps to improve the quality of ingredients by reducing the amount of soluble NSP thus improving broilers performance.

The starter negative control (NC) had more crude fiber than PC but there was no significance BW and BWG difference between PC, NC, N25 and N50. This shows that fiber inclusion level did not have much effect on performance. These results agree with (Hetland & Svihus, 2001; Hetland *et al*., 2002)). Who showed that performance in broiler and layer chicken is not affected by inclusion of ingredients in high fiber at moderate (40-100g/kg) levels in their diets.

Phytase and xylanase work synergistically to hydrolyse antinutritional factors in pigs (Kiarie *et al*., 2010; Kim *et al*., 2008) and broilers (Kiarie *et al*., 2014) with xylanase hydrolysing NSP and phytase working on phytate thus preventing the interaction of phytate with protein at the same time liberating bound phosphorus (Zijlstra *et al*., 2010). In the current study, phytase was added to all diets in equal amount (0.01%) to ensure they met phosphorous requirement in additional to monocalcium phosphate. Since NC and test diets were high in NDF and lower in ME compared with PC, phytase was also added to PC to prevent it from becoming another variable that would influence results other than the pre-treated ingredients. It's possible to reduce broiler crude protein with up to 3% from the recommended inclusion (21%) without affecting performance but the diet must be formulated based on equal total or digestible amino acids (Namround *et al*., 2008; Award *et al*., 2014). It was hypothesized that enzymatic pre-treatment would release nutrients trapped by fiber thus restoring the energy balance. To alleviate amino acids, diets were formulated based on equal and SID AA (from INRA database).

In the current study, NC and test diets had 16.35% maize and 31.67% of soybean being replaced with cheaper SM and WM and the mean performance of NC, N25 and N50 was not significantly different ($p>0.05$) compared with PC which shows that use of NC and our test diets up to N50 would save costs to a farmer. The cost of starter diets in USD/tonne were PC 623.4, NC 564.3, N25 565.6, N50 567.2, N75 568.1, N100 569.4 and finisher diets PC 601.4, NC 522.7, N25 524.6, N50 526.8, N75 528.4, N100 530.3 (these were current prices in Kenyan at the time of the study), treated ingredients were given a 10% premium value more than the untreated. Compared with PC, the test diets were the cheapest throughout the study ($P \leq 0.003$), The N50 BWG/kg FI was cheaper during the starter phase ($P = 0.020$), NC and N25 were cheapest during the finisher phase and overall study period (d0-42) ($P \leq 0.001$) cost/kg FI performance of NC, N25 and N50 throughout the trial were not different ($P>0.05$) (Table 4.5). Therefore, NC and N25 can be economically integrated into the feed formulation for the BW gain comparable to the current study PC. The by-products used in the current study are also readily available all year round and in large quantities in most developing countries (Alshelmani *et al*., 2021; Mbukwane *et al*., 2022).

While the efficacy of any feed enzyme is based on performance data obtained from feeding trial (Aftab & Bedford, 2018), it's of importance to note that pre-treatment has been shown to improve digestibility in *invitro* studies. For example, pre-treatment of maize-soybean based diets with probiotics and consequently oven drying and pelleting them has been shown to work and improve growth performance (Yeh *et al*., 2018). Therefore, more research is needed to develop an optimum heat processing standards for different pre-treated non-conventional ingredients as our study has suggested that oven drying of TSM and TWM at 60-65˚C reduces their nutritional value thus affecting broilers performance.

Table 4. 5. The economic analysis of cost of feed intake and cost of body weight gain per kilogram of FI

Treatments[1]	Starter	Finisher	overall	Starter Feed cost	Finisher feed cost	Overall Feed cost	Final BW, kg	Feed cost/ kg live BW
	FI, kg/bird	FI, kg/bird	FI, kg/bird	$/bird	$/bird	$/bird	Kg/bird	
PC	1.10	3.05	4.16	0.69^{a}	1.84^{a}	2.52^{a}	2.56^{a}	0.97
NC	1.12	3.04	4.16	0.63^{ba}	1.59^{ba}	2.22^{ba}	2.42^{a}	0.90
N25	1.08	2.77	4.17	0.63^{b}	1.61^{ba}	2.23^{ba}	2.34^{ba}	0.93
N50	1.11	3.07	3.97	0.62^{b}	1.51^{b}	2.13^{b}	2.35^{ba}	0.89
N75	1.10	2.87	3.77	0.60^{b}	1.43^{b}	2.03^{b}	2.18^{bc}	0.92
N100	1.06	2.71	3.86	0.62^{b}	1.47^{b}	2.09^{b}	2.11^{c}	0.97
SEM	0.02	0.13	0.14	0.14	0.07	0.08	52.8	0.03
P- value	0.635	0.217	0.213	0.003	0.002	<0.001	<0.0001	0.284

Prices were obtained from the Kenyan market on 21-march-2022
SEM-standard error of means
Values within a column without a common superscript differ significantly by LS means at 5% probability.
[1]PC; maize-soybean based positive control; NC, PC plus untreated sunflower meal (USM) and untreated wheat middling's (UWM); N25, NC with USM & UWM replaced with 25% of treated sunflower meal (TSM) and 25% of treated wheat middling's (TWM), respectively; N50, NC with USM & UWM replaced with 50% of TSM and 50% of TWM, respectively; N75, NC with USM & UWM replaced with75% TSM and 75% TWM, respectively; N100, NC with USM & UWM replaced with 100% TSM and 100% of TWM, respectively.

4.3.3 Organ weights

The spleen, bursa and gizzard weights were not significantly different ($p>0.05$) while small intestines showed a linear response on day 21. There was no difference in finisher small intestines, gizzard, liver and spleen while NC breast muscle was heavier than PC. Results are shown in ***Table 4.6***.

Broilers' chicken gut system respond very quickly depending on the structure and composition of the diet (Svihus, 2011). Mateos *et al*.,(2012) and Tejeda & Kim,(2020) showed that dietary fiber affects size of gizzard and small intestines, this being an adaptation to the increased volume of

contents inside them (Rezaei *et al*. 2018). This agrees with current study where the small intestines length showed a linear increased response on day 21. The NC and TDs were high in crude fiber compared with PC.

The liver has lipogenic and anti-toxigenic activity. In current study, liver relative weight linearly increased with inclusion of pre-treated materials, highest being at N100 during starter phase, which shows that the pre-treated ingredients had an effect beyond the gut, and this can be due to high concentration of xylose in the diet which has been shown to affect liver hepatic cells (Regassa *et al*., 2017b).

The N75 finisher breast weight was significantly lower ($p=0.044$) and the reason for this could not be explained.

Table 4. 6. Organ weights (% body weight) in broiler chickens fed maize-soybean meal-based diets with pre-treated sunflower meal and wheat middlings

Item	Treatments[1]							overal	Response of treated feedstuffs		contrast
	PC	NC	N25	N50	N75	N100	SE	P-value	Linear	Quadratic	PC vs. NC
d 21											
Gizzard	2.16	2.13	2.43	2.16	2.05	2.33	0.13	0.379	0.101	0.411	0.621
Liver	2.64^{b}	2.65^{ba}	2.86^{ba}	2.69^{ba}	2.42^{b}	3.14^{a}	0.16	0.074	0.017	0.059	0.247
Spleen	0.09	0.09	0.09	0.10	0.08	0.10	0.01	0.720	0.389	0.331	0.582
Bursa	0.27	0.26	0.32	0.29	0.28	0.34	0.03	0.397	0.041	0.438	0.688
Small	3.60	3.43	4.08	4.04	3.72	4.33	0.22	0.056	0.003	0.594	0.423
d 42											
Gizzard	1.25	1.36	1.53	1.48	1.29	1.44	0.07	0.073	0.010	0.486	0.423
Liver	2.45	2.31	2.45	2.32	1.82	2.28	0.18	0.142	0.949	0.164	0.481
Spleen	0.12	0.13	0.16	0.11	0.14	0.13	0.02	0.473	0.698	0.790	0.230
Small	2.15	2.27	2.55	2.27	2.33	2.22	0.15	0.520	0.370	0.316	0.123
Breast	20.3^{b}	24.02	21.54^{b}	21.54^{b}	18.53	20.0^{b}	1.17	0.044	0.563	0.678	0.360

SEM-standard error of means

Values within a row without a common superscript differ significantly by LS means at 5% probability.

[1]PC; maize-soybean based positive control; NC, PC plus untreated sunflower meal (USM) and untreated wheat middling's (UWM); N25, NC with USM & UWM replaced with 25% of treated sunflower meal (TSM) and 25% of treated wheat middling's (TWM), respectively; N50, NC with USM & UWM replaced with 50% of TSM and 50% of TWM, respectively; N75, NC with USM & UWM replaced with75% TSM and 75% TWM, respectively; N100, NC with USM & UWM replaced with 100% TSM and 100% of TWM, respectively.

4.3.4 Tibia attributes

Results of tibia characteristics are shown in ***Table 4.7***. Nutrient's density in diets is the main determinant for the growth and development of bones and any change in feed composition might affect these processes leading to locomotion disorders (Güz *et al*., 2019). Bone strength is determined by the degree of mineralization as supported by the data from Boivin & Meunier,(2002). Of late, leg problems have been the main challenge affecting broiler health and welfare issues (Authority, 2010). According to Julian, (1998) and (Dibner *et al*., 2007), tibia is the bone that bears most of the load hence its prone to disorders that affects locomotion.

In the current study, tibia length, diameter, relative weight and percentage ash were not different among all the treatments during the starter phase. This shows that available phosphorous was similar in all our diets as phosphorous plays the major role during bone mineralization (Fernandez *et al*., 2019).

During finisher phase, N75 and N100 Tibia length and diameter were significantly (P=0.005 and P= 0.01 respectively) lower than PC. As the PC BW was higher than the N75 and N100, the difference in length and diameter can be due to allometric growth. N75 tibia relative weight was significantly lower (p= 007) from PC, and this could not be explained. The relative weight of a bone is a better indicator of mineralization as opposed to its ash content (Li *et al*., 2015). There was no difference in ash content in the current trial.

Table 4. 7. Tibia characteristics in broiler chickens fed maize-soybean meal-based diets with pre-treated sunflower meal and wheat middlings

Length	Treatments[1]						SEM	Overall	Response of treated		contrast
	PC	NC	N25	N50	N75	N100		P-value	Linear	Quadratic	PC *vs.* NC
d 21											
Length, inch	2.83	2.87	2.86	2.79	2.78	2.86	0.04	0.525	0.746	0.253	0.965
Diameter, inch	0.24	0.23	0.23	0.23	0.22	0.23	0.01	0.824	0.519	0.264	0.686
Weight, %BW	0.27	0.29	0.32	0.27	0.26	0.31	0.26	0.075	0.033	0.182	0.817
Ash, %	59.7	56.8	58.3	59.7	61.2	59.4	1.54	0.469	0.913	0.812	0.623
d 42											
Length, inch	4.14^{a}	4.12^{ba}	4.05^{ba}	4.05^{ba}	3.92^{b}	3.92^{b}	0.05	0.005	0.004	0.598	0.023
Diameter, inch	0.39^{a}	0.38^{ba}	0.37^{ba}	0.35^{ba}	0.36^{b}	0.35^{b}	0.01	0.010	0.003	0.105	0.129
Weight, %BW	0.35^{a}	0.33^{ba}	0.36^{a}	0.32^{ba}	0.29^{b}	0.31^{ba}	0.01	0.007	0.623	0.201	0.012
Ash, %	47.4	48.5	46.1	45.2	46.6	47.1	0.88	0.162	0.184	0.190	0.398

SEM-standard error of means

Values within a column without a common superscript differ significantly by LS means at 5% probability.

[1]PC; maize-soybean based positive control; NC, PC plus untreated sunflower meal (USM) and untreated wheat middling's (UWM); N25, NC with USM & UWM replaced with 25% of treated sunflower meal (TSM) and 25% of treated wheat middling's (TWM), respectively; N50, NC with USM & UWM replaced with 50% of TSM and 50% of TWM, respectively; N75, NC with USM & UWM replaced with75% TSM and 75% TWM, respectively; N100, NC with USM & UWM replaced with 100% TSM and 100% of TWM, respectively.

4.4.0 Conclusions

Inclusion of enzymatically pre-treated sunflower meal and wheat middlings in a maize-soybean meal broiler chicken diets,- did not improve the growth performance, organ weights and tibia attributes of broiler chickens.

4.4.1 Recommendations

There is need to investigate whether inclusion of enzymatically pre-treated sunflower meal and wheat middlings, in a maize-soybean diets have any negative biochemical/physiological effect, which reduced the growth performance of broiler chicken.

Chapter Five: Effects of inclusion of sunflower meal and wheat middlings pre-treated with fiber degrading enzymes on apparent retention of components and concentration of mono-sugars and short chain fatty acids in ceca digesta of broiler chickens

5.0 Abstract

The effects of including fiber degrading enzymes pre-treated sunflower meal (TSM) and wheat middlings (TWM) on apparent retention (AR) of components, and ceca digesta concentration of sugars and short-chain fatty acids in broiler chickens was investigated. Diets and experimental design are as in chapter 4. All diets had titanium dioxide (0.3%) for AR determination. Birds had free access to feed and water. Excreta was collected and frozen (-20°C) per cage from d18 through to d20 for starter phase and from d39 to d41 for finisher phase. Relatively to PC, on day 21, the AR of NC crude protein (CP) and AMEn was not different ($P \geq 0.133$), NDF was greater ($P = 0.015$), GE and DM were lesser ($P \leq 0.029$). The NC excreta moisture was lesser than PC ($P = 0.033$). Relatively to PC, the test diets AR of CP and DM was not different ($P \geq 0.248$), NDF was greater ($P < 0.001$), GE was lesser ($P < 0.001$) while the AR of AMEn showed a linear decreased retention with N100 being the lowest ($P = 0.027$). The test diets excreta moisture was not different relative to PC ($P = 0.163$). On day 42, relatively to PC, the NC and test diets AR of CP, NDF was greater ($P < 0.001$), DM and AMEn were lesser $P \leq 0.024$). NC and test diets excreata moisture was not different from PC ($P \geq 0.215$), compared to PC, on day 21, NC and test diets cecal concentration of mono sugars and organic acids were not different ($P \geq 0.148$), except for NC lactic acid which was lesser ($P = 0.016$) and test diets propionic acid which was also lesser than PC ($P = 0.008$). The NC and test diets total sugars and organic acids were not different from PC ($P \geq 0.306$). On day 42, relatively to PC, NC arabinose was lesser ($P < 0.001$), lactic acid, acetic and propionic acids were also lesser ($P \leq 0.042$), the total mono sugars were not different ($P = 0.200$), while the total organic acids were lesser ($P = 0.04$). The test diets cecal concentration of arabinose was lesser than PC (P

<0.001), lactic acid and propionic acids were also lesser than PC (P ≤0.003), total mono sugars were not different (P =0.620), while the total organic acids were lesser (P=0.022). In conclusion the inclusion of TWM and TSM did not improve components retention, nor did they increase mono sugars and organic acids concentration in cecal contents.

Key words**: fiber, enzymes, components retention, broilers, organic acids**

5.1 Introduction

Globally poultry production is growing exponentially as people continue to demand for more healthier meat posing a challenge on conventional feed ingredients (Abdollahi & Ravindran, 2021). This challenge, for developing countries, has been due to increasing disposable income while in developed countries it has been occasioned by an over-reliance on the conventional ingredients such as soybean meal which is only produced by a few countries (Fitches *et al*., 2019).

Most by-products are high in fiber and thus their ME (Metabolizable Energy) is low, which limits their use in monogastric farm animals as they lack of endogenous enzymes to utilise them (Rojas & Stein, 2017). Wheat processing by-products, such as wheat bran, can it's their ME and phosphorous availability increased by up to 10% and 20% respectively by just steam pelleting (Kraler *et al*., 2014). The goal of any feed ingredients is to provide digestible nutrients that can be used for maintenance and production (Kiarie & Mills, 2019).

The beneficial use of enzymes to promote the growth and digestibility of nutrients in pigs has been well documented (Kiarie *et al*., 2013). Carbohydrases have shown inconsistency in their ability to improve the digestibility of feed components, with some having shown improved performance (Cowieson & Roos, 2016; Emiola *et al*., 2009; Yan *et al*., 2017) while others did not (Elangovan *et al*., 2004; Mikhail *et al*., 2013; Ahmed *et al*., 2017; Wu *et al*., 2005). Most enzymes are added during ingredient mixing; therefore, they need to be heat stable and stable in the gut of monogastrics to avoid a reduction in their activity (Rojas & Stein, 2017). Rojas & Stein, (2017)suggested that for better performance, exogenous enzymes may need to be used to pre-treat ingredients before being fed to pigs as there would be less variables to be considered.

The use of FDE to pre-treat maize distillers' grains with solubles (DDGS) has resulted in contradicting results. Jakobsen *et al*., (2015) reported a positive response while others reported no

improvement (Rho *et al*., 2018). However, there is little data on use of FDE to pre-treat high fibrous materials (HF) for use in broilers chicken diets. The aim of current study was to investigate the effect of including TSM and TWM in a low nutrients dense (LND) test diets on components retention and cecal metabolites production when compared with similar LND, negative control (NC) containing USF and UWM and a maize-soybean based positive control diet in broiler chickens.

5.2.0 Materials and Methods

5.2.1 Ingredients and Pre-Treatment

This was as described chapter 4.

5.2.2 Diets formulation

As described in chapter 4.

5.2.3 Samples Collection

On day 17, collection trays were placed under each cage for collection of starter phase excreta. The excreta were collected starting in the morning of d18 through to d20 and immediately stored at -20℃ after each collection day. Collection trays were also placed on day 38 and finisher phase excreta samples collected from d39 to 42 and stored as previously stated. On days 21 and 42, one bird per cage was randomly selected and euthanized by cervical dislocation and cecal content collected by taking the whole pair of ceca with its contents, put inside a whirl pack nylon bag and stored at -20℃ until required for analyses.

5.2.4 Samples Preparation and Chemical Analysis

Daily cage fecal samples within phase for each treatment were thawed, pooled, and placed in oven set at 65℃ for 4 days. The excreta were weighed before and after oven drying. The dried samples

were weighed and along with diet samples, ground into fine particles using a coffee grinder (CBG5 Smart Grind; Applica Consumer Products, Inc., Shelton, CT). The diets and excreta samples were analyzed for dry matter (DM), neutral detergent fiber (NDF), gross energy (GE), crude protein (CP, N x 6.25) and titanium. The DM was determined using method 930.15 (AOAC, 2004) and ash using method 942.05 (AOAC, 2004). The protein content (KjeldahlN×6.25) was determined (AOAC method 930.15) using an automatic analyser (FP-2000, LECO® Corporation, St Joseph, MI, USA). VanSoest method was used to analyze for NDF (AOAC, 2004). Adiabatic bomb calorimeter was used to determine GE (IKA Calorimeter System C 6000; IKA Works, Wilmington, NC). The concentration of SCFA (citric, lactic, formic, acetic, propionic, and butyric) and sugars in ceca digesta was analysed according to Kiarie *et al*., (2014). Briefly, the samples ware thawed and 0.1g weighed, mixed with 1ml of 0.005N sulfuric acid buffer, vortexed and spanned at high speed for 15 minutes. 400ul of supernatant were mixed with 400ul of the buffer and run on HPLC (Hewlett Packard 110, Germany) with Rezex ROA-Organic Acid LC column at 40C (Agilent 1260 Infinity RID from Agilent Technologies, Germany). A 20µL was injected into the column, column temperature was 60ºC and 0.005N sulfuric acid buffer mobile phase at 0.5ml/min for 35min. The detector temperature was 40ºC.

Titanium was determined as described by Myers *et al*. (2004). Briefly, porcelain crucible were weighed and tared, 4g of feed, 2g of excreta were weighed duplicate before and after placing in an oven set at 135ºC for 2h for DM determination. The samples were then ashed using a muffle furnace set at 580ºC for 10h and weighed. In a tared pyrex tube, 0.1g of feed ash, 0.05g of excreta ash were weighed and 0.8g of of anhydrous Na_2SO4 and 5ml of concentrated H_2SO_4 added to each tube. Each tube was capped and vortexed and placed in a heating block preheated at 120ºC for 20h with occasional vortexing. The samples were then transferred into 100ml volumetric flask and

topped to the mark using double distilled water, covered with parafilm, inverted 3 times and left to for overnight. 200µL of each titanium standard and sample were placed in a 96 well plate in duplicate and 20µL of hydrogen peroxide added into each well. Samples left for 15min for color reaction. Absorbance was measured on a plate reader at 408nm after 15sec shaking. Graph of titanium concentration against absorbance was plotted.

5.3.0 Calculations and Statistical Analysis

Coefficient of apparent components retention (AR) as shown in equation 1 as described by Kiarie *et al*., (2014)

$$AR = [\frac{\left(\frac{N_C}{TiO}\right)d - \left(\frac{N_C}{TiO}\right)e}{\left(\frac{N_C}{TiO}\right)d}] \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots..(1)$$

Where:

i. N_C is component of interest
ii. TiO is Titanium (II) Oxide as the indigestible marker
iii. d is diet
iv. e is excreta

Apparent metabolizable energy corrected for nitrogen (AMEn) was calculated using equation 2 as described by (Mwaniki & Kiarie, (2019)

$$AMEn\left(\frac{\frac{Kcal}{kg}}{dm}\right) = AME \ - (8.22 \times ANR \ldots\ldots\ldots\ldots\ldots\ldots\ldots.(2)$$

Where:

a. AME is apparent metabolizable energy

b. ANR was apparent nitrogen retained

The cage was the experimental unit. Outliers in the data were removed using PROC UNIVARIATE of SAS 9.4. Any values three standard deviations above or below the mean was identified and removed as outliers. The nutrients retention (DM, NDF, CP and GE), excreta moisture and SCFA data was analyzed using PROC GLIMIXX of SAS 9.4 with diets as fixed factors in the model. Least Square Means were separated using Tukey test, while pre-planned orthogonal and polynomial contrasts statements were used to compare the performance of NC against PC (PC vs NC) and response of diets containing test feedstuffs. The level of statistical significance was pre-set at $p \leq 0.05$. Linear equation is as in chapter 4.

5.4.0 Results and Discussion

Table 5.1 shows apparent retention (AR) of components in broiler chickens fed a maize-soybean based diet without or with untreated and pre-treated sunflower meal and wheat middling. On d21, compared with PC the AR of crude protein (CP) ($P=0.388$) and AMEn was not different ($P \geq 0.133$) compared to NC. This may be due to diets being formulated based on equal ileal digestible amino acids. The AR of NDF was greater ($P = 0.015$), while GE, and DM were lesser ($P \leq 0.029$). These results may be due to dietary fiber present in the diets as it has shown that insoluble fiber especially in sunflower meal promote retention of protein but with negative effect on energy, DM and ash retention (Kalmendal *et al*., 2011). Excreta moisture content can be used as a measure of litter quality (Kimiaeitalab *et al*., 2017), and in the current study excreta moisture for NC was also lower than PC ($P= 0.033$). Although the current study did not analyze particle size, the USM and UWM were not processed further after procurement, and coarse ingredients high in insoluble fiber

reduces excreta moisture (Jiménez-Moreno *et al*., 2013), USM contains most of its fiber as insoluble fiber. Hoeven-Hangoor *et al*., (2014), observed that adding a source of insoluble fiber (coarse oat hull) in a wheat-based broiler chicken diet reduced excreta moisture when compared to a maize-based control diet, although their study happened in pen floors and wood shavings might also have contributed to a significant source of insoluble fiber. There is also contrasting literature on effect of fiber on excreta moisture. Fiber particle size and shape may irritate the intestinal lumen wall and act as a laxative to stimulate water excretion. McRorie, (2015) observed that if a diet containing ingredients with high insoluble fiber, the insoluble fiber is not fermented, and it retains its high-water holding capacity leading to excreta of lower DM. Wheat processing by products such as wheat bran have been shown to contain insoluble fiber (McRorie, 2015).

In the current study, compared to PC, the AR of test diets (TD) CP was not different (P =0.815) but N75 and N100 retained 2.24% and 1.80% less indicating that pre-treated ingredients had a negative effect on CP retention at higher inclusion levels. The AR of NDF was greater (P <0.001) and this may be attributed to the effect of FDE solubilizing more fiber, as FDE has been shown to promote fiber digestibility (Moran *et al*., 2016b). The AR of GE, DM, AMEn linearly decreased with increased inclusion rate of pre-treated ingredients (P ≤0.027) which shows that, although the enzymes might have worked, the components solubilized were not utilized by the broiler chickens, the excreta moisture had a linear decrease with inclusion of pre-treated ingredients (P =0.032) which may be due to reduction of insoluble fiber by inclusion of treated ingredients and reduction of insoluble fiber leads to excreta of lower moisture (McRorie, 2015).

On d42, relative to PC the NC AR of CP was greater (P <0.001) and AMEn was lesser (P ≤0.001), in contrast to results observed during the starter phase. This could be due to the fact that relative to the starter, the finisher diets had more inclusion of USM (7.5 vs 8%) and UWM (10% vs 18.5),

AR of NDF was greater (P <0.001), the NC retained 4.01% and 5.58% less GE and DM respectively, which is as result of high fiber in the diet and the bird lacked endogenous enzymes to hydrolyze them (Singh & Kim, 2021a). The excreta moisture were not different between PC and NC (P =0.215).These results agree with Dunaway, (2019) who showed that diets with wheat middlings increases fiber contents and lowers the GE and DM retention when compared to a maize-soybean based diet control diet. There are various feed energy description concepts used by poultry nutritionist such as AMEn, total digestible energy, total digestible nutrients, true metabolizable energy and effective energy (Barzegar *et al*., 2020). It should be noted that AMEn is the most commonly and widely accepted method, but the method is affected by poultry species, age, and their feed intake (Barzegar *et al*., 2020). Adult broiler birds can utilise energy from different ingredients with less variation as compared to growers (Cozannet *et al*., 2010). Diet form and composition determines the AME values (Noblet *et al*., 2010). In the current study, on d42, the N75 and N100 AR of CP, GE, and DM were lesser than PC (P <0.001), all the test diets retained more NDF and AMEn than PC (P <0.001) the AMEn retention can be attributed to increased solubilization of fat by FDE , test diets had between 3- 3.9% more crude fat than PC (Table 5.2),the excreta moisture of N75 and N100 was lesser by 2.28% and 2.66 respectively.

The TD diets retention of components especially at the higher inclusion rate (N75 and N100) can be attributed to various factors; 1) the test diet were formulated based on book value without accounting for any nutrient that would have been liberated by enzymatic pre-treatment. It can therefore, be hypothesized that the extra nutrient such as amino acids that were solubilized by FDE might have caused an imbalance leading to their underutilisation. for example, N25 and N50 had 3.54% and 3.56% more crude fat than PC (summarized in table 5.2). 2) the xylose concentration in TWM and TSM were high 59.44 and 29.40 µmol/ml respectively, TSM was also high in

arabinose 4.01µmol/ml and these sugars have been shown to reduce lipids and energy utilization and causes liver damage since they are directly absorbed in the small intestines (Regassa *et al*., 2017b). Heat processing has had both negative and positive effects on nutrients digestibility (Herkelman *et al*., 1992). Hydrothermal pre-treatment of feed has been associated with increased feed intake (FI) and nutrients passage rate which affects nutrients retention and bioavailability especially that of protein and starch (Goodarzi Boroojeni *et al*., 2016). The TSM in the current study had lower soluble protein and increased neutral detergent fiber protein (NDF-P) and had higher NDF compared with USM. A contrary observation with TWM was that SP (Soluble Protein) was higher, NDF-P and NDF ware lower compared with UWM. The reduction of SP and increased level of NDF-P might have affected CP retention in the test diets as the protein was unavailable to the bird and most of it was excreted.

Different methods are used to process more than a billion metric tons of livestock feed/ingredients annually globally and these methods need to be evaluated on their effects on nutrients digestibility (Rojas & Stein, 2017). It has been hypothesized that during saccharification and the drying of wheat and maize by-products such as maize distillers dried grains with solubles (DDGS) and wheat DDGS, fiber and starch interact with protein to form complexes that are even resistant to hydrolysis by carbohydrases (Jha *et al*., 2015). The current study did not measure complexed lysine. As shown in chapter 4, despite composition TWM being better than UWM, this was not reflected by improved performance when it was used to replace UWM in test diets meaning it was not well utilised by the birds. It is of importance to note that Sunflower meal is high in polyphenol compounds (Zatari, 1989) and it has been shown that heating carbohydrates promotes formation of phenolic-carbohydrate complexes especially with starch that escape digestion (Jha & Mishra, 2021; Kandil *et al*., 2012); the complexes are deemed nutritionally damaged. Studies have shown

that heat pre-treatment (HP) increases starch availability to α-amylase for degradation in poultry thus improving digestibility through gelatinization and by breaking down cell wall and protein matrixes by use of shear force (Svihus *et al*., 2011a; Zaefarian *et al*., 2015). Other studies have shown that HP may decrease starch digestibility by encouraging formation of lipid-amylose complexes through increased gut viscosity because of increased solubility of fiber (Abdollahi *et al*., 2013; Svihus *et al*., 2011b).

Table 5. 1. Coefficients of apparent retention of components, metabolizable energy (AME) and excreta moisture content in broiler chickens fed maize-soybean meal-based diets with pre-treated sunflower meal and wheat middlings

		Treatments[1]						Overall	Response of		Contrast
	PC	NC	N25	N50	N75	N100	SEM	P-value	Linear	Quadratic	PC ~~*vs* N~~C
Day 21											
Crude protein	67.49	66.85	67.02	67.63	65.25	65.69	1.46	0.815	0.619	0.971	0.388
Neutral detergent fiber	28.92^{b}	38.32^{ba}	45.73^{a}	45.83^{a}	41.09^{a}	37.34^{ba}	2.36	<0.001	<0.001	<0.001	0.015
Gross energy Mcal/kg	74.86^{a}	72.71^{ba}	70.90b	73.86^{a}	72.89^{ba}	71.80^{ba}	0.91	0.051	0.013	0.750	0.022
Dry matter	70.45	69.02	67.53	69.17	68.08	67.14	1.04	0.248	0.027	0.801	0.029
AMEn Mcal/kg	2.55^{ba}	2.55^{ba}	2.30^{c}	2.63^{a}	2.59ba	2.49b	0.03	<0.001	<0.001	0.070	0.133
Excreta moisture	71.87	70.80	69.76	67.64	68.50	67.70	1.34	0.163	0.032	0.306	0.033
Day 42											
Crude protein	61.59^{b}	63.38^{b}	67.53^{a}	64.66^{ba}	61.91^{b}	62.39^{b}	0.90	<0.001	0.015	0.043	<0.001
Neutral detergent fiber	25.47^{d}	40.63b^{ac}	51.66^{a}	48.61^{ba}	33.46^{dc}	39.65^{bc}	2.78	<0.001	<0.001	<0.001	<0.001
Gross energy Mcal/kg	76.32^{a}	72.31^{c}	77.44^{a}	75.31^{ba}	73.31^{bc}	74.89^{bac}	0.62	<0.001	0.054	0.041	0.109
Dry matter	71.09^{ba}	65.51^{d}	71.92^{a}	69.76b^{ac}	66.49^{dc}	68.38^{bdc}	0.82	<0.001	0.200	0.054	0.024
AMEn Mcal/kg	2.69^{c}	2.60^{d}	2.89^{a}	2.73^{cb}	2.72^{cb}	2.80^{b}	0.02	<0.001	<0.001	0.444	<0.001
Excreta moisture	65.03	65.35	64.54	63.27	62.75	62.37	1.49	0.624	0.231	0.827	0.215

Values within a row without a common superscript differ significantly by LS means at 5% probability.
SEM- standard error of means
[1]PC; maize-soybean based positive control; NC, PC plus untreated sunflower meal (USM) and untreated wheat middling's (UWM); N25, NC with USM & UWM replaced with 25% of treated sunflower meal (TSM) and 25% of treated wheat middling's (TWM), respectively; N50, NC with USM & UWM replaced with 50% of TSM and 50% of TWM, respectively; N75, NC with USM & UWM replaced with75% TSM and 75% TWM, respectively; N100, NC with USM & UWM replaced with 100% TSM and 100% of TWM, respectively

Table 5. 2. Differences in the analyzed chemical composition of dietary treatments against positive control in starter and finisher phases on a DM basis**.**

Treatments[1]	**Starter**						**Finisher**					
Item	PC	NC vs PC	N25 vs PC	N50 vs PC	N75 vs PC	N100 vs PC	PC	NC vs PC	N25 vs PC	N50 vs PC	N75 vs PC	N100 vs PC
Gross energy, Mcal/kg	4.60	0.11	-0.11	0.16	0.13	0.08	4.59	0.16	0.29	0.17	0.24	0.25
Poultry ME (Mcal/kg)[2]	3.34	-0.12	-0.19	-0.27	-0.32	-0.10	3.38	-0.20	-0.18	-0.21	-0.20	-0.25
Starch, %	45.97	-5.77	-8.14	-8.89	-9.23	-6.51	47.06	-9.57	-10.22	-11.01	-11.53	-12.40
Crude protein, %	23.39	-1.24	-0.23	-0.88	-0.99	-0.54	21.95	-1.22	-0.78	-0.48	-0.15	-0.39
Crude fat, %	4.54	2.16	3.54	3.56	2.40	4.20	5.58	3.42	3.47	3.52	3.82	3.90
Neutral detergent fiber, %	8.37	4.69	5.41	4.64	4.98	4.41	8.34	8.54	8.24	8.39	5.40	6.60
Ash, %	6.08	0.29	0.50	0.53	0.56	0.55	5.26	0.65	0.37	0.72	0.65	0.90
Calcium, %	0.96	-0.09	-0.02	-0.03	-0.09	-0.09	0.75	-0.10	-0.11	-0.06	-0.12	-0.04
Phosphorous, %	0.77	0.04	0.08	0.07	0.05	0.09	0.63	0.14	0.15	0.16	0.14	0.18

[1]PC; maize-soybean based positive control; NC, PC plus untreated sunflower meal (USM) and untreated wheat middling's (UWM); N25, NC with USM & UWM replaced with 25% of treated sunflower meal (TSM) and 25% of treated wheat middling's (TWM), respectively; N50, NC with USM & UWM replaced with 50% of TSM and 50% of TWM, respectively; N75, NC with USM & UWM replaced with75% TSM and 75% TWM, respectively; N100, NC with USM & UWM replaced with 100% TSM and 100% of TWM, respectively

[2]Calculated by a commercial laboratory

Table 5.3 shows the concentration of sugars and SCFA in the cecal digesta. On day 21, relative to PC, NC glucose, xylose and arabinose were not different (P ≥0.148) and total sugars were also not different (P =0.148), lactic acid was lesser (P =0.016) which is an indication of reduced available glucose for fermentation. Acetic, propionic, butyric, iso-butyric, iso-valeric and valeric acid were not different (P ≥0.078) and total SCFA were not different (P =0.818), an indication that NC birds were unable to utilize the NSP present in USM and UWM. Compared with PC the TD glucose, xylose and arabinose were not different from PC (P ≥0.444) and the total sugars were also not different (P =0.750). Lactic, acetic, butyric, iso-butyric, iso-valeric and valeric acid were not different (P ≥0.102), propionic acid was lesser (P =0.008) and total SCFA were not different (P =0.306).

On day 42, compared with PC, NC glucose and xylose were not different (P ≥0.052), arabinose was lesser (P <0.001) and total sugars were not different (P =0.200). The lactic, acetic, and propionic acids were different (P ≤ 0.042) while butyric, iso-butyric, iso-valeric and valeric acid were not different (P ≥0.123) and total SCFA were lesser (P =0.040). Compared to PC, TD glucose and xylose were not different (P ≥0.185), arabinose was lesser (P <0.001) and total sugars were not different (P =0.620). The lactic acid, propionic, iso-butyric, and valeric acids were lesser (P ≤0.046) while acetic, butyric, iso-valeric acid were not different (P ≥0.144), lactic and propionic acids had a linear response (P ≤0.001) and total SCFA were also lesser (P =0.022).

Researchers have shown that gut bacteria play a significant role in digestion of feeds, bird immunity, energy utilization and vitamins synthesis (Klasing, 2007; Rakoff-Nahoum *et al*, 2004). Complex carbohydrates such as non-starch polysaccharides that are not degraded in the small intestines are later fermented in the large intestines to form SCFA which are absorbed and used as energy sources (Rinttilä & Apajalahti, 2013a). There is also a correlation between cecal microbiota

and efficiency of conversion of GE to ME which leads to enhanced FCR (Feed Conversion Ratio) (Rinttilä & Apajalahti, 2013a). Cecal short chain fatty acids (SCFA) also modulate the lumen pH which is a crucial factor for inhibiting proliferation of pathogenic bacteria such as *Enterobacteriaceae* which are acid pH sensitive (van der Wielen *et al*., 2000). Butyrate has been shown to be the most preferred energy source by enterocytes, as it has been proven to regulate proliferation and differentiation of intestinal cellular mucosa leading to intestinal weight increment (Fukunaga *et al*., 2003; Le Blay *et al*., 2000). Intestinal mucosa is the main barrier that prevents toxins from being absorbed into the bloodstream thus it plays a key role in the bird's immunity (Niba *et al*., 2009). Lactic acid plays a leading role in lowering the gut pH as it is the strongest acids produced among the SCFA (Belenguer *et al*., 2007). Inclusion of high fiber up to 7% (soy hull) have been shown to increase iso-butyric, butyric, acetic, and propionic acids while formic acid is reduced (Linden, 2012). In the current study, high fiber did not increase these SCFA, and it had been hypothesized that NC would have higher SCFA than PC. Dietary fiber has been shown to improve digestive physiology by increasing enzyme production and promoting small and large intestines integrity (Jha & Mishra, 2021).

Table 5. 3. Concentration (µmol/g) of sugars and short-chain fatty acids (SCFA) in ceca digesta of broiler chickens fed maize-soybean meal-based diets with pre-treated sunflower meal and wheat middlings

	Treatments[1]							Overall	Response of treated		ontrast
	PC	NC	N25	N50	N75	N100	SEM	P-value	Linear	Quadratic	PC *vs* NC
Day 21											
Sugars											
Glucose	0.38	0.62	0.28	0.65	0.52	0.79	0.24	0.708	0.568	0.922	0.227
Xylose	0.20	0.20	0.21	0.27	0.22	0.29	0.05	0.680	0.203	0.851	0.194
Arabinose	0.24	0.24	0.14	0.21	0.15	0.18	0.05	0.444	0.151	0.525	0.316
Total sugars	0.59	0.83	0.45	0.79	0.74	0.98	0.26	0.750	0.688	0.826	0.148
SCFA											
Lactic	4.44	3.69	3.03	3.98	3.09	2.93	0.44	0.102	0.020	0.642	0.016
Acetic	8.76	9.32	6.91	7.96	7.09	7.89	0.87	0.333	0.155	0.411	0.500
Propionic	1.22^{a}	1.27^{a}	1.06^{ba}	1.09^{ba}	0.87^{b}	1.05^{ba}	0.07	0.008	0.044	0.094	0.103
Iso-butyric	0.4	0.41	0.43	0.42	0.38	0.48	0.03	0.384	0.077	0.247	0.078
Butyric	1.94	1.8	1.55	1.51	1.46	1.52	0.27	0.782	0.221	0.417	0.279
Iso-valeric	0.27	0.31	0.24	0.32	0.30	0.35	0.05	0.766	0721	0.903	0.405
Valeric	0.44	0.39	0.29	0.32	0.36	0.32	0.05	0.384	0.039	0.350	0.123
Total SCFA*	14.8	17.1	12.9	13.4	11.7	14.2	1.66	0.306	0.336	0.332	0.818
Day 42											
Sugars											
Glucose	0.65	0.59	0.69	0.38	0.36	0.28	0.13	0.132	0.185	0.745	0.052
Xylose	0.20	0.14	0.21	0.17	0.20	0.20	0.03	0.58	0.351	0.540	0.822
Arabinose	0.38^{a}	0.27^{ba}	0.26^{ba}	0.21^{b}	0.19^{b}	0.16^{b}	0.03	<0.01	<0.01	0.043	<0.01
Total sugars	0.93	0.69	0.96	0.76	0.70	0.64	0.16	0.62	0.660	0.806	0.200
SCFA											
Lactic	0.9^{a}	0.86^{a}	0.79^{ba}	0.72^{ba}	0.7^{ba}	0.57^{b}	0.06	0.003	<0.01	0.932	<0.01
Acetic	10.75	9.27	10.73	10	9.1	9.0	0.59	0.13	0.487	0.662	0.042
Propionic	1.58^{a}	1.33^{ba}	1.16^{b}	1.45^{ba}	1.15^{b}	1.13^{b}	0.08	0.001	0.001	0.41	<0.01
Iso-butyric	0.45^{ba}	0.39^{b}	0.42^{ba}	0.48^{ba}	0.51^{a}	0.5^{a}	0.02	0.003	0.095	0.961	0.123
Butyric	1.69	1.89	2.63	1.87	1.62	1.60	0.29	0.141	0.411	0.445	0.824
Isovaleric	0.28	0.25	0.25	0.27	0.29	0.26	0.06	0.993	0.759	0.98	0.918
Valeric	0.36	0.33	0.45	0.48	0.33	0.36	0.04	0.046	0.144	0.236	0.989
Total SCFA*	16^{ba}	14.2^{ba}	16.5^{a}	15.1^{ba}	12.4^{b}	13.2^{ba}	0.92	0.022	0.585	0.522	0.04

5.4.0 Conclusion and recommendation

Conclusions

Pre-treatment of sunflower meal and wheat middlings with FDE did not improve the AR of crude protein and apparent metabolisable energy.

Pre-treatment of sunflower meal and wheat middlings had no effect on cecal mono-sugars and short chain fatty acids concentrations in broiler chicken.

Recommendation

There is need to investigate whether there is a step(s) in pre-treatment process that affected the nutrients liberated by enzymes negatively, or whether it is the pre-treated ingredients that have a negative effect on the broiler chicken gut, e.g altering the gut micro biota, thus reducing their apparent retention.

Chapter Six. General discussion, conclusions and recommendations

Poultry products form a vital source of human nutrients worldwide as chicken are widely accepted and kept by many people irrespective of race, religion, and other beliefs (Manning & Baines, 2004). The production of broiler chickens has seen a tremendous increase since the 1980s due to the awareness of the nutritional attributes of poultry meat compared to other meat protein sources. The broiler chicken meat also provides a quick source of quality protein within a short period due to its high growth rate due to advanced genetic selection (Saeedatu Nissa *et al*., 2018).

The poultry industry lags in developing countries compared to other middle-income and developed world. One of the reasons for this poor growth of the poultry industry in developing countries is the shortage of feed ingredients (both quality and quantity). Broiler chicken rations are mainly maize-soybean meal-based diets. The two components are primarily imported from a few countries, particularly Brazil, Argentina, Paraguay, and the United States of America (Williams & Thompson, 2022); the two are also used as human food, thus creating a competition between food and feed. Maize is also becoming limited due to other uses such as biofuels.

In contrast, many developing countries have had other agricultural products such as sunflower, wheat bran, wheat middlings, and cottonseed cake meal in plenty whose sources are grown both in tropics and temperate regions. These byproducts are mainly from plants grown in both tropical and temperate regions. These byproducts have not been fully incorporated into the commercial chicken feeds in high amounts due to anti-nutritional factors such as cellulose, hemicellulose, and non-starch-polysaccharides (NSP), for which poultry lacks endogenous enzymes that can hydrolyze (de Vries *et al*., 2012).

The use of enzymes has enabled the poultry industry to save billions of shillings and, at the same time, reduce environmental pollution from unutilized nutrients in poultry fecal wastes (Munir & Maqsood, 2013). Numerous studies have shown that poultry performance and feed conversion ratio are improved when diets containing ingredients with high NSP such as sorghum, barley, and wheat or triticale are supplemented with exogenous enzymes both from fungal and bacterial sources.

Whenever researchers conduct *in vitro* studies using these exogenous enzymes, the results have shown high digestibility compared to *in vivo* results; the reasons for this discrepancy is due to many limitations that they face inside the animal; 1) most of the enzymes have an optimum pH, and inside the broiler chicken, the pH keeps on changing, from 2-4 in the gizzard, 4-5 in the proventriculus, and 6-7.5 in the intestines, 2) whenever the enzyme gets to the organ where the pH is optimum, they also face a time limitation due to a short feed retention time by the broiler chicken which is usually less than 90 minutes, 3) when broiler chicken are selected for high growth rate, the gizzard which can grind coarse feed ingredients have been reduced in size, and it now operates more of a transit organ. This calls for pre-treating the ingredients prior to feeding where conditions can be controlled.

The *in vitro* pre-treatment of SM and WM showed that incubating these ingredients with water alone increases soluble protein. Protein is among the most expensive nutrients in broiler diets and therefore, steeping ingredients with water can reduce cost of production. The additional of FDE increased SP even more and also helped to solubilize fiber in SM and WM thus improving their compositional quality. Feeding pre-treated ingredients to broiler chicken did not affect feed intake, increased FCR, and reduced performance, especially in a high inclusion rate. Although *in vitro* data showed increased solubilisation of protein and crude fiber, and also showed increased

concentration of mono sugars and organic acids in TWM and TSM, the pre-treated ingredients did not improve the AR of CP and GE and cecal concentration of mono sugars and organic acids in broilers chicken.

6.1.0 General conclusions

- Pre-treatment of WM with FDE increased CP solubilisation and AD of crude fiber. The FDE also increased both mono sugars and organic acids concentrations.
- Pre-treatment of SM with FDE increased CP solubilisation and AD of crude fiber. Additional of protease to FDE increased CP solubilization while it had no effect on AD of crude fiber. The FDE also increased both mono sugars and organic acids concentrations. Additional of protease to FDE increased both concentration of mono sugars and organic acids.
- Pre-treatment of sunflower meal and wheat middlings with FDE did not improve the AR of crude protein and apparent metabolisable energy
- Pre-treatment of wheat middlings or sunflower meal did not affect concentration of cecal mono sugars and short chain fatty acids in broiler chicken.
- Inclusion of TSM and TWM in diets did not improve the growth performance and tibia attributes of broiler chicken

6.2.0 General recommendations

- There is need to investigate why pre-treatment of sunflower meal with water only increased NDF relative to untreated one.
- There is need to investigate whether inclusion of enzymatically pre-treated sunflower meal and wheat middlings, in a maize-soybean diets have any negative biochemical/physiological effect, which reduced the growth performance of broiler chicken.

- There is need to investigate whether there is a step(s) of pre-treatment process that affected the nutrients liberated by enzymes negatively, or whether it is the pre-treated ingredients that have a negative effect on the broiler chicken gut, eg altering the gut micro biota, thus reducing their apparent retention.

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