IN VITRO DUODENAL ENZYME ACTIVITY ASSAYS AND PROTEIN PROFILING AS FEED EVALUATION METHOD

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ABSTRACT

To determine the validity of *in vitro* feed evaluation method, 20 day-old broilers were divided into four (4) groups and assigned to four (4) treatments, specifically: a basal diet, basal diet + 10 ppm avilamycin, basal diet + 150 ppm plant extract containing thymol (*Thymus vulgaris*), eugenol (*Cinnamomum* spp.) and piperine (*Piper* spp.), and basal diet + 300 ppm plant extract, following Completely Randomized Design (CRD). All growth parameters were recorded. After slaughter, duodenal digesta samples were collected and subjected to α-amylase and total proteolytic activities assays using potato starch and bovine serum albumin as substrates, respectively. Subsequently, the protein profiles of the crude duodenal digesta were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Results showed no significant differences in all growth performance parameters, in duodenal α-amylase and total proteolytic activities of the broilers fed with the different dietary treatments (P>0.05). The SDS-PAGE electrophoretogram showed qualitative differences in band intensity of the different proteases and the α-amylase present in the duodenal digesta samples. Results on the growth trial and enzyme activity assays are in agreement indicating that duodenal enzyme activity assays may predict feed quality and digestibility. This study suggests that duodenal *in vitro* enzyme activity assays coupled with digesta protein profiling can be used as a tool in feed evaluation.

Key words: broilers, duodenum, enzyme activity, *in vitro* feed evaluation, SDS-PAGE protein profile

INTRODUCTION

Animal feeds contribute some 65-80% of the broiler chicken production cost. Since broiler chickens are now grown for as early as 28 days to reach market weight, feed ingredients used in the diets are limited to highly digestible starchy grains and legumes with good protein quality. For the past 20 years, technology development in poultry feeding is centered in the search for novel feed additives that could alter and improve gastro-intestinal function resulting in better feed nutrients digestibility. Most successful products added to broiler diets include exogenous enzymes that could augment endogenous enzyme secretion in digesting diverse substrates in the feed; antimicrobials and direct-fed microorganisms that regulate microbial consortia in the gastro-intestinal (GI) tract; and novel substances mostly of plant origins (e.g., secondary compounds, essential oils, etc.) that also regulate GI tract microbiota and enhance enzyme secretions (Jang *et al*., 2007; Zeng *et al*., 2015).

Since most improvements in the poultry industry had been focused on formulating cost-effective feeds that would provide adequate nutrients for the chickens (Dibner and Richards, 2004), rigid feed evaluation protocols are deemed necessary. Feed evaluation typically includes physical and chemical analyses of the feed ingredients; nutrient digestibility and balance studies; performance feeding trials and even toxicity assays (Osuji *et al*., 1993). Enzymes are essential in the process of digestion, and an increase in enzyme secretion is beneficial to the growth of chickens because it facilitates the digestion of carbohydrates, proteins and fats. If carbohydrates and proteins are not digested well, nutrient deficiencies may occur which can affect the animals' production performance. Studies on the activity of enzymes could aid in gathering more information about the functions of the dietary enzymes in the nutrition of poultry (Khattak *et al*., 2006). One way to test the carbohydrate and protein digestion capacity of the broilers is by conducting enzymatic assays on the secretions of the organs in the GI tract of the chickens (Gorrill and Thomas, 1967).

Gracia *et al*. (2014) reported that incorporation of plant extract or essential oils would result in higher enzyme secretions and activities in the GI tract. This study examined the effects of a mixture of plant extract/ essential oils, such as thymol, eugenol, piperine, as feed additives in diets of broiler chickens on their growth performance. It also aimed to compare duodenal digesta's α-amylase and proteases activities, and protein profiles of broilers fed diets with an antibiotic growth promoter (avilamycin) and varying levels of a mixture of the abovementioned compounds. The results of the *in vitro* assay were compared with the feeding trial results. The potential of using *in vitro* enzyme assays of the duodenal digesta samples was also evaluated in an attempt to develop an alternative feed evaluation method that is possibly cheaper and reliable. The digestibility of the feed's energy and amino acid is important for growth of the animal; thus, an assay procedure that can describe the duodenal enzyme secretion and activity may indicate nutrient absorption and metabolism.

To the best of the authors' knowledge, this study is the first in the country to report on the optimization and utilization of a potential feed evaluation method using *in vitro* enzyme activity assays, profiling of duodenal enzymes via electrophoresis and evaluation of feed additive effects on broiler growth, since *in vitro* methods are less tedious and time consuming than traditional feed evaluation protocols (Makkar, 2004).

MATERIALS AND METHODS

Twenty day-old chicks (DOC) were assigned to four (4) dietary treatments and were replicated five times with one DOC per replicate following a Completely Randomized Design (CRD). The dietary treatments formulated based on Cobbs 500 nutrient recommendations were as follows: Treatment 1 (basal diet – negative control), Treatment 2 (Treatment 1 + 10 ppm avilamycin – positive control), Treatment 3 (Treatment 1 + 150 ppm plant extract containing thymol (*Thymus vulgaris*), eugenol (*Cinnamomum* spp.) and piperine (*Piper* spp.) (Crina® Plus, DSM Nutritional Products, Makati, Philippines), and Treatment 4 (Treatment 1 + 300 ppm plant extract). Isocaloric and isonitrogenous diets were fed *ad libitum* with booster (days 1 to 10), starter (days 11 to 24) and finisher (days 25 to 35) diets. Clean water was made available at all times.

After 35 days, the broilers were slaughtered, and the small intestine, specifically, the duodenum of the chickens were then excised. The duodenal digesta were obtained by gently pressing out and scraping the surface of the duodenum and were placed directly in properly labeled 50-ml falcon tubes. The amount of duodenal digesta collected from each broiler sample was recorded and was immediately diluted to 5X using Phosphate Buffer Saline (PBS), pH 7.2.

Protein content determination

Duodenal digesta samples were mixed in a magnetic stirrer for 1 h to solubilize the proteins. The samples were centrifuged at 8,000 rpm for 30 min at 4ºC, and 1 ml of

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supernatant was collected. This was further diluted with PBS buffer to 300X.

The protein contents of the samples were measured using the Bradford protein content assay as modified in Bio-Rad Laboratory's Quick Start Bradford Protein Assay (2007). After the preparation of the different concentrations of bovine serum albumin (BSA) as protein standards, 20 µl was taken from each of the standards, and was transferred to another tube with the addition of 1 ml of the prepared Bradford reagent. The mixture was homogenized using a vortex mixer, and incubated at room temperature for 5 min. After incubation, the mixture was read at 595 nm using Shimadzu UV Mini-1240 spectrophotometer. The calibration curve with a minimum coefficient of linearity (r^2) of 0.98 was used to establish a linear equation with absorbance as dependent variable (Y) and concentration as independent variable (X).

Alpha-amylase activity assay

The enzyme assay used in the study was based on the revised procedures of Patil and Muskan (2009), Varalakshmi *et al*. (2012), and Sigma-Aldrich's Enzymatic Assay of α-AMYLASE (EC 3.2.1.1) (1997), Worthington and Worthington's Amylase, A-Assay (2011). Glucose standards were prepared from the serial dilution of a stock solution containing 1 mg/ml glucose. After boiling for 5 min, the tubes were cooled to room temperature and then 1 ml distilled H_2O was added and absorbance was read at 540 nm. For the duodenal digesta samples, three sets of conditions were made: first, the control tube that contained 1 ml of 1% starch solution and 1 ml of PBS; second, the tube that contained 1 ml of duodenal digesta and 1 ml of 1% starch solution; and lastly, the tube that contained 1 ml of duodenal digesta only. The mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 1.0 ml of 1 N NaOH. Further, 1.0 ml of 3,5-dinitrosalicylic acid (DNSA) reagent was added to each test tube and kept in boiling water bath for 10 min. The samples were vortexed briefly and absorbances were read at 540 nm. The data were interpolated from the 1 mg/ml glucose standard solution and the concentrations of glucose released from the hydrolyzed starch by the α-amylase enzyme in 10 min were also determined.

Total protease activity assay

A modification of the Bradford protein content assay was used to determine the proteolytic activity of the proteases present in the duodenal digesta samples with the use of BSA as substrate. Four sets of conditions were made: first, the control tube that contained 0.5 ml PBS; second, the tube that contained 0.5 ml BSA and 0.5 ml duodenal digesta; third, the tube that contained 0.5 ml PBS and 0.5 ml duodenal digesta; lastly, the tube that contained 0.5 ml PBS and 0.5 ml BSA. The mixture was incubated at 37°C, for 10 min and stopped by adding 0.5 ml of 10% trichloroacetic acid (TCA). The mixture was again incubated at room temperature for 5 min after adding 0.5 ml of the Bradford reagent. Absorbance was measured at 595 nm.

Protein quality determination and profiling via electrophoresis

To check the quality of the proteins extracted from the duodenal digesta, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using 12% resolving gel and 6% stacking gel that were prepared according to the method of Laemmli (1970). Eight (8) µl of the protein extracts were loaded onto the gel while 5 µl of the Wide Molecular Weight Range SigmaMarker® (Sigma-Aldrich, St. Louis, MO, USA) (6500 to 205,000 Da) protein standard was added in the last well and ran for around 1-2 h at 100 V with 1X Tris-borate running buffer. Afterwards, gel was washed with distilled water and stained with Coomassie Brilliant Blue R-250, Thermo Scientific® Pierce Coomassie Brilliant Blue R-250 Dye (Waltham, MA, USA) for 30 min on a rotary shaker at medium speed. This was then de-stained with a de-staining solution containing 10% acetic acid and 50% methanol for 2-3 h. After de-staining, the gel was visualized, scanned and image saved as a JPEG file.

Data recorded were: 1) average feed intake, 2) gain in weight, 3) feed conversion ratio, 4) percent livability, 5) protein concentration of duodenal digesta (in mg/ml), 6) α-amylase activity of duodenal digesta with unit activity defined as the amount of enzyme which releases 1 µmole of reducing sugar as glucose in 10 min, at pH 7.2 and 37°C, and 7) proteolytic activity of duodenal digesta with the unit activity defined as mg of BSA hydrolyzed per ml of duodenal digesta in 10 min, at pH 7.2 and 37°C.

All data were subjected to analysis of variance (ANOVA) in completely randomized design (CRD) using SAS Software, version 9.1.3 portable, (SAS Institute Inc., Cary, NC, USA). Comparison of treatment means was done using Fisher's Least Significant Difference (LSD). The level of statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Performance of broilers

Table 1 shows the average growth performance of the broilers fed with diets supplemented with avilamycin and varying levels of plant extract. There were no significant differences ($P > 0.05$) among the average feed intake of the broilers. The results conform with the studies of Lee *et al*. (2003), Angeles *et al*. (2011), Gracia *et al*. (2013), and Reyes (2015), showing no significant effect on the feed intake of broilers upon addition of plant extracts.

There were no significant differences ($P > 0.05$) on the average body weight gain of the broilers (Table 1). The results indicate that the different treatments did not influence the growth rate of broilers during the entire feeding period. These observations are similar to the reports of various authors who used plant extracts as feed additive in broiler diets, such as the effect of thymol essential oils on growth performance, the effect of thyme extract on growth performance, the use of coated plant extracts and organic acids as alternatives (Arriesgado, 2009; Pourmahmoud *et al*., 2013; Lippens *et al*., 2005). The differences in the results of body weight gain can be attributed to the differences in the composition of essential oils, level of supplementation, the kind of diets or ingredients and the strain and age of broilers used (Perez, 2007). Botsoglou *et al*. (2004) suggested that the dietary plant extracts as growth stimulants could not give positive results when chickens are kept at optimal condition such as highly digestible diets and clean environment.

Table 1. Summary of growth performance of broilers fed diets with 10 ppm avilamycin, 150 and 300 ppm of plant extract (PE) containing thymol (*Thymus vulgaris*), eugenol (*Cinnamomum* spp.) and piperine (*Piper* spp.).

Parameters	TREATMENTS				Mean	%CV
	1) Basal-negative control	2) Basal + 10 ppm avilamycin	3) Basal + 150 ppm PE	4) Basal + 300 ppm PE		
Ave. initial 11d BW^{ns}, g	313	318	303	323	314	8.43
Ave. final 35d BW^{ns} ,g	2050	1920	2190	2020	2045	8.54
Ave. 11-35 d BW gain^{ns}, g	1737	1602	1887	1697	1731	9.72
Ave. 11-35 feed intake^{ns}, g	2856	2840	2986	2909	2898	3.86
Ave. FCR	1.66	1.77	1.58	1.72	1.69	6.55
Livability^{ns}, %	100	100	100	100	100	-

^{ns}, no significant differences ($P>0.05$) FCR=Feed Conversion Ratio

Feed conversion ratios of the broilers were not significantly different (P > 0.05) suggesting that the broilers fed with different treatments can convert almost the same amount of feeds to produce a kilogram of meat (Table 1). These results are in agreement with those reported by Abudabos and Alyemni (2013) in a study of the effects of the essential oil blend in feed on broiler performance and gut microbiology and those examined in the studies of Lee *et al*. (2003), Gracia *et al*. (2013) and Reyes (2015).

There were no recorded mortalities throughout the study, thus livability of all the treatments was reported at 100% (Table 1). According to Gracia *et al*. (2013), the essential oils-supplemented diets fed to broilers reduced the levels of toxic microorganisms in their gut which eventually enhanced the broilers' livability.

Protein concentration and enzymatic activity of broilers' duodenal digesta

The mean protein concentration, total protease activity, and the α-amylase activity of the duodenal extracts of the broilers fed avilamycin and varying plant extract concentrations are shown in Table 2. The comparable protein concentrations show that the duodenal digesta samples contained statistically invariable amounts of protein (P > 0.05). This suggests that the protein content including duodenum and pancreatic secretions and other housekeeping proteins in the duodenum were not affected by the treatments. Statistical analysis also showed that there were no significant differences (P > 0.05) in the average proteolytic activity of the duodenal digesta of the broilers (Table 2). These results coincide with the study of Lee and co-workers in 2003, which reported that the essential oil inclusions did not affect the activities of the pancreas and its secretions.

The overall α-amylase activities among treatments were not significantly different with each other (P > 0.05) (Table 2). These results conform with that of the study of Lee *et al*. (2004) wherein there were no significant differences in amylase activities fed with diets. Nutrient digestibility of feeds and environmental conditions can also be attributed to the results. According to Botsoglou *et al*. (2004), feed additives are less effective if the health condition, environment, and management were in good conditions.

The results from the growth performance of the broilers could be partially explained by the results of the α-amylase activity and proteolytic activity assays; since there were no significant differences found in the activities of these enzymes, these would not have affected the broilers' growth performance.

Protein profile of crude duodenal digesta using SDS-PAGE

Figures 1 and 2 present the summary of the protein profiles of the crude proteases,

Table 2. Average protein concentration, proteolytic and amylolyticactivities of the crude duodenal digesta of broilers fed avilamycin and different inclusion rates of plant extract (PE) containing thymol (*Thymus vulgaris*), eugenol (*Cinnamomum* spp.) and piperine (*Piper* spp.).

Treatment	[Protein] of Duodenal digesta^{ns} (mg/ml)	[BSA] Hydrolyzed in 10 min ^{ns} (mg/10 mins.)	[Glucose] Produced in 10 min^{ns} (µmol/ml)
Basal Diet (- Control)	188.04	131.43	920.89
Basal Diet + avilamycin	173.02	132.34	981.69
Basal Diet + 150ppm PE	165.91	130.64	1161.70
Basal Diet + 300ppm PE	220.86	165.68	1135.86
Mean	186.96	140.02	1050.04
CV, %	16.91	19.33	18.02

^{ns}, no significant differences among treatments(P>0.05)

and crude α-amylase, respectively to verify their presence in the duodenal digesta samples.

Trypsin was detected in all of the samples, with an average calculated weight of 23,268 Da, corresponding to the theoretical weight of trypsin which is 23,300 Da (Worthington and Worthington, 2011). Trypsin was observed to be more visible in lanes of treatments 1 and 3, compared to those of Treatments 2 and 4. Chymotrypsin appeared in all Treatments, but was very faint in both Treatments 2 and 3. The average molecular weight of chymotrypsin derived from the four (4) treatments was 19,947 Da, which is near the theoretical value of 20,000 Da (Sturkie, 1986). In the study of Guyonnet and co-workers in 1999, they reported that the molecular weight of elastase is 25,700 Da, which is relatively close to the average molecular weight obtained in the study (26,001 Da). Elastase was also present with clear bands in all of the treatments but was least distinct at Treatment 4 (Figure 1). Jamadar *et al*. (2003) showed that aminopeptidase is a heterodimer with two subunits, with sizes 94,000 Da, and 66,000 Da. Bands with sizes close to these values were also observed (Figure 1). The bands for the heavier subunit (94,000 Da) were clearer and very distinct in all of the lanes, except for the first lane of Treatment 1. The 66,000 Da subunit bands were already very faint especially for Treatment 1. Lastly, the carboxypeptidase's theoretical molecular weight is 34,700 Da (Worthington and Worthington, 2011), and a 34,074 Da protein band was found in all of the samples, although the bands from Treatments 1 and 2 were indistinguishable.

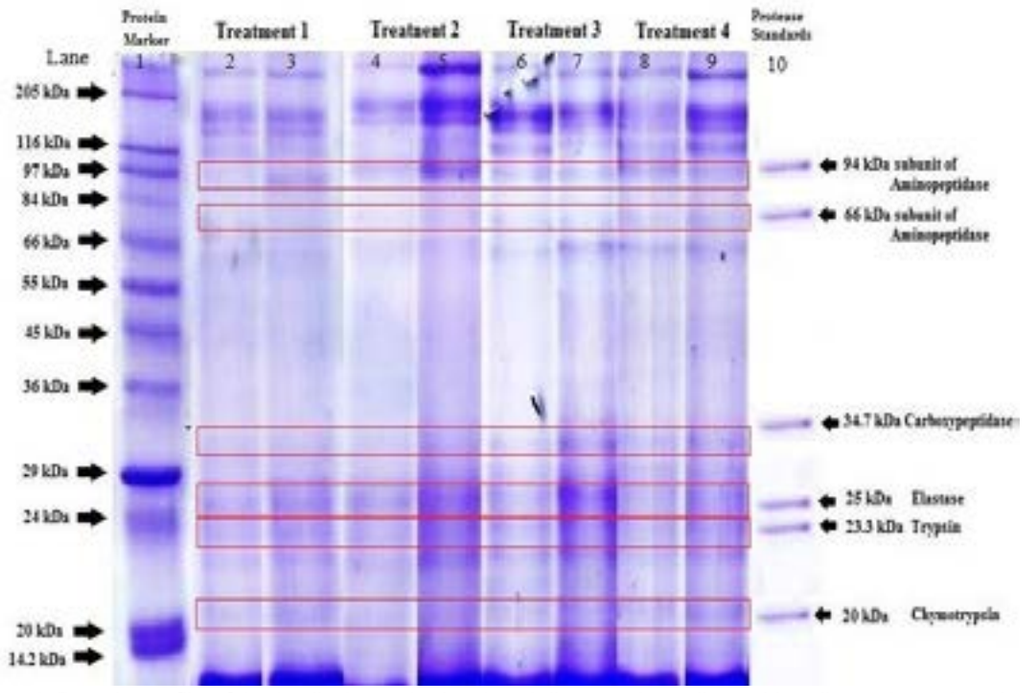

Figure 1. Protein profile of the broiler's crude duodenal digesta given diets with avilamycin and a mixture of plant extract (PE), determined by SDS-PAGE with 12% resolving gel; Lane 1: SigmaMarkerTM Protein Ladder; Lane 2: T1 (Basal diet) replicate 1; Lane 3: T1 (Basal diet) rep. 2; Lane 4: T2 (Basal diet + 10 ppm avilamycin) rep. 1; Lane 5: T2 (Basal diet + 10 ppm avilamycin) rep. 2; Lane 6: T3 (Basal diet + 150 ppm PE) rep 1; Lane 7: T3 (Basal diet + 150 ppm PE) rep. 2; Lane 8: T4 (Basal diet + 300 ppm PE) rep. 1; Lane 9: T4 (Basal diet + 300 ppm PE) rep. 2; Lane 9: Protease standards.

The molecular weight of the monomeric α-amylase in the pancreas of chickens, as shown in the study of Lehrner and Malacinski (1975), is 55,000 Da. In the electrophoretogram shown in current study, the computed average molecular weight of α-amylase from different treatments was 55,980 Da, which is near the standard computed of the pure pancreatic α-amylase which is 54,040 Da. The α-amylase protein band appeared in all the treatments but with varying band thickness and intensities. Treatment 3 exhibited the most distinct protein bands of α-amylase. The feed additives used in this study did not contain α-amylase as a component but primarily had plant extracts which were previously reported to stimulate α-amylase secretion in the broiler's duodenum (Williams and Lisa, 2001) and this may explain the intense α-amylase protein bands obtained in Treatments 3 and 4.

Some bands in the electrophoresis gels appeared very thin and faint. This may be due to some degree of hydrolysis of the enzymes during the storage period and upon the homogenization of the samples. Although there were no statistical differences found in the growth performance and enzymatic activities of the broilers, there were distinct qualitative variations in the band intensity of the protein profiles of the crude duodenal digesta from the four different treatments.

Unlike traditional feed evaluation methods that mostly focus on measuring the levels of proteins and carbohydrates in the feeds, this study assessed the potential of in vitro techniques (enzyme assays and protein profiling using electrophoresis) in testing

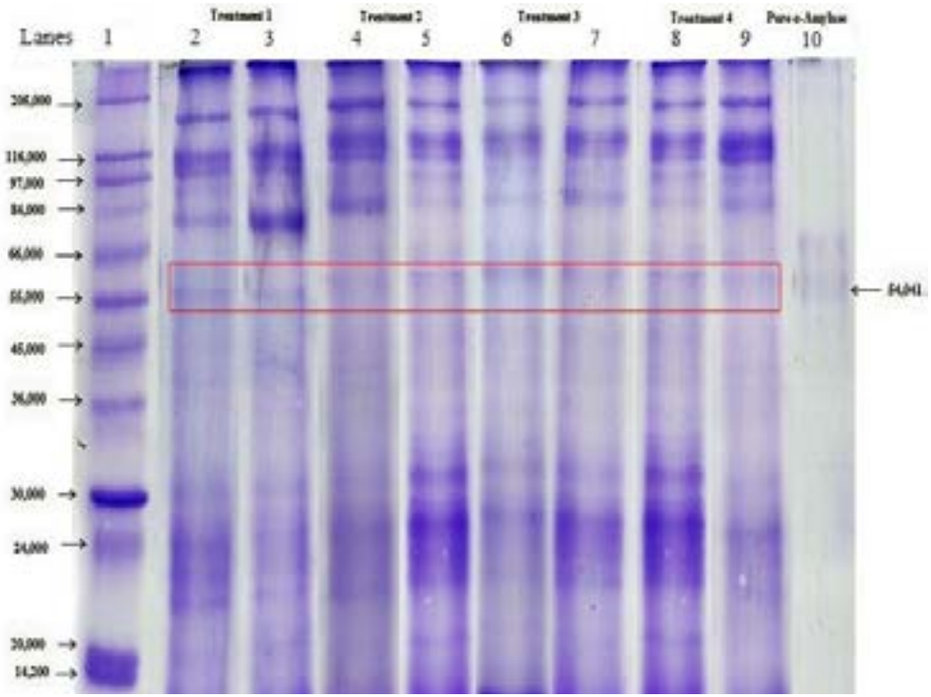

Figure 2. SDS-PAGE (12% resolving gel) of the α-amylase from crude duodenal digesta of different treatments. The α-amylase was visualized by Coomassie Brilliant Blue staining. Lane 1, Wide Molecular Weight Range SigmaMarkerTM; Lane 2: T1 (Basal diet) replicate 1; Lane 3: T1 (Basal diet) rep. 2; Lane 4: T2 (Basal diet + 10 ppm avilamycin) rep. 1; Lane 5: T2 (Basal diet + 10 ppm avilamycin) rep. 2; Lane 6: T3 (Basal diet + 150 ppm PE) rep 1; Lane 7: T3 (Basal diet + 150 ppm PE) rep. 2; Lane 8: T4 (Basal diet + 300 ppm PE) rep. 1; Lane 9: T4 (Basal diet + 300 ppm PE) rep. 2; Lane 10 contains pure pancreatic α-amylase.

the effects of the feed additives on the broiler's performance and growth through the analysis of enzyme secretion and activity, in relation to substrate digestibility and nutrient metabolism.

CONCLUSIONS AND RECOMMENDATIONS

The study showed no significant effects of added plant extracts as feed additives in broiler diets on their growth performance parameters, activity of crude duodenal enzymes (i.e. proteases and α-amylase) and average feed intake. However, examination of electrophoretograms of duodenal crude protein extracts showed differences in the protein profiles of the various treatments suggesting induction of duodenal enzyme secretion. Further purification of the proteins prior to SDS-PAGE analysis and enzyme activity assays is recommended. Improving replicability, reproducibility and varying the levels and composition of the feed additives may be useful in expanding the capability of this potential feed evaluation method.

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