Contribution of purified soybean trypsin inhibitor and exogenous protease to endogenous amino acid losses and mineral digestibility

A. S. Aderibigbe ,* A. J. Cowieson,† K. M. Ajuwon,* and O. Adeola*,[1]

*Department of Animal Sciences, Purdue University, West Lafayette IN 47907, USA; and †DSM Nutritional Products, Kaiseraugst 4303, Switzerland

ABSTRACT The primary objective of the current study was to evaluate the impact of trypsin inhibitor (**TI**) and exogenous protease supplementation on endogenous loss of amino acids (**AA**) in broiler chickens. A total of 384 Cobb-500 broiler chicks were allocated to 4 nitrogen-free diets, each with 8 replicate cages and 12 birds per replicate. The diets were arranged as a 2 × 2 factorial with factors being dietary TI (0 or 8,000 TIU/g) and exogenous protease (0 or 15,000 PROT/kg). Desired dietary TI concentration was achieved by addition of commercially available, purified soybean TI. There was no effect of TI or exogenous protease or their interaction on growth performance of birds. However, the endogenous loss of nitrogen (**N**) and all AA increased ($P < 0.05$) due to dietary TI concentration except for Cys. The increase in endogenous AA due to TI ranged from 17% for Thr to 52.2% for Trp. Exogenous protease had no effect on endogenous loss of N and all AA. There was no effect of TI or exogenous protease or their interaction on the AID of P, however AID of Ca, Fe, Mg, Mn, and Cu was reduced ($P < 0.05$) due to dietary TI. The AID of Cu ($P < 0.01$) and K ($P < 0.05$) improved with exogenous protease supplementation. Significant interactions ($P < 0.05$) between exogenous protease and TI existed for Zn, Mg, Cu, and Na. The concentration (g/kg DM intake) of crude mucin and sialic acid increased ($P < 0.05$) with increased dietary TI. Decreased trypsin ($P < 0.001$) and increased chymotrypsin ($P < 0.001$) activity in the pancreas were observed as a result of exogenous protease supplementation. In conclusion, the current study showed that TI increases the endogenous loss of AA and reduced the digestibility of minerals in broiler chickens. Furthermore, exogenous protease did not affect endogenous AA flow, irrespective of added purified dietary TI.

Key words: amino acid, endogenous loss, mineral, protease, trypsin inhibitor

2021 Poultry Science 100:101486
https://doi.org/10.1016/j.psj.2021.101486

INTRODUCTION

During the digestive process in the gastrointestinal tract, endogenous proteins from digestive secretions (saliva, bile, gastric, and pancreatic secretions as well as intestinal secretions), mucoproteins, sloughed intestinal epithelial cells, serum albumin, and amides (Nyachoti et al., 1997; Ravindran and Bryden, 1999) are continuously secreted into the lumen of the intestine. A previous estimate (Nasset and Ju, 1961) reveals that endogenous protein secretion may be five times as abundant as those of dietary origin and, about 60 to 79% of this gross endogenous secretion may be reabsorbed (Krawielitzki et al, 1990; Souffrant et al., 1993). However, the degree of reabsorption will vary depending on the relative ratio of individual endogenous components and their point of entry into the gut (Souffrant, 1991). Regardless of the intestinal site, net endogenous losses are the result of total secretion, reabsorption, and reutilization of reabsorbed endogenous protein. The unabsorbed portion is inevitably lost to the animal and referred to as basal (or diet-independent) endogenous losses, representing the amino acids that are lost irrespective of whether the animal is fed protein.

The level of endogenous amino acid (**EAA**) flow may also depend on the presence of antinutritive factors in the gut (Barth et al., 1993; Schulze, 1994). Schulze (1994) studied the effect of various inducing agents such as trypsin inhibitors (**TI**), lectins, and fiber, on EAA flow at the terminal ileum in pigs and found that endogenous nitrogen (**N**) secretions increased by 7.3, 5.2, and 0.04 g per g lectins, TI, and fiber respectively. For TI and fiber, there were linear increases in EAA flow with increasing amounts of the inducing agent (Schulze, 1994). While soybean meal (**SBM**) provides

Received April 27, 2021.
Accepted September 9, 2021.

[1]Corresponding author: ladeola@purdue.edu

an excellent source of protein for nonruminants, it contains variable amounts of TI depending on processing conditions (Newkirk, 2010). This may lead to increased TI intake which affects dietary protein digestion and amino acid utilization in nonruminants by inhibiting the activation of the proteolytic enzymes in the gut (Rawlings et al., 2004; Erdaw et al., 2017). On the other hand, exogenous protease supplementation is a widely accepted strategy to improve dietary protein utilization in nonruminants (Clarke and Wiseman, 2005; Erdaw et al 2017; Aderibigbe et al., 2020). Additionally, exogenous protease has been shown to improve EAA recovery in birds (Cowieson and Roos, 2016). Likewise, an in vitro trial (Huo et al., 1993) showed that exogenous protease can directly degrade TI in raw and low-temperature extruded soybean.

There are limited number of studies that report the direct impact of TI on EAA flow, and to the best of our knowledge, these have not been reported for broiler chickens. Therefore, the hypothesis of the current study was that TI and exogenous protease will affect the loss of endogenous protein in broiler chickens. To test this hypothesis, specific objectives were to 1) determine the contribution of purified soybean TI and exogenous protease to EAA flow and mineral digestibility in broiler chickens fed nitrogen-free diet (**NFD**); 2) evaluate the effect of exogenous protease in diets containing purified soybean TI on EAA flow and mineral digestibility in broiler chickens fed NFD.

MATERIALS AND METHODS

Protocols of animal experiments were reviewed and approved by the Purdue University Animal Care and Use Committee.

Experimental Diets and Birds

A total of 384 male 0-d-old Cobb 500 broiler chicks (Cobb-Vantress, Siloam Springs, AR) were procured from a commercial hatchery. Birds were individually tagged, weighed, raised in heated battery brooders (model SB 4 T; Alternative Design Manufacturing, Siloam Springs, AR) and fed a nutritionally adequate corn-SBM based diet (210 g/kg CP; 3,100 kcal/kg ME) until d 20 post hatching. Temperature and lighting were maintained as previously described by Aderibigbe et al. (2020). Twelve hours prior to experimental diet allocation, birds were fasted to empty the gut of residual dietary N. Subsequently on d 21 post hatching, using a 2 × 2 factorial arrangement, with 2 concentrations of dietary TI (0 or 8,000 TIU/g) and exogenous protease (0 or 15,000 PROT/kg; Ronozyme ProAct, DSM Nutritional Products, Kaiseraugst, Switzerland) the chicks were allotted to 4 experimental NFD (Table 1) in a randomized complete block design, with BW as a blocking factor, resulting in 8 blocks per treatment. Each diet comprised of 8 replicates cages and 12 birds per replicate cage. The TI used in the current study is a commercially available purified form of soybean TI (Sigma Aldrich, St. Louis, MO; EC 2329069). Titanium dioxide was included as an indigestible marker.

Table 1. Ingredient and calculated nutrient composition of experimental diets, as-fed basis.

Protease, PROT/kg:	0		15,000	
Purified trypsin inhibitor, TIU/g:	0	8,000	0	8,000
Ingredients, g/kg				
Dextrose	615.5	565.5	605.5	555.5
Cornstarch	200.5	200.5	200.5	200.5
Soybean oil	50.0	50.0	50.0	50.0
Solka floc	50.0	50.0	50.0	50.0
Ground limestone[1]	15.5	15.5	15.5	15.5
Monocalcium phosphate[2]	21.0	21.0	21.0	21.0
Potassium carbonate	2.6	2.6	2.6	2.6
Magnesium oxide	2.0	2.0	2.0	2.0
Sodium bicarbonate	7.5	7.5	7.5	7.5
Choline chloride	2.5	2.5	2.5	2.5
Potassium chloride	2.9	2.9	2.9	2.9
Vitamin-mineral premix[3]	5.0	5.0	5.0	5.0
Trypsin inhibitor premix[4]	0.0	50.0	0.0	50.0
Ronozyme ProAct premix[5]	0.0	0.0	10.0	10.0
Titanium dioxide premix[6]	25.0	25.0	25.0	25.0
Total	1,000	1,000	1,000	1,000
Calculated composition				
MEn, kcal/kg	3,606	3,606	3,606	3,606
Crude protein, g/kg	0.60	0.60	0.60	0.60
Ether extract, g/kg	48.50	48.50	48.50	48.50
Ca, g/kg	9.10	9.10	9.10	9.10
Available P, g/kg	4.52	4.52	4.52	4.52
DEB, mEq/kg diet[7]	90.98	90.98	90.98	90.98
Analyzed composition				
Crude protein, g/kg	1.16	3.15	1.24	2.58
Trypsin inhibitor, TIU/g[8]	LOD	6,244	LOD	5,921

[1]38% Ca.
[2]16% Ca, 21% P.
[3]Supplied the following per kg diet: vitamin A, 8,575 IU; vitamin D_3, 4,300 IU; vitamin E, 28.58 IU; menadione, 7.30 mg; riboflavin, 9.15 mg; D-pantothenic acid, 18.33 mg; niacin, 73.5 mg; choline chloride, 1,285 mg; vitamin B_{12}, 0.02 mg; biotin, 0.09 mg; thiamine mononitrate, 3.67; folic acid, 1.65 mg; pyridoxine hydrochloride, 5.50 mg; I, 1.85 mg; Mn, 178.5 mg; Cu, 7.40 mg; Fe, 73.5 mg; Zn, 178.5 mg.
[4]Purified trypsin inhibitor (PTI) from soybeans product contains 9,000,000 TIU/g. 1g of STI added to 55.25 g of corn supplies 160,000 TIU/ g of premix. 50 g/kg premix delivers 8,000,000 TIU/kg in feed.
[5]Product contained 75,000 PROT/g. 1 g Protease added to 49 g ground corn supplied 1,500 PROT/ g premix. 10 g premix delivered 15,000 PROT/ kg feed.
[6]1 g of Titanium dioxide added to 4 g of dextrose.
[7]Dietary electrolyte balance (mEq/kg diet) was calculated as Na+K-Cl.
[8]LOD, limit of detection.

Sampling Procedures

Birds were fed experimental diets on an ad libitum basis for a 3-d period, and mortality records was taken daily. On d 24 post hatching, feed intake was measured per cage and birds were individually weighed. Gain to feed ratio was calculated and corrected for the body weight of any bird that died or was culled during the experimental period. Subsequently, all birds were euthanized by CO_2 asphyxiation and eviscerated for sample collection. Ileal digesta was collected from the distal two-thirds of the section of the ileum between the Meckel's diverticulum and approximately 2-cm cranial to the ileocecal junction. The ileal digesta was pooled within

cage by flushing with distilled water into plastic containers and stored at −20°C prior to nutrient analyses.

Digestive Enzyme, Crude Mucin, and Sialic acid Assays

The pancreas was collected from 2 median bird per replicate cage, frozen in liquid nitrogen and stored at −80°C until required for enzyme assay. Enzymes activities were determined as previously described by Aderibigbe et al. (2020) using a commercially available assay kit (Sigma Chemical Co, St. Louis, MO). Crude mucin and sialic acid concentration were determined based on methods previously described by Horn et al. (2009). Briefly, excreta samples were freeze-dried for 96 h and subsequently ground to pass through a 0.5-mm screen (Retsch ZM 100, GmbH, Haan, Germany). Subsequently, 3 g of the lyophilized excreta sample was placed in a 50-mL plastic centrifuge tube. Twenty milliliters of chilled NaCl solution (0.15 M NaCl, 0.02 NaN_3, kept at 4°C) was added to the excreta sample and homogenized for 30 s (T25 Basic, IKA Corp., Staufen, Germany). The mixture was then centrifuged at 12,000 × *g* for 20 min at 4°C, and the soluble supernatant was decanted into a 50-mL pre-weighed tube. For extraction of mucin proteins, 15 mL of chilled (4°C) absolute ethanol was added to the supernatant and allowed to sit overnight at −20°C. The mixture was then centrifuged at 1,400 × *g* for 10 min at 4°C and the mucin pellet was retained. The mucin pellet was washed with a mixture of 10 mL of chilled NaCl solution (0.15 M NaCl, 0.02 NaN_3, kept at 4°C) and 15 mL of chilled absolute ethanol and the sample was extracted overnight at −20°C. Subsequently, the mixture was then centrifuged at 1,400 × *g* for 10 min at 4°C and the wash-extraction cycle was repeated until a clear supernatant was obtained. Water was removed from the mucin pellet by suction and the pellet was then weighed to obtain crude mucin yield. The pellet was then dissolved in 2 mL of distilled water and immediately frozen at −40°C.

Sialic acid concentration was determined from the purified crude mucin samples. Briefly, 100 μL of crude mucin solution was diluted with 100 μL of distilled water in a 1.5-mL microcentrifuge tube and 200 μL of Bial reagent (Ward's Science, Rochester, NY) was added to the samples and heated for 15 min at 100°C in a water bath (Precision, GCA Corp., College Park, MD). The samples were cooled in room temperature water for 5 min. One mL of isoamyl alcohol (Sigma Chemical Co) was added to the sample, vigorously vortexed, and chilled on ice for 5 min. The samples were then centrifuged at 1,200 × g for 1 min and 200 μL of the upper color phase was gently transferred to a 96-well plate. Absorbance was measured at 560 nm on a UV spectrophotometer (Spark 10M, TECAN, Baldwin Park, CA). Sialic acid concentration was determined from regression of the standard (N-acetylneuraminic acid; Sigma Chemical Co, St. Louis, MO) vs. absorbance.

Chemical Analyses and Calculations

Ileal digesta were freeze-dried for 96 h and subsequently ground to pass through a 0.5-mm screen (Retsch ZM 100, GmbH). The dry matter (**DM**) content of diets and digesta were analyzed by drying overnight at 105°C (Precision Scientific Co., Chicago, IL; method 934.01; AOAC, 2006). Nitrogen content was determined by combustion using a LECO FP-428 nitrogen analyzer (LECO Corp., St. Joseph, MI) with EDTA as a calibration standard. Samples for amino acid (**AA**) analysis were prepared using a 24-h hydrolysis in 6 N hydrochloric acid at 110°C under an atmosphere of N. Samples were oxidized in performic acid prior to acid hydrolysis for methionine and cysteine analyses. Samples for tryptophan analysis were hydrolyzed using barium hydroxide. Amino acids in hydrolysates were determined by cation-exchange chromatography coupled with post-column derivatization (AOAC, 2000; method 982.30 E [a, b, c]). Following wet digestion using a mixture of nitric and perchloric acid, concentrations of Ca, Fe, Zn, Mg, Mn, Cu, Na, and K in diets and lyophilized ileal digesta samples was determined with appropriate standards using an atomic absorption spectrometer (AAnalyst 300; Perkin Elmer, Norwalk, CT; method 985.01; AOAC, 2006). Concentration of P was determined by spectrophotometry at 620 nm (Spectra Count, model AS1000, Packard, Meriden, CT; AOAC, 2006). Titanium concentration was measured on a UV spectrophotometer (Spark 10M, TECAN) following the method of Short et al. (1996).

The index method was used to calculate the endogenous flow of N and AA in the ileal digesta, crude mucin, and sialic acid in the excreta and apparent ileal digestibility (**AID**) of macro and trace minerals, according to the following equations:

$$\text{Endogenous loss, g/kg DM intake} = P_o \times (Ti_i/Ti_o);$$

$$\text{AID, \%} = 100 - [(Ti_i/Ti_o) \times (Q_o/Q_i) \times 100]$$

where Ti_i and Ti_o are the respective titanium concentrations (g/kg DM) in experimental diets and output (ileal digesta or excreta); P_o is the concentration of N, AA, crude mucin, or sialic acid (g/kg DM) in the ileal digesta or excreta; Q_i and Q_o are the respective concentrations (g/kg DM) of macro and trace minerals in experimental diets and excreta.

Ileal endogenous energy flow (**IEEF**) was calculated according to the following equations:

$$\text{IEEF, kcal/kg DM intake} = (F_{aa} \times G_{aa})/1000$$

where F_{aa} is the endogenous flow of AA (g/kg DM intake) in the ileal digesta and G_{aa} is the gross energy of individual AA (kcal/kg) adapted from Boisen and Verstegen (2000).

Statistical Analyses

Data were analyzed as a randomized complete block design using the GLM procedures of SAS (SAS Inst.

Inc., Cary, NC). Initial body weight was used as the blocking criterion. Block was treated as a random factor. The main effects of TI and exogenous protease and the interaction were tested accordingly and an α level of 0.05 was considered significant. Where interactions exist, Tukey's mean separation test was used to make pairwise comparisons.

RESULTS

Overall, there were 11 mortalities during the 3-d trial period, and on-site postmortem examinations did not reveal any direct link to specific dietary treatment. Although the mortality was relatively high, the exact cause was unclear, and is more likely related to the non-physiological nature of the NFD. The effect of purified TI and exogenous protease on growth performance of broiler chickens NFD are shown in Table 2. There were no effects of TI or exogenous protease or their interaction on growth performance of birds. As shown in Table 3, there was no interaction between TI and exogenous protease on EAA loss in birds. Exogenous protease had no effect on endogenous loss of N and all AA. The calculated energetic losses associated with the endogenous protein flows are presented in Table 4. Similar to AA, the ingestion of TI increased ($P < 0.05$) the energy loss associated with EAA loss, except Cys and exogenous protease addition did not reduce energy loss in EAA. Table 5 shows the AID of minerals in broiler chickens fed an NFD containing purified TI and exogenous protease. There were no effects of TI or exogenous protease or their interaction on the AID of P. However, the AID of Ca, Fe, Mg, Mn, and Cu were reduced ($P < 0.05$) due to dietary TI. Exogenous protease improved the AID of Cu ($P < 0.01$) and K ($P < 0.05$). Furthermore, significant interactions ($P < 0.05$) between exogenous protease and TI existed for Zn, Mg, Cu, and Na. Added TI reduced ($P < 0.05$) the AID of Zn reduced by 43.7% in birds fed diets containing protease. Exogenous protease increased

Table 2. Growth performance of broiler chickens fed nitrogen-free diets supplemented with protease (PROT/kg) and purified trypsin inhibitor (TIU/g), from d 21 to 24 post hatching.

	Protease											
	0		15,000									
	TI		TI			Protease		TI		*P*-value		
Item[1]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
D 21 BW, g	631.6	631.2	631.7	631.6	0.44	631.4	631.7	631.7	631.4	0.614	0.614	0.664
D 24 BW, g	550.1	550.8	551.1	552.7	4.09	550.4	551.9	550.6	551.8	0.723	0.776	0.903
BW gain, g	−81.2	−81.3	−80.6	−79.2	4.07	−81.2	−79.9	−80.9	−80.3	0.751	0.872	0.863
Feed intake, g	204.5	240.4	231.1	248.9	18.1	222.4	239.9	217.8	244.6	0.342	0.151	0.622
G:F, g/kg	−424.7	−347.9	−365.7	-318.3	44.4	−386.4	−341.9	-395.2	−333.1	0.329	0.177	0.744
N	8	8	8	8		16	16	16	16			

[1]Abbreviations: G:F, gain to feed ratio; P, protease; TI, trypsin inhibitor.

Table 3. Ileal endogenous flow (mg/kg DMI) of nitrogen and amino acid in broiler chickens fed nitrogen-free diets supplemented with exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g), at d 24 post hatching.

	Protease											
	0		15,000									
	TI		TI			Protease		TI		*P*-value		
Item[1]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
Nitrogen	1,975	2,215	1,725	2,341	157.5	2,095	2,033	1,850	2,278	0.699	0.013	0.244
Indispensable AA												
Arg	434	575	376	607	44.5	505	492	405	591	0.773	< 0.001	0.325
His	208	262	189	280	17.6	235	235	199	271	0.993	< 0.001	0.310
Ile	523	622	452	669	43.2	573	561	487	646	0.787	0.001	0.185
Leu	791	960	681	1,019	66.6	876	849	736	989	0.701	0.001	0.217
Lys	414	552	388	591	47.9	483	490	401	571	0.890	0.002	0.499
Met	146	169	119	190	14.8	158	155	133	179	0.851	0.004	0.120
Phe	461	569	399	596	40.7	515	497	429	583	0.667	0.001	0.290
Thr	860	908	747	973	64.2	884	860	804	941	0.713	0.045	0.179
Trp	74	102	65	108	10.4	88	87	69	105	0.885	0.003	0.491
Val	887	982	743	1,044	73.2	934	893	815	1,013	0.585	0.013	0.174
Dispensable AA												
Ala	500	582	429	634	42.1	541	531	465	608	0.823	0.003	0.156
Asp	1,008	1,203	883	1,294	85.1	1,105	1,088	945	1,248	0.844	0.002	0.218
Cys	474	493	381	518	41.9	483	449	427	506	0.436	0.076	0.176
Glu	1,142	1,359	981	1,477	103.0	1,251	1,229	1,062	1,418	0.838	0.002	0.193
Gly	625	712	531	773	52.6	668	652	578	742	0.759	0.005	0.155
Pro	689	748	572	814	55.6	719	693	631	781	0.648	0.013	0.114
Ser	703	798	599	873	63.9	751	736	651	835	0.819	0.009	0.178
Tyr	368	434	324	469	30.9	401	397	346	452	0.893	0.003	0.215
N	8	8	8	8		16	16	16	16			

[1]Abbreviations: AA, amino acid; P, protease; TI, trypsin inhibitor.

Table 4. Calculated ileal endogenous energy flow (kcal/kg DMI)[1] associated with endogenous amino acids in broiler chickens fed nitrogen-free diets supplemented with exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g), at d 24 post hatching.

	Protease											
	0		15,000									
	TI		TI			Protease		TI		*P*-value		
Item[2]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
Indispensable AA												
Arg	2.22	2.94	1.93	3.11	0.227	2.58	2.52	2.07	3.02	0.773	< 0.001	0.325
His	1.08	1.36	0.98	1.45	0.091	1.22	1.22	1.03	1.40	0.993	0.001	0.310
Ile	3.45	4.11	2.98	4.42	0.285	3.78	3.70	3.21	4.26	0.787	0.001	0.185
Leu	5.22	6.33	4.49	6.72	0.439	5.78	5.61	4.86	6.53	0.701	0.001	0.217
Lys	2.33	3.10	2.18	3.32	0.269	2.71	2.75	2.25	3.21	0.890	0.002	0.499
Met	0.65	0.75	0.53	0.85	0.065	0.70	0.69	0.59	0.80	0.851	0.004	0.120
Phe	3.11	3.84	2.69	4.02	0.275	3.47	3.35	2.90	3.93	0.667	0.001	0.290
Thr	3.54	3.73	3.07	4.00	0.264	3.63	3.54	3.30	3.87	0.713	0.045	0.179
Trp	0.49	0.67	0.43	0.71	0.068	0.58	0.57	0.46	0.69	0.885	0.003	0.491
Val	5.29	5.87	4.44	6.24	0.437	5.58	5.34	4.87	6.05	0.585	0.013	0.174
Dispensable AA												
Ala	2.18	2.53	1.87	2.76	0.183	2.35	2.31	2.02	2.64	0.823	0.003	0.156
Asp	2.91	3.48	2.55	3.74	0.246	3.20	3.15	2.73	3.61	0.844	0.002	0.218
Cys	2.08	2.17	1.68	2.28	0.185	2.13	1.98	1.88	2.22	0.436	0.076	0.176
Glu	4.17	4.97	3.59	5.40	0.377	4.57	4.50	3.88	5.19	0.838	0.003	0.193
Gly	1.93	2.19	1.64	2.38	0.162	2.06	2.01	1.78	2.29	0.759	0.005	0.155
Pro	3.91	4.23	3.24	4.61	0.315	4.07	3.92	3.57	4.42	0.648	0.014	0.114
Ser	2.32	2.63	1.98	2.88	0.211	2.48	2.43	2.15	2.76	0.819	0.009	0.178
Tyr	2.19	2.58	1.93	2.79	0.184	2.39	2.36	2.06	2.69	0.893	0.003	0.215
N	8	8	8	8		16	16	16	16			

[1]Calculated using the flow of each AA in Table 3 and the gross energy of AA adapted from Boisen and Verstegen (2000). Gross energy estimates (kcal/kg) for individual AA from Boisen and Verstegen (2000) are as follows: Arg, 5,115; His, 5,186; Ile, 6,597; Leu, 6,597; Lys, 5,617; Met, 4,446; Phe, 6,740; Thr, 4,111; Trp, 6,568; Val; 5,975; Ala, 4,350; Asp, 2890; Cys, 4,398; Glu, 3,657; Gly, 3,083; Pro, 5,664; Ser, 3,298; Tyr, 5,951.
[2]Abbreviations: AA, amino acid; P, protease; TI, trypsin inhibitor.

($P < 0.05$) the AID of Mg irrespective of dietary TI concentration. Although exogenous protease increased ($P < 0.05$) the AID of Cu, there was no change in effect when supplemented to diets containing added TI. The AID of Na increased ($P < 0.05$) with added TI, similar to the effect of exogenous protease. However, exogenous protease did not affect the AID of Na in diets containing added TI.

Table 6 shows the effect of purified TI and exogenous protease on excretion of crude mucin and sialic acid in birds fed NFD. Dietary TI concentration significantly increased ($P < 0.05$) the excretion (g/kg DM intake) of crude mucin and sialic acid. As shown in Table 7, pancreas weight was unaffected by TI or exogenous protease. Exogenous protease resulted in a reduction in trypsin activity ($P < 0.001$) but increased pancreatic chymotrypsin activity ($P < 0.001$).

DISCUSSION

The primary objective of this study was to evaluate the direct contribution of TI to EAA losses in broiler chickens, and whether this is affected by exogenous protease supplementation. The impact of soybean TI on growth performance and nutrient digestibility in broiler chickens is widely reported (Clarke and Wiseman,2005; Erdaw et al., 2017; Aderibigbe et al., 2020). Currently, there are no published data on the effect of TI on

Table 5. Apparent ileal digestibility (%) of minerals in broiler chickens fed nitrogen-free diets supplemented with exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g), at d 24 post hatching.

	Protease											
	0		15,000									
	TI		TI			Protease		TI		*P*-value		
Item[1]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
P	54.9	62.6	63.8	57.5	3.59	58.8	60.7	59.4	60.1	0.612	0.839	0.065
Ca	59.2	53.3	70.5	49.4	5.95	56.2	59.9	64.9	51.3	0.541	0.034	0.215
Fe	−25.7	−82.9	1.72	−84.5	22.09	−54.3	-41.4	−11.9	−83.7	0.965	0.004	0.519
Zn	48.9[ab]	56.6[a]	60.2[a]	33.9[b]	5.48	52.8	47.1	54.6	45.2	0.313	0.104	0.006
Mg	−82.6[b]	−80.1[b]	−21.3[a]	−90.6[b]	14.74	−81.3	−55.9	−51.9	−85.3	0.101	0.034	0.024
Mn	90.4	86.4	89.6	85.4	1.39	88.4	87.5	90.0	85.9	0.522	0.008	0.933
Cu	−31.3[bc]	−63.8[c]	14.9[a]	−10.1[ab]	10.92	−47.5	2.4	−8.2	−37.0	< 0.001	0.015	0.039
Na	35.2[b]	62.7[a]	65.2[a]	51.8[ab]	6.15	48.9	58.5	57.2	50.2	0.136	0.267	0.003
K	−9.62	−23.9	18.1	9.59	13.0	−16.8	13.8	4.2	−7.2	0.029	0.391	0.825
N	8	8	8	8		16	16	16	16			

[1]P, protease; TI, trypsin inhibitor.
[a-c]Means within the same row with different superscripts are different at $P < 0.05$.

Table 6. Effect of exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g) on crude mucin and sialic acid excretion in broiler chickens, at d 24 post hatching.

	Protease											
	0		15,000									
	TI		TI			Protease		TI		*P*-value		
Item[1]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
CM, g/kg excreta	317.2	342.3	312.2	353.1	25.56	329.8	332.7	314.7	347.7	0.911	0.211	0.759
CM, g/kg DMI	70.2	71.8	57.5	77.4	4.97	71.0	67.4	63.8	74.6	0.479	0.042	0.081
SA, mg/kg excreta	187.8	270.0	189.1	241.3	23.73	228.9	215.2	188.4	255.7	0.568	0.010	0.534
SA, mg/kg DMI	40.2	50.7	34.1	48.5	5.56	45.3	41.3	37.1	49.6	0.477	0.035	0.739
N	8	8	8	8		16	16	16	16			

[1]Abbreviations: CM, crude mucin; P, protease; SA, sialic acid; TI, trypsin inhibitor.

Table 7. Effect of exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g) on pancreas weight and proteolytic enzyme secretion in broiler chickens, at d 24 post hatching.

	Protease											
	0		15,000									
	TI		TI			Protease		TI		*P*-value		
Item[1]	0	8,000	0	8,000	SEM	0	15,000	0	8,000	Protease	TI	P × TI
Pancreas wt., g	1.39	1.47	1.35	1.32	0.075	1.42	1.33	1.36	1.39	0.231	0.698	0.504
Pancreas wt., g/kg BW	2.55	2.66	2.57	2.50	0.143	2.60	2.54	2.56	2.58	0.652	0.909	0.532
Trypsin, U/mg	5.78	5.89	5.11	4.62	0.247	5.83	4.86	5.44	5.26	< 0.001	0.438	0.252
Chymotrypsin, U/mg	4.53	4.04	5.25	5.34	0.240	4.28	5.30	4.88	4.69	< 0.001	0.466	0.240
N	8	8	8	8		16	16	16	16			

[1]Abbreviations: P, protease; TI, trypsin inhibitor.

endogenous secretion in birds. Because NFD is a commonly used assay for estimating EAA flow in broiler nutrition, we also evaluated the digestibility of macro and trace minerals in an experimental NFD and whether or not this is affected by TI concentration. Our data showed that growth performance of birds was unaffected by dietary TI concentration or exogenous protease supplementation. This is not surprising because all the birds were fed NFD, which is not expected to support tissue accretion. Compared to conventional diets, feeding NFD, irrespective of protease or TI addition, resulted in losses in BW and feed efficiency over the 3-d trial period. The BW losses observed for birds in the current trial may be attributed to increased body protein degradation because of the sustained dietary protein deficiency, which becomes critical for sustenance of biological functions under NFD feeding. As previously noted, the primary purpose of the current study was not to evaluate the growth performance of birds, as the sensitivity of the performance variables may be affected by the protein deficiency. An important observation, however, is the lack of effect of TI on feed intake response of birds. This is especially crucial because variances in feed intake will influence endogenous nutrient losses between dietary treatments (Boisen and Moughan, 1996; Adedokun et al., 2011) and potentially confounding any TI effect.

Endogenous AA secretion in the current study falls within the range of previously published data typical of the NFD assay (Ravindran, 2021) and was strongly affected by added dietary TI. This is similar to a previous report by Barth et al. (1993) who observed a 7.8-fold increase in endogenous protein secretion when pigs were fed casein meal containing 3,000 mg of purified TI per test meal. This observation suggests that the influence of TI on metabolic amino acid economy may be quantitatively more affected by a loss of endogenous protein. However, it is difficult to ascertain the specific contribution of the sources of secreted endogenous protein, because digesta collected at the terminal ileum represents the net result of the digestive dynamics of endogenous sources along the entire digestive tract (Hee et al., 1988; Nyachoti et al., 1997; Ravindran and Bryden, 1999). Indeed, Souffrant et al. (1993) estimated that about 79% of the gross endogenous secretion is reabsorbed, but the degree of reabsorption will vary depending on the relative ratio of individual endogenous components and their point of entry into the gut. For instance, when digestive enzymes dominate the endogenous flow, the proteins pass through the duodenum and jejunum where there is greater opportunity for digestion and absorption. However, opportunity for digestion is lower and relatively higher endogenous losses will be inevitable at the ileal level if mucus secretion or desquamation is significant, which particularly occurs more distal to the duodenum.

In the current study, and similar to previous observations (Adedokun et al., 2007), 4 AA (Glu, Asp, Ser, and Thr) were predominantly identified in endogenous AA, which could represent up to 34% of total AA flow in the broiler fed an NFD (Adedokun et al., 2007). In the current study, the average increase in EAA loss attributable to TI was 32.3% and was more apparent in Trp (52.2%), Arg (45.9%), and Lys (42.4%). Generally, the

impact of TI on EAA secretion was higher for indispensable AA, which may have great nutritional consequences in birds. Interestingly, pancreatic enzyme secretion or pancreas weight was not affected by TI, indicative of no TI-induced hypertrophy. This is partly expected due to a lack of dietary protein needed for constant stimulation of the pancreas through the inhibitory actions of TI on trypsin. This also suggests that the pancreatic juice may not be a significant source for TI-induced EAA flow, however, this inference only applies to NFD conditions. Notwithstanding, digestive enzymes are degraded during the digestion process, absorbed into blood, reaccumulated by the pancreas, and reutilized (Rothman et al., 2002). Indeed, it is argued that a large fraction of the intact digestive enzymes secreted by the pancreas are absorbed and recycled in an enteropancreatic circulation, instead of being reduced to their constituent amino acids in the intestines (Rothman et al., 2002). Perhaps TI disrupts the digestive enzyme recycling process that may result in a surge in EAA flow, however, this need to be investigated.

Supplemental protease had no impact on the reduction or recovery of EAA secretion irrespective of dietary TI concentration, and contrary to a previous report (Huo et al., 1993) did not appear to be involved in inactivation or degradation of the TI. However, in vitro trials such as the report by Huo et al. (1993) may not be easily replicated in vivo. Perhaps fluctuations in the in vivo environment, especially in birds fed a protein-free nonphysiological diet like the NFD, interferes with the exogenous protease ability to recognize and degrade the TI. However, these postulations have to be experimentally verified. Even more importantly, most commercially available proteases are chymotrypsin-like semi-alkaline endopeptidases and compared with an acid protease, may have limited capacity to degrade TI and other antigenic proteins (Hessing et al., 1996; Rooke et al., 1998). Although alkaline, the protease used in the current study may likely hydrolyze TI, but this reaction would more likely occur further down the gut (pH optima of Ronozyme ProAct is >6; Cowieson and Roos, 2016). However, this might be too late to prevent TI-induced EAA flow, especially if most of the secretions originated from the upper gastrointestinal tract (e.g., HCl, pepsin). Asides from lack of substrate with the use of NFD, the exogenous protease action may be limited by the low total EAA flow compared with a conventional diet. Furthermore, using data from Boisen and Verstegen (2000), which reported that the gross energy (**GE**) of amino acids ranges from 2,890 kcal/kg for Asp to 6,740 kcal/kg for Phe, we calculated the GE of the lost proteins based on their amino acid composition and gross flow. Similar to phytic acid (Cowieson et al., 2008), endogenous protein loss from the terminal ileum of broilers as a result of TI ingestion had considerable energetic consequences for the host. This is without considering the substantial amount of energy required in the synthesis of the endogenous proteins in the first place.

A major source of EAA secretion is the intestinal mucus glycoprotein. In the current study, and similar to phytic acid (Cowieson et al., 2004), TI increased the excretion of crude mucin and its metabolic marker, sialic acid (Jourdian et al., 1971) indicative of continuous sloughing of the mucus layer. However, contrary to previous reports (Peek et al., 2009; Cowieson and Roos, 2014) exogenous protease did not alleviate loss of mucoproteins from the gut. Cowieson and Roos (2014) showed a significant correlation between the AA profile of intestinal mucin and the effect of exogenous protease on AA digestibility and submits that part of the beneficial effect of exogenous protease may be mediated via a reduction in the loss of mucoprotein from the intestine. Although the mechanism by which this may occur is not clear, it was suggested (Cowieson and Roos, 2014) that exogenous protease may likely reduce the secretion of HCl and pepsin in the gastric phase of digestion, reducing the need for mucin as a protective agent in the intestine. However, as previously discussed, the observed lack of mucoprotective capacity by protease may be related to the nonphysiological nature of the NFD or the absence of protein in the feed or both. Intestinal mucoprotein is highly resistant to enzymatic hydrolysis (Montagne et al., 2004), compared to pancreatic secretions, which hinders AA recycling and is predominated by Thr, Pro, Ser, and Glu in the ileal flow (Lien et al., 2001; Ravindran, 2021). This disruption in thickness and fluidity of the mucosal layer by TI would also disrupt nutrient digestion, absorption and intestinal barrier function (Smirnov et al., 2004). Interestingly, a previous study (Sambeth et al., 1967) identified TI as a stimulant of gall bladder contraction in birds. The resultant increase in bile production will increase EAA flow, especially for taurine (data not included), which almost exclusively conjugates with bile acid in birds (Bremmer, 1958; Ravindran, 2021).

The current study also provides novel data on mineral digestibility in broiler chickens fed an experimental NFD and offers valuable insight into possible interactions of macro and trace minerals with TI. Feeding birds with NFD resulted in negative digestibility values for Fe, Mg, Cu, and K indicating increased endogenous flow at the terminal ileum, which was further exacerbated by added TI. Increased endogenous mineral excretion may be explained by the interactions of various cations with the mucus components, where divalent ions have a greater affinity than monovalent ions for the mucins (Powell et al., 1999). Similarly, Cowieson et al. (2004) and Woyengo et al. (2009) previously reported increased endogenous excretion of Fe, Mg, Na, and S in broiler chickens and pigs due to phytic acid ingestion. Phytic acid is a potent chelator of mineral ions (Maenz et al., 1999) and the observations in the current study suggests similar propensity of TI to form insoluble complexes with minerals, which is eventually excreted. Exogenous protease improved the digestibility of Cu, irrespective of dietary TI concentration in the current study. This is an interesting observation because aside from the primary function in enzyme systems within cells, Cu intake is the main determinant of bone strength (Roughead and Lukaski, 2003). This is ascribed to a Cu-containing

enzyme, lysyl oxidase, which is responsible for enhancing bone strength (Ilich and Kerstetter, 2000). In fact, Cu and Fe deficiency inhibits bone growth and decreases bone strength even when Ca and P levels are adequate (Medeiros et al., 1997). With few exceptions, the negative digestibility values observed in the current study suggest that the impact of protease may be more related to reductions in endogenous flow rather than improved dietary retention of the minerals. This may be a more appropriate description of the exogenous protease effect because of the consistent weight loss of birds as a result of being fed a NFD. It is unclear why addition of exogenous protease affected the digestibility of Zn and Mg in birds fed NFD containing TI. It is pertinent to state that a careful interpretation of these data may be required because mineral intake through the water source was unaccounted for in the current study. However, drinking water may not be a major source of minerals for broilers and due to high variability of individual daily water consumption, it is impossible to calculate mineral intakes from drinking water (Underwood, 1999). More importantly, complex interactions among minerals exist in the gut, which is a major cause of variation in availability and utilization or endogenous secretion. For instance, Zn absorption is impaired by Fe (Solomons and Jacob, 1981), while excess Zn interferes with Fe incorporation into ferritin (Settlemire and Matrone, 1967).

It can be concluded that the ingestion of TI by broilers increased the excretion of EAA, minerals, crude mucin, and sialic acid. Although supplementing with protease differentially affected proteolytic enzyme secretion by the pancreas, it did not reduce EAA losses, irrespective of added purified dietary TI. However, there were significant interactions between exogenous protease and TI for the AID of Zn, Mg, Cu, and Na. In particular, the AID of Cu and Mg increased with protease supplementation but protease reduced or caused no change in other mineral utilization in diets containing added TI. It should be noted that the effects observed in the current study were obtained in birds fed NFD, and it is unclear how these will be mediated in birds fed on conventional diets, containing SBM with variable TI concentrations. However, these results emphasize the impact of trypsin inhibitor on endogenous nutrient loss and hence should be considered when formulating poultry diets.

ACKNOWLEDGMENTS

The authors thank Cobb-Vantress, Monticello, KY for donating the chicks and Pat Jaynes for her technical assistance and help with this study.

DISCLOSURES

The authors declare that there is no conflict of interest.

REFERENCES

Adedokun, S. A., O. Adeola, C. M. Parsons, M. S. Lilburn, and T. J Applegate. 2011. Factors affecting endogenous amino acid flow in chickens and the need for consistency in methodology. Poult. Sci. 90:1737–1748.

Adedokun, S. A., C. M. Parsons, M. S. Lilburn, O. Adeola, and T. J Applegate. 2007. Endogenous amino acid flow in broiler chicks is affected by the age of birds and method of estimation. Poult. Sci. 86:2590–2597.

Aderibigbe, A., A. J. Cowieson, J. O. Sorbara, G. Pappenberger, and O. Adeola. 2020. Growth performance and amino acid digestibility responses of broiler chickens fed diets containing purified soybean trypsin inhibitor and supplemented with a mono-component protease. Poult. Sci. 99:5007–5017.

AOAC. 2000. Official Methods of Analysis. 17th ed. Assoc. Off. Anal. Chem., Arlington, VA.

AOAC. 2006. Official Methods of Analysis. 18th ed. Assoc. Off. Anal. Chem., Gaithersburg, MD.

Barth, C. A., B. Lunding, M. Schmitz, and H. Hagemeister. 1993. Soybean trypsin inhibitor (s) reduce absorption of exogenous and increase loss of endogenous protein in miniature pigs. J. Nutr. 123:2195–2200.

Boisen, S., and P. J. Moughan. 1996. Dietary influences on endogenous ileal protein and amino acid loss in the pig—a review. Acta. Agr. Scand. A-An. 46:154–164.

Boisen, S., and M. W. A. Verstegen. 2000. Developments in the measurement of the energy content of feeds and energy utilisation in animals. Pages 57−76 in Feed Evaluation: Principles and Practice. Wageningen Pers, Wageningen, the Netherlands.

Bremmer, J. 1958. Species differences in the conjugates of free bile acids. Biochem. J. 63:507–513.

Clarke, E., and J. Wiseman. 2005. Effects of variability in trypsin inhibitor content of soya bean meals on true and apparent ileal digestibility of amino acids and pancreas size in broiler chicks. Anim. Feed Sci. Technol. 121:125–138.

Cowieson, A. J., T. Acamovic, and M. R. Bedford. 2004. The effects of phytase and phytic acid on the loss of endogenous amino acids and minerals from broiler chickens. Br. Poult. Sci. 45:101–108.

Cowieson, A. J., V. Ravindran, and P. H. Selle. 2008. Influence of dietary phytic acid and source of microbial phytase on ileal endogenous amino acid flows in broiler chickens. Poult. Sci. 87:2287–2299.

Cowieson, A. J., and F. F. Roos. 2014. Bioefficacy of a mono-component protease in the diets of pigs and poultry: a meta-analysis of effect on ileal amino acid digestibility. J. Appl. Anim. Nutr. 2:13–21.

Cowieson, A. J., and F. F. Roos. 2016. Toward optimal value creation through the application of exogenous mono-component protease in the diets of non-ruminants. Anim. Feed. Sci. Tech. 221:331–340.

Erdaw, M. M., S. Wu, and P. A. Iji. 2017. Growth and physiological responses of broiler chickens to diets containing raw, full-fat soybean and supplemented with a high-impact microbial protease. Asian-Austr. J. Anim. Sci. 30:1303.

Hee, J., W. C. Sauer, and R. Mosenthin. 1988. The measurement of pancreatic secretions in the pig with the pouch technique. J. Anim. Physio. Nutr. 60:241–248.

Hessing, G. C., H. Van Laarhoven, J. A. Rooke, and A. Morgan. 1996. Quality of soyabean meals (SBM) and effect of microbial enzymes in degrading soya antinutritional compounds (ANC). Pages 8 −13 in 2nd International Soyabean Processing and Utilization `Conference.

Horn, N. L., S. S. Donkin, T. J. Applegate, and O. Adeola. 2009. Intestinal mucin dynamics: response of broiler chicks and White Pekin ducklings to dietary threonine. Poult. Sci. 88:1906–1914.

Huo, G. C., V. R. Fowler, J. Inborr, and M. R. Bedford. 1993. The use of enzymes to denature antinutritive factors in soyabean. Pages 517−521 in 2nd Workshop on Antinutritional Factors in Legume Seed.

Ilich, J. Z., and J. E. Kerstetter. 2000. Nutrition in bone health revisited: a story beyond calcium. J. Am. Coll. Nutr. 19:715–737.

Jourdian, G. W., L. Dean, and S. Roseman. 1971. A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides. J. Biol. Chem. 246:430–435.

Krawielitzki, K., T. Zebrowska, R. Schadereit, J. Kowalczyk, U. Hennig, J. Wünsche, and U. Herrmann. 1990. Determining of nitrogen absorption and nitrogen secretion in different sections of the pig's intestine by digesta exchange between 15N labelled and unlabeled animals. Arch. Anim. Nutr. 40:25–37.

Lien, K. A., W. C. Sauer, and J. M. He. 2001. Dietary influences on the secretion into and degradation of mucin in the digestive tract of monogastric animals and humans. J. Anim. Feed Sci. 10:223–245.

Maenz, D. D., C. M. Engele-Schaan, R. W. Newkirk, and H. L. Classen. 1999. The effects of minerals and mineral chelators on the formation of phytase-resistant and phytase-susceptible forms of phytic acid in solution and in a slurry of canola meal. Anim. Feed Sci. Technol. 81:177–192.

Medeiros, D. M., J. Ilich, J. Ireton, V. Matkovic, L. Shiry, and R. Wildman. 1997. Femurs from rats fed diets deficient in copper or iron have decreased mechanical strength and altered mineral composition. J. Trace. Elem. Exp. Med. 10:197–203.

Montagne, L., C. Piel, and J. P. Lalles. 2004. Effect of diet on mucin kinetics and composition: nutrition and health implications. Nutr. Rev 62:105–114.

Nasset, E. S., and J. S. Ju. 1961. Mixture of endogenous and exogenous protein in the alimentary tract. J. Nutr. 74:461–465.

Newkirk, R. 2010. Page 48 in Feed Industry Guide. 1st ed Canadian International Grains Institute, Winnipeg, Canada Soybean.

Nyachoti, C. M., C. D. Lange, B. W. McBride, and H. Schulze. 1997. Significance of endogenous gut nitrogen losses in the nutrition of growing pigs: a review. Can. J. Anim. Sci. 77:149–163.

Peek, H. W., J. D. Van der Klis, B. Vermeulen, and W. J. Landman. 2009. Dietary protease can alleviate negative effects of a coccidiosis infection on production performance in broiler chickens. Anim. Feed Sci. Technol. 150:151–159.

Powell, J. J., R. Jugdaohsingh, and R. P. H. Thompson. 1999. The regulation of mineral absorption in the gastrointestinal tract. P. Nutr. Soc. 58:147–153.

Ravindran, V. 2021. Progress in ileal endogenous amino acid flow research in poultry. J. Anim. Sci. Biotechno. 12:1–11.

Ravindran, V., and W. L. Bryden. 1999. Amino acid availability in poultry—in vitro and in vivo measurements. Aust. J. Agr. Res. 50:889–908.

Rawlings, N., D. Tolle, and A. Barrett. 2004. Evolutionary families of peptidase inhibitors. Biochem. J. 378:705–716.

Rooke, J. A., M. Slessor, H. Fraser, and J. R. Thomson. 1998. Growth performance and gut function of piglets weaned at four weeks of age and fed protease-treated soya-bean meal. Anim. Feed. Sci. Tech. 70:175–190.

Rothman, S., C. Liebow, and L. Isenman. 2002. Conservation of digestive enzymes. Physiol. Rev. 82:1–18.

Roughead, Z. K., and H. C. Lukaski. 2003. Inadequate copper intake reduces serum insulin-like growth factor-I and bone strength in growing rats graded amounts of copper and zinc. J. Nutr. 133:442–448.

Sambeth, W., M. C. Nesheim, and J. Serafin. 1967. Separation of soybean whey into fractions with different biological activities for chicks and rats. J. Nutr. 92:479–490.

Schulze, H. 1994. Ph.D. Thesis. Wageningen Agric. Univ., Wageningen, the Netherlands.

Settlemire, C. T., and G. Matrone. 1967. In vivo interference of zinc with ferritin iron in the rat. J. Nutr. 92:153–158.

Short, F. J., P. Gorton, J. Wiseman, and K. N. Boorman. 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Anim. Feed Sci. Technol. 59:215–221.

Smirnov, A., D. Sklan, and Z. Uni. 2004. Mucin dynamics in the chick small intestine are altered by starvation. J. Nutr. 134:736–742.

Solomons, N. W., and R. A. Jacob. 1981. Studies on the bioavailability of zincin humans: effects of heme and non-heme iron on the absorption of zinc. Am. J. Clin. Nutr. 34:475–482.

Souffrant, W. B. 1991. Endogenous nitrogen losses during digestion in pigs. Pages 147−166 Proc. of the 5th International Symposium on Digestive Physiology in Pigs. 24−26 April 1991. M. W. A. Verstegen, J. Huisman and L. A. den Hartog, eds. .

Souffrant, W. B., A. Rérat, J. P. Laplace, B. V. Darcy, R. Kohler, T. Corring, and G. Gebhardt. 1993. Exogenous and endogenous contributions to nitrogen flaxes in the digestive tract of pigs fed a casein diet. III. Recycling of endogenous nitrogen. Reprod. Nutr. Devel. 33:373–382.

Underwood, E. J. 1999. Natural sources of minerals. Pages 17−36 in The mineral nutrition of livestock. EJ Underwood and NF Suttle, eds. CABI Publishing, New York, NY.

Woyengo, T. A., A. J. Cowieson, O. Adeola, and C. M. Nyachoti. 2009. Ileal digestibility and endogenous flow of minerals and amino acids: responses to dietary phytic acid in piglets. Brit. J. Nutr. 102:428–433.