Growth performance and amino acid digestibility responses of broiler chickens fed diets containing purified soybean trypsin inhibitor and supplemented with a monocomponent protease

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ABSTRACT Trypsin inhibitors (**TI**) resident in soybeans affects protein utilization. While heat treatment influences residual TI, it simultaneously affects the structure and solubility of the soybean proteins and confounds any response to exogenous proteases. Using purified TI, the effect of exogenous protease to TI can be dissociated from changes in the soybean protein. Thus, the current study was designed to evaluate the growth performance and protein utilization responses of broiler chickens to purified TI and exogenous protease. Soybean meal (**SBM**) was preanalyzed for basal TI (2,996 TIU/g SBM), formulated into nutritionally adequate experimental diets to contain 1,033 TIU/g diet, and purified TI was added at 9,000 TIU/g diet. A total of 320 Cobb-500 broiler chicks were allocated to 4 diets, each with 8 replicate cages and 10 birds per replicate. The experimental diets were arranged as a 2 × 2 factorial with factors being dietary TI (1,033 or 10,033 TIU/g) and exogenous protease (0 or 15,000 PROT/kg). On day 7, 14, and 21 posthatching, protease supplementation improved the BW gain ($P < 0.01$) and gain to feed ratio ($P < 0.05$) of birds. On day 14 and 21 posthatching, the relative weight of pancreas increased ($P < 0.05$) with added TI but was reduced ($P < 0.001$) with protease supplementation. Apparent ileal digestibility of all amino acids, except methionine, decreased ($P < 0.001$) with added TI but increased ($P < 0.05$) with protease supplementation. Jejunal MUC-2 was downregulated ($P < 0.01$) and SCL7A-2 was upregulated ($P < 0.05$) by protease supplementation. Duodenal trypsin and chymotrypsin activities reduced ($P < 0.05$) with added TI but increased ($P < 0.01$) with protease supplementation. Exogenous protease produced longer villi ($P < 0.05$) and deeper crypts ($P < 0.01$) in the jejunal tissue. In conclusion, dietary addition of purified TI negatively affects nutrient utilization by broiler chickens. Furthermore, the study showed that the efficacy of the exogenous protease might be independent of dietary TI concentration.

Key words: broiler, gene expression, protease, soybean meal, trypsin inhibitor

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INTRODUCTION

Although soybean meal (**SBM**) is an excellent protein feed ingredient for nonruminants, it contains several antinutritional factors, which may contribute to variance in its nutritional value. Among these are the protease inhibitors, specifically of the Bowman-Birk and Kunitz types. Both inhibitors have considerable antinutritive effects which impedes the activation of gastrointestinal proteolytic enzymes, thereby affecting dietary protein digestion. In soybeans, the Kunitz-type inhibitor is bigger (>20 kDA), found in much larger concentrations, and acts by forming stable stoichiometric complexes with the digestive enzyme trypsin and chymotrypsin (Liener, 1994). The resulting noncovalent complex renders the proteases inactive and significantly reduces the digestibility and utilization of proteins and amino acids (**AA**) by nonruminants (Rawlings et al., 2004). Although the inhibitors are heat labile and can be deactivated by heat treatment, excessive processing can negatively influence the nutritional quality of SBM, necessitating care in the management of processing conditions (Newkirk, 2010).

Supplementing poultry diets with exogenous proteases may be a complementary strategy to improve the digestibility of SBM for nonruminant animals (Ghazi et al., 2002, 2003; Clarke and Wiseman, 2005; Costa

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et al., 2008; Erdaw et al. 2017). One potential mode of action of microbial protease is by outcompeting the trypsin inhibitor (**TI**) for active sites, thereby improving the overall protein and AA utilization. Alternatively, the microbial protease could also destroy or inactive the TI. Huo et al. (1993) found that fungal and bacterial protease enzymes could inactivate TI in raw soybean and low-temperature extruded soybean in vivo. Rooke et al. (1998) incubated soybean meal using 0.1% acid protease for 3 h at 50°C and pH 4.5 and reported fewer antigenic proteins compared with the nonprotease treatments. In another study, dietary addition of monocomponent protease reduced the SBM-specific antibodies in serum of broiler chicks fed a corn–SBM–based diet (Ghazi et al., 2002)

Previous work to explore the potential for microbial protease to reduce the antinutritional effect of TI in SBM have been conducted using variably heat-treated SBM. Thus, the effect of the exogenous protease on utilization of the soybean protein may be confounded by the level and or method of processing. Hence, by the dietary addition of a purified source of soybean TI, it becomes possible to clearly delineate any direct effects of the exogenous protease on growth performance and nutrient utilization of the birds.

To our knowledge, there has not been any previous reports in literature that assess the efficacy of exogenous protease on nutrient utilization of birds fed diets containing purified TI. Therefore, the hypothesis of the current study was that purified soybean TI and exogenous protease will not affect the nutrient utilization and performance of broiler chickens. To test this hypothesis, specific objectives were set out to 1) determine the effect of dietary addition of purified soybean TI on growth performance, nutrient utilization, intestinal morphology, and enzyme secretion of broiler chickens from day 0 to 21 posthatching and 2) evaluate the effect of exogenous protease administration in diets containing purified TI on growth performance, nutrient utilization, intestinal morphology, and enzyme secretion of broiler chickens from day 0 to 21 posthatching.

MATERIALS AND METHODS

Protocols of animal experiments were reviewed and approved by the Purdue University Animal Care and Use Committee (#: 1112000389).

Diets and Experimental Birds

A batch of SBM obtained from a local supplier was set aside, subsampled, and subsequently analyzed (Eurofins Scientific, Des Moines, IA) for quality indices (Table 1). Given the basal concentration of TI in the SBM, basal diets were supplemented with 0 or 9,000 TIU/g of a commercially available purified form of soybean TI (Sigma Aldrich, St. Louis, MO; EC 2329069). This produced either a low TI diet or a high TI diet, which is 10 times as concentrated in dietary TI as the low TI diet.

A total of 320 male 0-day-old broiler chicks (Cobb 500, Siloam Springs, AR) were purchased from a commercial hatchery. Birds were individually tagged, weighed, and raised in heated battery brooders (model SB 4 T; Alternative Design Manufacturing, Siloam Springs, AR) with temperature and lighting maintained as previously described by Park et al. (2017). Using a 2 × 2 factorial arrangement, with 2 concentrations of dietary TI (1,033 or 10,033 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 diets (Table 2) in a randomized complete block design, each diet with 8 replicates cages and 10 birds per replicate cage. All diets were corn–SBM–based, formulated to meet breeder nutrient specifications, and fed as mash. All diets were formulated to be nutritionally equivalent in terms of energy, protein, calcium, phosphorus, and dietary electrolyte balance. All diets contained phytase (RONOZYME HiPhos, DSM Nutritional Products) at 1,000 FYT/kg, and titanium dioxide was included as an indigestible marker.

Sampling Procedures

For growth performance assessment, feed and water was available *ad libitum* during the 21-D experimental period. Body weights and feed intake was recorded weekly on day 7, 14, and 21 posthatching, and mortality records was taken daily. Gain to feed ratio was calculated and corrected for the body weight of any bird that died or was culled during the experimental period. On day 7 and 14 posthatching, 2 birds (heaviest and lightest) from each cage were selected and euthanized by CO_2 asphyxiation. The pancreas, liver, and duodenal loop were excised for respective weight and length measurements. On day 21 posthatching, the 6 birds remaining in each cage were euthanized by CO_2 asphyxiation. Ileal digesta was collected from the distal two-thirds of the ileum (i.e., from the Meckel's diverticulum to approximately 2 cm cranial to the ileocecal junction), by flushing with distilled water into plastic containers and stored at −20°C before nutrient analyses.

Intestinal Morphological Analysis

On day 21 posthatching, mid-jejunal segments were collected from 1 bird per replicate with median BW, flushed with ice-cold 10% phosphate-buffered saline (VWR International, Radnor, PA) and fixed in 10% neutral buffered formalin (VWR International) for approximately 30 D. Samples were subsequently dehydrated with ethanol (VWR International), cleared with Sub-X (Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO). The segments (5 μm) were stained with hematoxylin and eosin at the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN). Villus height and crypt depth were measured from 4 complete, vertically oriented villi per slide and villus height to crypt depth ratio was calculated. Villus

Table 1. Analyzed values of nutrient composition and quality measuring parameters of soybean meal used in the study.

Items	Amount
Composition	
Dry matter, g/kg	866.3
Crude protein, g/kg (N × 6.25)	480.6
Ether extract, g/kg	6.1
Gross energy, kcal/kg	4,113
Quality measuring parameters	
Protein solubility in KOH, g/kg	781.8
Trypsin inhibitor, TIU/g	2,996
Urease activity, ΔpH	0.02
Amino acid, g/kg	
Arg	34.9
His	12.5
Ile	22.7
Leu	36.5
Lys	30.2
Met	6.2
Cys	6.6
Phe	24.2
Tyr	17.5
Thr	18.1
Trp	6.1
Val	23.4

length is defined as the length from the villus tip to the valley between each villus, whereas crypt depth is defined as the length between the crypt opening and base. All measurements were performed under a binocular light microscope (National Optical and Scientific Instruments, Inc., Schertz, TX).

Digestive Enzyme Assay

Duodenal digesta and the pancreas was collected from 1 bird per replicate with median BW day 21 posthatching, frozen in liquid nitrogen, and stored at −80°C until required for assay. Enzymes activities were determined using a commercially available assay kit (Sigma Chemical Co, St. Louis, MO). The absorbance of the colorimetric final product was measured in a UV/visible spectrophotometer, and the concentration of the respective enzymes was calculated accordingly. For duodenal digesta, the samples were centrifuged at 13,000 rpm at 4°C for 10 min, and aliquots of the supernatant was used for enzyme assay. The activity of the pancreatic enzymes was determined after the whole organ was homogenized in appropriate buffers and centrifuged at 13,000 rpm at 4°C for 10 min to get a clear supernatant. Trypsin activity (EC 3.4.21.4) was determined with benzoyl-DL-arginine-p-nitroanalide as substrate (Sigma Aldrich, CN MAK290). The product of p-nitroaniline was measured at an absorbance of 405 nm. One activity unit of trypsin was expressed as nanomoles of p-nitroaniline released per minute per milligram of protein. Chymotrypsin activity (EC 3.4.4.5) was determined with N-Benzoyl-L-tyrosine ethyl ester as the enzyme substrate and absorbance was measured at 405 nm. Amylase activity (EC 3.2.1.1) was determined using a coupled enzyme assay, and absorbance of ethylidene-pNP-G7 cleaved by the amylase was measured at 405 nm. One unit is the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 μmol of *p*-nitrophenol per minute at 25°C. Lipase activity (EC 3.1.1.3) was determined at an absorbance of 570 nm using a coupled enzyme reaction. One unit of lipase is the amount of enzyme that will generate 1.0 μmol of glycerol from triglycerides per minute at 37°C.

Total RNA Extraction and Reverse Transcription

On day 21 posthatching, a section of the jejunum from 1 bird per replicate with median BW was removed and flushed with ice-cold PBS (VWR International), cut longitudinally in half exposing the lumen, and mucosal contents were scraped with a metal spatula. Mucosal contents were immediately placed in 2 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at −80°C until RNA isolation. Total RNA was extracted from the tissues using Trizol reagent (Invitrogen) following the manufacturer's protocol. RNA concentrations were determined by NanoDrop 1000 (Thermo Scientific, Waltham, MA), and RNA integrity was verified by 1% agarose gel electrophoresis. To prevent the contamination of the DNA, extracted RNA was purified with DNA-*free* DNase Treatment and Removal Kit (Ambion, Austin, TX). Afterwards, 2 mg of total RNA from each sample were reverse transcribed into cDNA using the MMLV reverse transcription system (Promega, Madison, WI). The cDNA was then diluted 1:10 with nuclease-free water (Ambion) and stored at −20°C until use.

Quantitative Real-time PCR Analysis

Real-time PCR was performed with Bio-Rad iCycler with the Faststart SYBR green-based mix (Life Technologies, Carlsbad, CA). PCR programs for all genes were designed as follows: 10 min at 95°C; 40 cycles of 95°C for 30 s, primer-specific annealing temperature for 30s, and 72°C for 30s, followed by melting curve analysis. The primer sequences used in the current study are listed in Table 3. Primer specificity and efficiency were verified, subsequently the samples were analyzed in duplicate, and a difference lesser than or equal to 5% was acceptable. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with normalization against glyceraldehyde 3-phosphate dehydrogenase as the housekeeping gene (Tan et al., 2014).

Chemical Analyses

Ileal digesta samples were freeze-dried for 96 h and subsequently ground to pass through a 0.5-mm screen (Retsch ZM 100, GmbH, Haan, Germany). For DM analysis, diets and ileal digesta samples were analyzed by drying overnight at 105°C (Precision Scientific Co., Chicago, IL; method 934.01; AOAC, 2006), and the nitrogen (**N**) content of the samples was subsequently determined by combustion using a LECO FP-428 nitrogen analyzer (LECO Corp., St. Joseph, MI) with EDTA

Table 2. Ingredient and calculated nutrient composition of experimental diets, as-fed basis.

Purified trypsin inhibitor, TIU/g:	0		9,000	
Protease, PROT/kg:	0	15,000	0	15,000
Ingredients, g/kg				
Corn	562.6	552.6	512.6	502.6
Soybean meal	345.0	345.0	345.0	345.0
Soybean oil	18.0	18.0	18.0	18.0
Monocalcium phosphate[1]	11.0	11.0	11.0	11.0
Limestone[2]	13.5	13.5	13.5	13.5
Salt	2.8	2.8	2.8	2.8
Vitamin-mineral premix[3]	3.0	3.0	3.0	3.0
$_{DL}$-Methionine	2.1	2.1	2.1	2.1
$_{L}$-Lysine HCl	2.2	2.2	2.2	2.2
Threonine	1.5	1.5	1.5	1.5
Tryptophan	0.3	0.3	0.3	0.3
$NaHCO_3$	3.0	3.0	3.0	3.0
Trypsin inhibitor premix[4]	0.0	0.0	50.0	50.0
Ronozyme ProAct premix[5]	0.0	10.0	0.0	10.0
Ronozyme HiPhos premix[6]	10.0	10.0	10.0	10.0
Titanium dioxide premix[7]	25.0	25.0	25.0	25.0
Total	1,000	1,000	1,000	1,000
Calculated composition				
Crude protein, g/kg	222.34	222.34	222.34	222.34
ME, kcal/kg	3,000	3,000	3,000	3,000
Ca, g/kg	7.95	7.95	7.95	7.95
P, g/kg	6.11	6.11	6.11	6.11
Nonphytate P, g/kg	3.54	3.54	3.54	3.54
Ca:total P	1.30	1.30	1.30	1.30
Na, g/kg	2.34	2.34	2.34	2.34
K, g/kg	9.66	9.66	9.66	9.66
Cl, g/kg	3.72	3.72	3.72	3.72
Dietary electrolyte balance, mEq/kg	244.49	244.49	244.49	244.49
Digestible amino acids, g/kg				
Arg	13.23	13.23	13.23	13.23
His	5.20	5.20	5.20	5.20
Ile	8.34	8.34	8.34	8.34
Leu	17.38	17.38	17.38	17.38
Lys	12.24	12.24	12.24	12.24
Met	5.19	5.19	5.19	5.19
Cys	3.81	3.81	3.81	3.81
Phe	9.49	9.49	9.49	9.49
Tyr	7.36	7.36	7.36	7.36
Thr	8.61	8.61	8.61	8.61
Trp	2.89	2.89	2.89	2.89
Val	9.04	9.04	9.04	9.04
Total sulfur amino acids	9.00	9.00	9.00	9.00
Phe + Tyr	16.85	16.85	16.85	16.85
Analyzed composition				
Crude protein, g/kg	232.1	233.0	237.8	238.6
Crude fiber, g/kg	24.7	25.2	25.2	25.0
Ether extract, g/kg	36.5	35.7	36.2	34.1
Protease, PROT/kg[8]	LOD	16,760	LOD	17,010
Trypsin inhibitor, TIU/g	1,181	1,218	8,882	8,833

[1]16% Ca, 21% P.
[2]38% Ca.
[3]Supplied the following per kg diet: vitamin A, 5,484 IU; vitamin D3, 2,643 ICU; vitamin E, 11 IU; menadione sodium bisulfite,4.38 mg; riboflavin, 5.49 mg; pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B12, 13.2 ug; biotin, 55.2 ug; thiamine mononitrate, 2.2 mg; folic acid, 990 ug; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug.
[4]Purified trypsin inhibitor (PTI) from soybeans product contains 9,000,000 TIU/g. 1 g of PTI added to 49 g of corn supplied 180,000 TIU/g of premix. 50 g premix delivered 9,000,000 TIU/kg 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]Phytase product contained 5,000 units/g. 1 g of phytase added to 49 g of ground corn supplied 100 units/g of premix. 10 g premix delivered 1,000 units/kg feed. 1,000 units/kg supplied 1.5 g P/kg and 1.7 g of Ca/kg.
[7]1 g of Titanium dioxide added to 4 g of corn.
[8]LOD = limit of detection.

as a calibration standard. Samples for 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 before acid hydrolysis for methionine and cysteine analyses. Samples for tryptophan analysis were hydrolyzed using barium hydroxide.

Table 3. Primers used in real-time quantitative PCR.

Genes	Primer sequence (5′to 3′)	Gene Bank ID	Reference
Housekeeping gene			
GAPDH	F: TCCTAGGATACACAGAGGACCA R: CGGTTGCTATATCCAAACTCA	ENSGALG00000014442[1]	Grenier et al., 2015
Markers of inflammation			
IL-1β	F: GCATCAAGGGCTACAAGCTC R: CAGGCGGTAGAAGATGAAGC	NM_204524	Adedokun et al., 2012
IL-8	F: GCGGCCCCCACTGCAAGAAT R: TCACAGTGGTGCATCAGAATTGAGC	ENSGALG00000011670[1]	Grenier et al., 2015
IL-10	F: GCTGAGGGTGAAGTTTGAGG R: AGACTGGCAGCCAAAGGTC	ENSGALG00000000892[1]	Grenier et al., 2015
Marker of gut integrity			
MUC-2	F: GCTACAGGATCTGCCTTTGC R: AATGGGCCCTCTGAGTTTTT	XM_421035	Adedokun et al., 2012
Markers of nutrient transport			
ASCT-1	F: TTGGCCGGGAAGGAGAAG R: AGACCATAGTTGCCTCATTGAATG	XM_001232899.4	Paris and Wong (2013)
SLC7A-2	F: TGCTCGCGTTCCCAAGA R: GGCCCACAGTTCACCAACAG	NM_001199102.1	Gilbert et al. (2007).

Abbreviations: ASCT-1, neutral amino acid transporter-1; F, forward primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; MUC2, mucin 2; R, reverse primer; SLC7A-2, cationic amino acid transporter-2.
[1]Sequence obtained from Ensembl chicken genome data resources.

Amino acids in hydrolysates were determined by cation-exchange chromatography coupled with postcolumn derivatization (AOAC, 2000; method 982.30 E [a, b, c]). Titanium concentration was measured on a UV spectrophotometer following the method of Short et al. (1996).

The index method was used to calculate the apparent ileal digestibility (**AID**) of N and AA, according to the following equation:

$$\text{AID, } \% = 100 - [(\text{Ti}_\text{I} / \text{Ti}_\text{O}) \times (\text{P}_\text{O} / \text{P}_\text{I}) \times 100]$$

where Ti_I is Titanium concentration in diets; Ti_O is Titanium concentration in output (ileal digesta); P_I is N or AA concentration in diets; and P_O is N or AA output in ileal digesta.

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. The main effects of dietary TI and protease concentrations 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.

Table 4. Growth performance of broiler chickens fed diets supplemented with protease (PROT/kg) and purified trypsin inhibitor (TIU/g), from day 0 to 21 posthatching.

	TI											
	0		9,000									
	Protease		Protease			Protease		TI		*P*-value		
Item	0	15,000	0	15,000	SEM	0	15,000	0	9,000	Protease	TI	P × TI
BW, kg												
Day 0	36.3	36.3	36.3	36.3	0.01	36.3	36.3	36.3	36.3	0.238	0.728	0.728
Day 7	141	148	132	145	2.29	137	146	144	138	<0.001	0.017	0.159
Day 14	415	432	394	429	6.36	405	430	423	411	0.001	0.074	0.172
Day 21	899	943	870	936	11.63	884	940	921	903	<0.001	0.144	0.356
Day 0 to 7												
BW gain, g	105	111	96	108	2.29	100	110	108	102	<0.001	0.017	0.159
Feed intake, g	134	137	132	132	2.35	133	134	136	132	0.634	0.126	0.594
G:F, g/kg	783	813	727	824	20.20	755	819	798	776	0.005	0.285	0.114
Day 0 to 14												
BW gain, g	379	395	358	392	6.36	368	394	387	375	0.001	0.044	0.169
Feed intake, g	466	465	465	463	10.66	466	464	466	464	0.873	0.879	0.957
G:F, g/kg	813	851	773	854	23.74	793	853	832	813	0.019	0.429	0.379
Day 0 to 21												
BW gain, g	862	906	834	876	15.32	848	891	884	855	0.011	0.046	0.946
Feed intake, g	967	983	970	971	10.29	969	977	975	971	0.452	0.692	0.431
G:F, g/kg	892	922	860	902	14.46	876	912	907	881	0.019	0.087	0.656
N	8	8	8	8		16	16	16	16			

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

Table 5. Organ response of broiler chickens at day 21, fed diets supplemented with protease (PROT/kg) and purified trypsin inhibitor (TIU/g).

	TI											
	0		9,000									
	Protease		Protease			Protease		TI		*P*-value		
Item	0	15,000	0	15,000	SEM	0	15,000	0	9,000	Protease	TI	P × TI
Day 7, Absolute												
Pancreas, g	0.64	0.59	0.68	0.62	0.029	0.66	0.61	0.62	0.65	0.074	0.266	0.838
Liver, g	6.22	6.11	5.63	5.45	0.200	5.92	5.78	6.17	5.54	0.477	0.005	0.854
Duodenal loop, cm	14.37	14.31	14.29	14.99	0.389	14.33	14.65	14.34	14.64	0.415	0.448	0.338
Day 7, Relative												
Pancreas, g/kg BW	4.75	4.24	4.99	4.63	0.233	4.88	4.44	4.50	4.81	0.072	0.189	0.736
Liver, g/kg BW	45.39	42.36	41.62	41.35	1.872	43.50	41.85	43.87	41.48	0.388	0.216	0.469
Duodenal loop, cm/kg BW	110.1	103.50	109.1	114.9	3.89	109.6	109.2	106.8	112.0	0.919	0.195	0.127
Day 14, Absolute												
Pancreas, g	1.58^{b}	1.55^{b}	1.92^{a}	1.58^{b}	0.063	1.75	1.57	1.56	1.75	0.009	0.008	0.027
Liver, g	13.67^{b}	14.94^{a,b}	15.13^{a}	13.70^{b}	0.455	14.40	14.32	14.30	14.42	0.861	0.799	0.007
Duodenal loop, cm	19.22^{b}	20.73^{a}	19.62^{a,b}	19.09^{b}	0.454	19.42	19.91	19.98	19.36	0.289	0.188	0.036
Day 14, Relative												
Pancreas, g/kg BW	4.20	3.73	4.75	3.82	0.149	4.47	3.78	3.97	4.28	<0.001	0.046	0.137
Liver, g/kg BW	36.67	35.80	37.38	33.59	1.583	37.03	34.70	36.23	35.49	0.156	0.642	0.367
Duodenal loop, cm/kg BW	52.44	50.64	49.59	46.68	1.796	51.02	48.66	51.54	48.14	0.204	0.072	0.759
Day 21, Absolute												
Pancreas, g	2.74^{b}	2.78^{b}	4.31^{a}	2.86^{b}	0.113	3.53	2.82	2.76	3.59	<0.001	<0.001	<0.001
Liver, g	27.00	25.62	28.10	25.86	0.694	27.55	25.74	26.31	26.98	0.016	0.346	0.545
Duodenal loop, cm	21.56	21.31	21.57	21.41	0.451	21.56	21.36	21.43	21.49	0.662	0.902	0.924
Day 21, Relative												
Pancreas, g/kg BW	3.10^{b}	3.04^{b}	4.78^{a}	3.17^{b}	0.128	3.94	3.11	3.07	3.97	<0.001	<0.001	<0.001
Liver, g/kg BW	30.44	27.96	31.06	28.46	0.781	30.75	28.21	29.20	29.76	0.004	0.485	0.942
Duodenal loop, cm/kg BW	24.37	23.49	23.93	23.91	0.699	24.15	23.70	23.93	23.92	0.530	0.990	0.521
N	8	8	8	8		16	16	16	16			

[a,b]Means within the same row with different superscripts are significantly different ($P < 0.05$).
Abbreviations: P, protease; TI, trypsin inhibitor.

RESULTS

The analyzed nutrient composition and quality characteristics of the soybean meal are presented in Table 1, and the ingredient composition and nutrient provision of the experimental diets are presented in Table 2. The effect of exogenous protease and dietary TI concentration on growth performance of the birds are presented in Table 4. There were no interactions between protease and TI on growth performance. Supplementation of the diets with exogenous protease resulted in an increase in BW gain ($P < 0.01$) and gain to feed ratio ($P < 0.05$) in all experimental phases. Increased dietary TI resulted in a reduction in BW gain ($P < 0.05$) in all experimental phases but had no effect on the gain to feed ratio. There was no effect of any of the experimental treatments on feed intake.

Table 5 shows the effect of exogenous protease and TI supplementation of diets on pancreas weight, liver weight, and length of the duodenal loop. On an absolute basis, addition of TI to diets reduced ($P < 0.01$) the liver weight of birds only on day 7 posthatching. Notably, exogenous protease supplementation reduced ($P < 0.01$) the relative weight of the liver on day 21 posthatching. On the other hand, absolute pancreas weight on day 14 and 21 increased ($P < 0.01$) in response to dietary TI increase. Relative to BW, the increase in dietary TI led to 8 and 29% increases in pancreas weight on day 14 ($P < 0.05$) and day 21 ($P < 0.01$) posthatching, respectively. Addition of exogenous protease to the diet resulted in a reduction ($P < 0.01$) in both absolute and relative pancreas weight on day 21 to a greater extent in the diet that contained supplemental TI compared with the diet with low TI, resulting in a protease by TI interaction ($P < 0.01$).

Added dietary TI reduced ($P < 0.01$) the AID of all AA (Table 6). There were no interactions between dietary TI concentration and exogenous protease on AA digestibility. Protease supplementation increased the AID of N ($P < 0.01$) and all AA ($P < 0.05$), with the exception of Met. The increase in Cys digestibility by exogenous protease supplementation was only marginal ($P = 0.057$). Overall, the magnitude of the relative protease effect was numerically greater in the low TI diet for most of the AA than the high TI diet (Figure 1). For example, the protease effect on Lys digestibility was +1.6% in the low TI diet and +1.2% in the high TI diet.

The effects of exogenous protease and dietary TI concentration on histology of jejunal tissue and mRNA expression of markers of inflammation, integrity of the intestinal wall, and nutrient transport in the mucosa of the chicken jejunum are presented in Table 7. There were no interactions between dietary TI concentration and exogenous protease. The expression of interleukin (IL)-1β, IL-8, IL-10, and neutral amino acid transporter-1 were not affected by dietary TI or exogenous protease supplementation. However, increased dietary TI downregulated ($P < 0.05$) the expression of

Table 6. Effect of exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g) concentration on apparent ileal digestibility (%) of nitrogen and amino acids in broiler chickens, at day 21 posthatching.

	TI											
	0		9,000			Protease		TI		*P*-value		
	Protease		Protease									
Item	0	15,000	0	15,000	SEM	0	15,000	0	9,000	Protease	TI	P × TI
Nitrogen	84.8	86.3	80.9	83.6	0.43	82.8	84.9	85.5	82.3	<0.001	<0.001	0.152
Indispensable AA												
Arg	89.6	90.7	87.5	88.1	0.38	88.5	89.4	90.1	87.8	0.034	<0.001	0.541
His	86.6	87.9	83.8	84.7	0.43	85.2	86.3	87.2	84.3	0.019	<0.001	0.624
Ile	84.5	86.0	81.0	82.0	0.47	82.8	84.0	85.2	81.5	0.017	<0.001	0.573
Leu	85.9	87.2	82.7	84.0	0.42	84.3	85.6	86.5	83.4	0.006	<0.001	0.904
Lys	87.7	89.1	85.2	86.2	0.44	86.5	87.7	88.4	85.7	0.012	<0.001	0.706
Met	92.9	93.3	90.8	90.7	0.29	91.8	92.0	93.1	90.8	0.609	<0.001	0.315
Phe	85.2	86.6	82.3	83.6	0.46	83.8	85.1	85.9	83.0	0.009	<0.001	0.915
Thr	80.3	82.3	76.0	77.2	0.51	78.2	79.8	81.3	76.6	0.005	<0.001	0.442
Trp	86.9	88.3	82.8	84.4	0.43	84.8	86.3	87.6	83.6	0.002	<0.001	0.763
Val	82.8	84.4	78.8	80.2	0.48	80.8	82.3	83.6	79.5	0.004	<0.001	0.819
Dispensable AA												
Ala	84.8	86.2	81.4	82.5	0.45	83.1	84.3	85.5	82.0	0.013	<0.001	0.825
Asp	82.6	84.2	78.6	79.7	0.50	80.6	81.9	83.4	79.2	0.015	<0.001	0.606
Cys	73.7	75.7	65.5	66.8	0.82	69.6	71.3	74.7	66.2	0.057	<0.001	0.675
Glu	89.0	90.0	86.9	87.5	0.36	87.9	88.8	89.5	87.2	0.028	<0.001	0.528
Gly	80.1	82.2	75.9	76.7	0.55	78.0	79.5	81.1	76.3	0.014	<0.001	0.277
Pro	85.0	86.5	82.0	83.3	0.41	83.5	84.9	85.7	82.6	0.002	<0.001	0.799
Ser	82.6	84.3	78.5	79.9	0.46	80.6	82.1	83.5	79.2	0.003	<0.001	0.745
Tyr	84.8	86.3	80.9	83.6	0.43	82.8	84.9	85.5	82.3	<0.001	<0.001	0.152
N	8	8	8	8		16	16	16	16			

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

cationic amino acid transporter-2 (**SLC7A-2**). Protease supplementation downregulated ($P < 0.01$) the expression of mucin-2 and upregulated ($P < 0.05$) the expression of SLC7A-2 in the jejunum. Exogenous protease increased the villus height ($P < 0.05$) and crypt depth ($P < 0.01$) in the jejunal tissue of birds.

Table 8 shows the effect of exogenous protease and dietary TI concentration on digestive enzyme activities. In the pancreas, there were no effects (or interactions) of exogenous protease and dietary TI concentration on digestive enzyme activities. Despite this, there was a reduction in trypsin activity ($P < 0.05$) in the duodenal digesta in response to increased dietary TI. However, duodenal trypsin activity increased ($P < 0.01$) with exogenous protease supplementation. Similarly, chymotrypsin activity was reduced ($P < 0.01$) by increased dietary TI concentration but increased ($P < 0.01$) with exogenous protease supplementation.

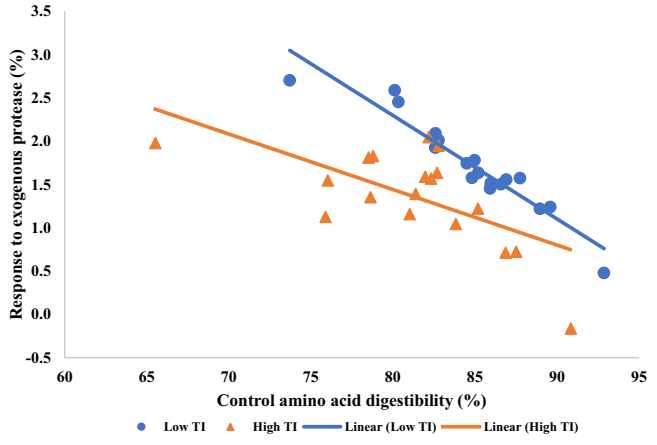

Figure 1. Correlation between inherent amino acid digestibility in the control diet (%) and response to exogenous protease (% change relative to the control diet without added protease). Solid blue circles represent data points from the low TI diet group, and solid orange triangles represent data points from the high TI diet group. Solid and dashed linear lines indicate the respective best fit model for the low and high TI groups. Abbreviation: TI, trypsin inhibitor.

DISCUSSION

There is a substantial body of work on the nutritional implications of TI in broiler chickens. To the authors' knowledge, the data obtained from the current study are the first to test the efficacy of a monocomponent protease on a purified source of TI in broiler chickens. This approach becomes valuable for optimization and indeed validation of the protease enzyme as a useful additive for improvements in the nutrient utilization of broiler chickens.

In the current study, the effect of increased dietary TI on the final BW of birds diminished with age, although there were improvements because of protease supplementation. This was evident with marked improvements in BW gain and feed efficiency over the entire feeding period and are similar to previous reports (Wang et al., 2008; Barekatain et al., 2013). However, there have also been reports of a lack of positive effect of exogenous protease on BW gain, even though the feed conversion significantly improved (Ghazi et al., 2002; Freitas et al., 2011). The diminishing effect of increased dietary TI on live BW agrees with previous authors (Erdaw et al., 2017) at 24 D or 35 D posthatching. However, Ruiz and De Belalcázar (2005) previously reported

Table 7. Relative gene expression[1] of cytokines, mucosa, amino acid transporter proteins in jejunal mucosa, and histology of jejunal tissue of broiler chickens fed diets containing exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g), at day 21 posthatching.

	TI											
	0		9,000									
	Protease		Protease			Protease		TI		*P*-value		
Item	0	15,000	0	15,000	SEM	0	15,000	0	9,000	Protease	TI	P × TI
Genes												
IL-1β	1.18	0.96	0.96	0.95	0.246	1.07	0.95	1.07	0.95	0.638	0.651	0.683
IL-8	1.23	1.51	1.02	1.14	0.258	1.12	1.33	1.37	1.08	0.435	0.278	0.763
IL-10	1.14	0.95	0.92	0.69	0.164	1.03	0.82	1.05	0.81	0.217	0.154	0.913
MUC-2	1.31	1.02	1.25	0.57	0.130	1.28	0.79	1.16	0.91	0.001	0.062	0.155
ASCT-1	1.11	1.18	0.79	1.06	0.206	0.95	1.12	1.14	0.92	0.407	0.301	0.629
SLC7A2	1.01	1.41	0.52	0.93	0.177	0.77	1.17	1.21	0.73	0.035	0.014	0.945
Histology												
Villus height, µm	1,057.5	1,273.8	849.4	1,084.0	88.88	953.5	1,178.9	1,165.7	966.7	0.019	0.036	0.919
Crypt depth, µm	100.6	130.5	96.7	112.8	6.75	98.7	121.7	115.6	104.8	0.003	0.125	0.322
VH:CD ratio	10.7	9.8	8.8	9.7	0.78	9.8	9.7	10.2	9.3	0.968	0.216	0.244
N	8	8	8	8		16	16	16	16			

Abbreviations: ASCT-1, neutral amino acid transporter-1; CD, crypt depth; IL, interleukin; MUC2, mucin 2; P, protease; SLC7A-2, cationic amino acid transporter-2; TI, trypsin inhibitor; VH, villus height.

[1]Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated with GAPDH as the endogenous control.

that the impact of dietary TI may simply be intake related, and as age approaches 21 D and older, excess TI intake could cause moderate to severe rapid feed passage syndrome and a consequent drop in overall growth performance of the birds. In the current study, there were no differences in feed intake between birds fed the low or high TI diets at 21 D posthatching which could open up an avenue for exogenous protease in complementing the functions of the endogenous proteases (Noy and Sklan, 1995).

Addition of purified TI to the diets increased both absolute and relative weight of the pancreas. This was especially true for birds at day 14 and 21 posthatching and is indicative of pancreatic hypertrophy or hyperplasia (Embaby, 2010). Owing to an intrinsic negative feedback control, enzyme secretion by the pancreas is inversely related to the enzyme activity in the gut. The ingested TI acts by forming an irreversible complex with trypsin in the small intestine, which limits the concentration and function of trypsin and leads to overproduction of digestive enzymes and thus, enlargement of the pancreas (Cabrera-Orozco et al., 2013). It is surprising that increased dietary TI had no effect on the relative pancreas weight at day 7 posthatching, even though the growth performance data suggest that birds were more susceptible to TI at this age. One possible explanation is that although dietary TI was high, low feed intake in the first week of the bird's life could limit total TI intake, which might not be enough to elicit major morphological changes in the pancreas. This is corroborated by a previous report by Clarke and Wiseman (2007) who showed a strong dose-dependent response of relative pancreas weight to dietary TI intake. However, Erdaw et al. (2017) reported that the pancreas of younger birds was more sensitive to dietary TI concentrations, but it is pertinent to state that responses in that study was for 10-day-old birds. Interestingly, the magnitude of change in the relative weight of the pancreas to dietary TI tends to increase with age, and this might be partly because of increased dietary intake of TI.

Table 8. Effect of exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g) concentration on enzyme activity in the duodenal digesta (units/mL) and Pancreas (units/mg), at day 21 posthatching.

	TI											
	0		9,000									
	Protease		Protease			Protease		TI		*P*-value		
Item	0	15,000	0	15,000	SEM	0	15,000	0	9,000	Protease	TI	P × TI
Duodenal digesta												
Trypsin	10.13	11.69	7.76	10.40	0.699	8.95	11.04	10.91	9.08	0.007	0.016	0.446
Chymotrypsin	5.49	6.77	3.79	5.41	0.123	4.64	6.09	6.13	4.60	<0.001	<0.001	0.178
Amylase	121.49	115.10	117.99	119.44	3.284	119.74	117.27	118.30	118.71	0.460	0.900	0.247
Lipase	1.30	1.32	1.41	1.35	0.047	1.35	1.33	1.31	1.38	0.682	0.140	0.393
Pancreas												
Trypsin	5.81	5.67	6.02	5.40	0.333	5.92	5.53	5.74	5.71	0.263	0.915	0.473
Chymotrypsin	4.28	4.31	4.12	4.21	0.076	4.20	4.26	4.29	4.16	0.433	0.102	0.708
Amylase	34.91	33.59	35.66	35.13	1.331	35.28	34.36	34.25	35.39	0.495	0.399	0.770
Lipase	0.95	0.98	1.07	1.00	0.045	1.01	0.99	0.97	1.03	0.670	0.143	0.325
N	8	8	8	8		16	16	16	16			

Abbreviations: P, protease; TI, trypsin inhibitor.

These results contradict findings by Erdaw et al. (2017), that the relative weight of the chicken pancreas fed extruded full fat SBM compared with the control, decreased with age. This suggests that purified TI exerts a more critical effect on the pancreas than the native TI in full fat SBM. In the current trial, birds were fed diets containing high-quality SBM with highly digestible AA, and this might increase the negative effects of the added purified TI. On the other hand, previous experiments that fed raw SBM, full-fat SBM, or underprocessed SBM used SBM with inherently low AA digestibility with a lot of residual TI. Therefore, it is possible that the TI as an antinutrient tends to exert different influences depending on the inherent quality of the diet that it is associated with. Although both lectin and TI contained in raw or full fat soybean may have complementary effects on pancreatic function (Grant, 1989), TI seems a more relevant antinutritional factor in young broilers (Douglas et al., 1999). Although lectin can increase pancreas weight (Liener, 1994) by stimulating the accumulation of polyamines (Pusztai et al., 1995), Fasina et al. (2004) reported no trophic changes in pancreas weights of turkey poults fed diets containing increasing concentrations of purified soybean lectin. Nevertheless, the current study showed that the exogenous protease was effective in ameliorating the negative effect of increased dietary TI on pancreas weight.

In the current study, increased dietary TI as a result of the addition of purified TI decreased the AID of N and all of the reported AA. This was at least a 4% drop in AID of Thr and Trp and up to 8% decrease for Cys. This result is similar to reports by Schulze (1994), who observed a significant decrease in N digestibility and increase in ileal endogenous N flow when growing pigs were fed diets containing increasing concentrations of purified TI. However, relative to the control, protease supplementation improved the overall N digestibility by 2.5%. This is similar to observations of Cowieson and Ravindran (2008) when a multienzyme complex, containing protease, was used. Angel et al. (2011) also reported an increased N digestibility with increasing dietary protease concentration. Bertechini et al. (2009) reported increased true AA digestibility for soybean meal, and Carvalho et al. (2009) reported increased true AA digestibility for corn, in the presence of a monocomponent protease. This indicates that variable quantities of N that could escape digestion and absorption were partially captured by the protease and made available to the birds. The relative effect of protease on control AA digestibility were consistent with those in the review of 25 independent experiments published by Cowieson and Roos (2014).

These improvements in growth performance responses can also be attributed to the observed improvements in the digestibility of AA. This is because birds that more readily reach their target intake of digestible AA have improved feed efficiency compared with the corresponding controls. In the current study, we observed a lack of effect of exogenous protease on methionine digestibility, which is also consistent with previous reports (Ravindran et al., 2005; Angel et al., 2011; Cowieson and Roos, 2014). This may be related to the typically high AID of methionine (90–95%) in a corn–SBM–based diet. However, there are some reports of significant improvements in AID of methionine when protease was supplemented at dietary concentrations of 400 mg/kg or higher (Angel et al., 2011). Although relatively low, but similar to a previous report (Cowieson, 2010), the AID of Cys in birds was improved by protease supplementation. These data on AA digestibility indicate a reliance by the exogenous protease on the inherent digestibility of dietary AA or "starting material" (Cowieson and Roos, 2014). However, in diets with high TI concentration, the protease effect was not large enough to counteract the drastic drop in digestibility of AA in the control diet. This suggests that the exogenous protease may not directly act on the TI, and any improvements in AA digestibility, attributable to the exogenous protease, is largely independent of dietary TI concentration. The reason for this observation is not clear but might be ascribed, in part, to a lack of an effective enzyme-substrate specificity, interference with other dietary components, or interference of endogenous proteolytic activity by the exogenous protease effect (Yuan et al., 2015).

Exogenous protease downregulated the expression of mucin-2 in the jejunum of birds fed diets with increased dietary TI concentration. This agrees with reports (Cowieson and Roos, 2014; Cowieson et al., 2017) that noted a beneficial effect of exogenous protease on mucin secretion in the intestine of the chicken. This is usually associated with a reduction in mucin secretion or an increase in the autolytic recovery of mucin or both. It is possible that the protease acts by reducing the metabolic demand for mucoprotein by reducing the erosion of the mucosal layer by the incoming feed matrix. Hence, there is usually a marked reduction in the mucin layer thickness, goblet cells, and endogenous protein losses. For example, birds fed diets supplemented with protease show significant reductions in the digesta sialic acid concentration (a component of mucin) and goblet cell numbers (Peek et al., 2009; Cowieson et al., 2017). It is also possible that a portion of the beneficial effect of protease on AA digestibility is conferred through a reduction in the loss of mucoprotein from the intestine, with possible implications for gut health.

In addition, mucin being a family of mucus glycoproteins have some essential AA in the core structure (e.g., Trp and Thr), and it is possible that an increase in the digestibility of these AA, conferred by the exogenous protease, would correlate with improvements in the integrity of mucus layer. Protease supplementation also upregulated (almost doubling) the expression of the Na-independent cationic AA transporter, SLC7A-2. Consistent with the reports of Cowieson et al. (2017), this suggests an effect of the exogenous protease in modulating protein absorption, which may also be because of the availability of more AA released in the gut. However, there was no protease effect on the expression of Na-dependent neutral AA transporter, amino acid transporter-1, and the reason for this observation is not totally clear. The exogenous protease also increased the villus height and crypt depth in the jejunal

tissue of the birds. This suggests a better absorptive capacity of dietary nutrients and may corroborate the observed improvements in growth performance and nutrient utilization responses previously noted. This observation is similar to reports by Wang et al. (2008) in broilers and Zuo et al. (2015) in piglets.

In the current study, increased dietary TI and exogenous protease supplementation had no effect on pancreatic enzyme activities. Although, birds fed high TI diet showed signs of pancreatic hypertrophy, it is unclear why the pancreatic enzyme activity was not different from birds fed the low TI diet. This is in dissonance with reports by Erdaw et al. (2017), who observed reduced activities of pancreatic trypsin and chymotrypsin of 24-day-old birds fed diets with increasing levels of raw soybean meal. However, in the duodenum, the activities of trypsin and chymotrypsin decreased as a result of increased dietary TI but increased with exogenous protease administration. The reduction in enzymatic activity might be because TI not only has inhibitory specificity to trypsin but will also affect the activity of chymotrypsin, which is activated by trypsin. On the other hand, the improvements in intestinal trypsin and chymotrypsin activity because of protease supplementation suggest a complementarity or additivity between the exogenous and endogenous proteases. This is because the enzyme activity in the small intestine is composed of both exogenous and endogenous components.

Given the foregoing, the current study shows that increased dietary TI affected the growth performance and digestibility of AA and that exogenous protease administration improves these responses in the birds. Furthermore, responses to protease administration seem more likely to have arisen from a general improvement in protein digestion. Pronounced pancreatic hypertrophy was identified in birds fed diets with added purified TI, which was ameliorated by exogenous protease administration. The data from the current study suggest that the efficacy of dietary exogenous protease might be independent of dietary TI concentration.

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REFERENCES

Adedokun, S. A., K. M. Ajuwon, L. F. Romero, and O. Adeola. 2012. Ileal endogenous amino acid losses: response of broiler chickens to fiber and mild coccidial vaccine challenge. Poult. Sci. 91:899–907.

Angel, C. R., W. Saylor, S. L. Vieira, and N. Ward. 2011. Effects of a mono-component protease on performance and protein utilization in 7- to 22-day-old broiler chickens. Poult. Sci. 90:2281–2286.

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.

Barekatain, M. R., C. Antipatis, M. Choct, and P. A. Iji. 2013. Interaction between protease and xylanase in broiler chicken diets containing sorghum distillers dried grains with solubles. Anim. Feed Sci. Technol. 182:71–81.

Bertechini, A. G., J. C. C. Carvalho, F. R. Mesquita, S. F. Castro, C. Meneghetti, and J. O. B. Sorbara. 2009. Use of a protease to enhance the utilization of soybean meal amino acids by broilers. Poult. Sci. 88(Suppl. 1):69 (Abstr.).

Cabrera-Orozco, A., C. Jiménez-Martínez, and G. Dávila-Ortiz. 2013. Soybean: non-nutritional factors and their biological functionality. Pages 387–410 in Soybean: Bio-Active Compounds. H. A. El-Shemy, ed. IntechOpen, London, UK.

Carvalho, J. C. C., A. G. Bertechini, R. L. Rios, F. R. Mesquita, E. M. C. Lima, and J. O. B. Sorbara. 2009. Use of a protease to enhance the utilization of corn amino acids by broilers. Poult. Sci. 88(Suppl. 1):69–70 (Abstr.).

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.

Clarke, E., and J. Wiseman. 2007. Effects of extrusion conditions on trypsin inhibitor activity of full fat soybeans and subsequent effects on their nutritional value for young broilers. Br. Poult. Sci. 48:703–712.

Costa, F. G., C. C. Goulart, D. F. Figueiredo, C. F. Oliveira, and J. H. Silva. 2008. Economic and environmental impact of using exogenous enzymes on poultry feeding. Int. J. Poult. Sci. 7:311–314.

Cowieson, A. J. 2010. Strategic selection of exogenous enzymes for corn/soy-based poultry diets. J. Poult. Sci. 47:1–7.

Cowieson, A. J., H. Lu, K. M. Ajuwon, I. Knap, and O. Adeola. 2017. Interactive effects of dietary protein source and exogenous protease on growth performance, immune competence and jejunal health of broiler chickens. Anim. Prod. Sci. 57:252–261.

Cowieson, A. J., and V. Ravindran. 2008. Effect of exogenous enzymes in maize-based diets varying in nutrient density for young broilers: growth performance and digestibility of energy, minerals and amino acids. Br. Poult. Sci. 49:37–44.

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.

Douglas, M. W., C. M. Parsons, and T. Hymowitz. 1999. Nutritional evaluation of lectin-free soybeans for poultry. Poult. Sci. 78:91–95.

Embaby, H. E. 2010. Effect of heat treatments on certain anti-nutrients and in vitro protein digestibility of peanut and sesame seeds. Food Sci. Tech. Res. 17:31–38.

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.

Fasina, Y. O., J. D. Garlich, H. L. Classen, P. R. Ferket, G. B. Havenstein, J. L. Grimes, M. A. Qureshi, and V. L. Christensent. 2004. Response of Turkey poults to soybean lectin levels typically encountered in commercial diets. 1. Effect on growth and nutrient digestibility. Poult. Sci. 83:1559–1571.

Freitas, D. M., S. L. Vieira, C. R. Angel, A. Favero, and A. Maiorka. 2011. Performance and nutrient utilization of broilers fed diets supplemented with a novel mono-component protease. J. Appl. Poult. Res. 20:322–334.

Ghazi, S., J. A. Rookie, H. Galbraith, and M. R. Bedford. 2002. The potential for the improvement of the nutritive value of soybean meal by different proteases in broiler chicks and broiler cockerels. Br. Poult. Sci. 43:70–77.

Ghazi, S., J. A. Rooke, and H. Galbraith. 2003. Improvement of the nutritive value of soybean meal by protease and a-galactosidase treatment in broiler cockerels and broiler chicks. Br. Poult. Sci. 44:410–418.

Gilbert, E. R., H. Li, D. A. Emmerson, K.E. Webb, Jr, and E. A. Wong. 2007. Developmental regulation of nutrient

transporter and enzyme mRNA abundance in the small intestine of broilers. Poult. Sci. 86:1739–1753.
Grant, G. 1989. Anti-nutritional effects of soyabean: a review. Prog. Food Nutr. Sci. 13:317–348.
Grenier, B., H. E. Schwartz-Zimmermann, S. Caha, W. D. Moll, G. Schatzmayr, and T. J. Applegate. 2015. Dose-dependent effects on sphingoid bases and cytokines in chickens fed diets prepared with *Fusarium verticillioides* culture material containing fumonisins. Toxins 7:1253–1272.
Huo, G. C., V. R. Fowler, J. Inborr, and M. R. Bedford. 1993. The use of enzymes to denature antinutritive factors in soybean. Pages 517–521 in Proceedings of the Second International Workshop on 'Anti-nutritional factors (ANFs) in Legume seeds. A. F. B. Van der Poel, J. Huisman, and H. S. Saini, eds. Wageningen Pers, Wageningen, the Netherlands.
Liener, I. E. 1994. Implications of antinutritional components in soybean foods. Crit. Rev. Food Sci. 34:31–67.
Livak, K. J., and D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$Ct method. Methods 25:402–408.
Newkirk, R. 2010. Soybean. Feed Industry Guide. 1st ed. Canadian International Grains Institute, Winnipeg, Canada.
Noy, Y., and D. Sklan. 1995. Digestion and absorption in the young chick. Poult. Sci. 74:366–373.
Paris, N., and E. Wong. 2013. Expression of digestive enzymes and nutrient transporters in the intestine of Eimeria maxima-infected chickens. Poult. Sci. 92:1331–1335.
Park, C. S., A. Helmbrecht, J. K. Htoo, and O. Adeola. 2017. Comparison of amino acid digestibility in full-fat soybean, two soybean meals, and peanut flour between broiler chickens and growing pigs. J. Anim. Sci. 95:3110–3119.
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.
Pusztai, A., G. Grant, D. S. Brown, S. Bardocz, S. W. B. Ewen, K. Baintner, W. J. Peumans, and E. J. M. Van Damme. 1995. Lectins binding to the gut wall are growth factors for the pancreas: nutritional implications for transgenic plants. Pages 141–182 in Lectins: Biomedical Perspectives. A. Pusztai and S. Bardocz, eds. Taylor and Francis, Bristol, PA.
Ravindran, V., L. I. Hew, G. Ravindran, and W. L. Bryden. 2005. Apparent ileal digestibility of amino acids in dietary ingredients for broiler chickens. Anim. Sci. 81:85–97.
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. Technol. 70:175–190.
Ruiz, N., and F. De Belalcázar. 2005. Field observation: trypsin inhibitors in soybean meal are correlated with outbreaks of feed passage in broilers. Poult. Sci. 84:70.
Schulze, H. 1994. Endogenous Ileal Nitrogen Losses in Pigs: Dietary Factors. Ph.D. Thesis. Wageningen Agricultural University, Wageningen, the Netherlands.
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.
Tan, J. Z., T. J. Applegate, S. Liu, Y. Guo, and S. D. Eicher. 2014. Supplemental dietary L-arginine attenuates intestinal mucosal disruption during a coccidial vaccine challenge in broiler chickens. Br. J. Nutr. 112:1098–1109.
Wang, H., Y. Guo, and J. C. H. Shih. 2008. Effects of dietary supplementation of keratinase on growth performance, nitrogen retention and intestinal morphology of broiler chickens fed diets with soybean and cottonseed meals. Anim. Feed Sci. Technol. 140:376–384.
Yuan, L., S. Q. Wang, Z. X. Wang, H. Zhu, and K. Huang. 2015. Effects of exogenous protease supplementation on endogenous trypsin activity and gene expression in broilers. Genet. Mol. Res. 14:13633–13641.
Zuo, J., B. Ling, L. Long, T. Li, L. Lahaye, C. Yang, and D. Feng. 2015. Effect of dietary supplementation with protease on the growth performance, nutrient utilization, intestinal morphology, digestive enzymes and gene expression of weaned piglets. Anim. Nutr. J. 1:276–282.