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Protease and phytase supplementation of broiler diets in which soybean meal is partially or completely replaced by raw full-fat soybean

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Abstract

The nutrient composition and enzymatic *in vitro* nutrient digestibility of raw full-fat soybean (RFSB) were assessed prior to evaluating the influences of enzyme supplementation in diets in which commercial soybean meal (SBM) was partially (phase 1) or completely (phase 2) replaced by RFSB. A 2 x 2 + 1 arrangement was used in a two-phase feeding trial. In phase 1 (0 - 25 days) a positive control, commercial-type diet (PC), without RFSB or enzymes, and a negative control diet (NC), with 75 g RFSB/kg were used. The NC diet was supplemented with or without protease (0 or 15000 PRPOT/kg) and phytase (0 or 2000 FYT/kg). In phase 2 (26 - 31 days), RFSB (NC) or SBM (PC) was used as the sole source of crude protein (CP) for birds. Each treatment was replicated six times. Phases 1 and 2 had the same enzyme supplementation and treatment arrangements. The results showed that the concentration of trypsin inhibitors in RFSB as an ingredient was 16 564 TIU/g, and its CP *in vitro* digestibility was improved significantly by protease and protease plus phytase. Birds fed on PC and on NC plus protease and phytase finally consumed higher quantities of feed and had the highest bodyweight gain (BWG). Protease improved feed intake (FI), BWG and feed conversion ratio (FCR) by approximately 5.3%, 22.5% and 11.4%, respectively, in phase 2. Birds on the diet with protease plus phytase were 11.7% and 24.8% superior in BWG and FCR, respectively, to NC between 26 and 31 days. Supplementation with both enzymes reduced the weight of the pancreas. Supplementing NC with protease and phytase marginally improved the apparent ileal digestibility (AID) of protein and some amino acids (AA) at 25 and 31 days. Overall, BWG and feed efficiency of birds were improved by supplementation of both enzymes and, to a lesser extent, by protease on its own.

Keywords: anti-nutritional factors, ileal digestibility, pancreas, trypsin inhibitors, sole source crude protein and amino acids

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Introduction

Soybean meal (SBM) is the most important and preferred source of quality protein in animal feeds (Banaszkiewicz, 2011). Among the oil seeds, SBM contains the highest crude protein (CP) and has the best AA profile, with only 6% crude fibre (Dei, 2011). The AAs in SBM are highly digestible by poultry (Newkirk, 2010). Based on the CP content, SBM can generally be classified as high protein (47% - 50%), or low protein (44% - 46%). Popescu & Criste (2003) suggested that full-fat SBM could completely replace conventional commercial SBM and supplemental oils in diets for broiler chickens, but these full-fat products require heat treatment. Untreated soybeans contain several anti-nutritional factors (ANFs), including trypsin inhibitors, lectins and phytate (Liu, 1998; Newkirk, 2010). Trypsin inhibitors and lectins inhibit protease and other enzymes in the digestive tract, particularly the activity of trypsin and chymotrypsin, and contribute to reducing protein digestion in non-ruminant animals and young ruminants (Leiner, 1994).

Birds that are fed on diets that contain raw soybean experience low FI, slow body growth and increased weight of pancreas (Mogridge *et al*., 1996; Perez-Maldonado *et al*., 2003; Newkirk, 2010). In

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recent studies, the authors examined the potential of improving the quality of diets containing RFSB for broiler chickens through supplementation with high-potency protease and phytase products. The enzymes reduced the negative impact of RFSB, although productivity was still poorer than that observed with a control diet that was devoid of RFSB (Erdaw *et al*., 2017a; 2017b). In previous studies, protease alone, and especially in combination with phytase, improved the *in vitro* digestion of protein (Erdaw *et al*., 2016). The phytate content of RFSB was also reduced. The mechanisms behind these *in vitro* and *in vivo* tests were not fully understood, particularly in the absence of a control diet that contained RFSB without microbial enzyme supplementation. The direct effect on soy protein of protease or in combination with phytase was not assessed either. Therefore, the objectives of this study were to evaluate the attributes of individual and combined (synergistic) effects on protein digestibility and growth of broiler chickens of microbial protease and phytase in diets containing RFSB. The authors hypothesized that there would be a reduction in the negative effects of ANFs in soya, trypsin inhibitors (TI) on birds through supplementation with microbial protease and/or phytase enzymes.

Materials and Methods

The seeds of RFSB were purchased from a local supplier in northern New South Wales, Australia, cleaned and hammer-milled to pass through a 2-mm sieve. This milled ingredient was then incorporated in diets, as described below. The samples and diets were further milled and then analysed for CP content (AOAC, 2006a), amino acid (AA) profiles (AOAC, 2006b) and available lysine contents (AOAC, 2006b), urease activities (UA) (AOCS, 2011a), TI concentrations (AOCS, 2011b) and protein solubility (KOH) (Araba & Dale, 1990).

A sub-sample of the RFSB was milled to pass through a 0.2-mm screen. About 1.0 g of this sample was incubated with 0.2 mg microbial protease, 0.2 mg phytase or a combination (0.2 mg each) of the two enzymes to determine the *in vitro* digestibility of protein in a two-step enzymatic procedure (Boisen & Fernández, 1997; Park *et al.,* 2012). Additionally, the material was incubated in an enzyme-free medium.

The RFSB that was milled to 2-mm particle size was used to replace commercial SBM at 25% (7.5% of diet) that was fed as such (NC) or supplemented with microbial protease (0 or 15000 PROT/kg) (NC-Pro), phytase (0 or 2000 FYT/kg) (NC-Phy). A positive control (PC) diet was prepared without RFSB or enzyme. The enzyme recovery rates are shown in Table 1.

Table 1 Recovery rates of test enzymes in diets (containing 75 g of raw, full-fat soybean per kg of diet (grower diet 10 - 24 days) or raw, full-fat soybean used as a sole source of crude protein and amino acids (special diet 25 - 31 days)

	Enzyme level g/kg	Protease PROT/kg	CV %	Phytase U/kg	CV %
Grower diets (10 - 24 d)					
RFSB + no enzymes	0	ND	-	ND	-
RFSB + protease	0.2	10850	4.0	-	-
RFSB + phytase	0.2	ND	-	2135	10.0
RFSB + protease + phytase	0.2+0.2	12540	3.0	2431	9.0
No RFSB + no enzymes	0	ND	-	ND	-
Special diets (26 - 31 d)					
RFSB+ no-enzyme	0	ND	-	ND	-
RFSB + protease	0.2	13440	11.0		
RFSB + phytase	0.2	ND	-	2498	9.0
RFSB + protease + phytase	0.2+0.2	17140	5.0	2639	13.0
SBM + no enzymes	0	ND	-	ND	-

RFSB: raw, full-fat soybean; SBM: commercial soybean meal; protease (0.2 g/kg of diet), equivalent to 15000 PROT/kg of diet; Phytase (0.2 g/kg of diet), equivalent to 2000 FYT/kg of diet. ND: no data

In general, the recovery rate of protease was slightly lower than calculated, with a coefficient of variation (CV) of between 3% and 11%. Phytase recovery was slightly higher than calculated (CV, 9 - 13%).

A positive control (PC) diet without RFSB or enzyme was also prepared (Tables 2 and 3). The diets were offered to birds in crumbled form for starters (0 - 10 days), in pelleted form for growers (11 - 25 days) and as mash (26 - 31 days). Each diet was replicated six times, with nine birds per replicate. A completely randomized design with a 2 x 2 +1 arrangement was used in both phases. Three sets of tests were conducted in this study:

1. *In vitro* nutrient digestibility test on RFSB
2. A feeding trial between zero and 25 days (Phase 1)
3. A feeding trail between 26 and 31 days (Phase 2)

A total of 270 one-day-old Ross 308 male broiler chicks (initial bodyweight 40.54 ± 0.18 g) were obtained from a local commercial hatchery (Baiada Poultry Pty., Ltd., Tamworth, Australia). Groups of nine chicks were allocated to each of 30 cages (60 x 42 x 23 cm). The birds were raised in a climate-controlled room and were offered corn-soybean-based diets (starter and grower) formulated to Aviagen standards for Ross 308 broilers (Aviagen, 2014).

Table 2 Ingredient composition (g/kg) of starter, grower and special diets of broiler chickens

	Starter diets (0 - 10 days)		**Grower diets (10 - 26 days)**		**Special diets (26 - 31 days)**	
	NC	**PC**	**NC**	**PC**	**NC^{Ø}**	**PC^{Δ}**
Corn	596.5	597	577.2	586.7	0	0
Soybean meal	225	300	225	300	0	460
Raw full-fat soybean	75	0	75	0	510	0
Meat meal	72.3	68.8	51	45	0	0
Corn starch	0	0	0	0	197.2	224
Dextrose	0	0	0	0	185.1	204
Cellulose	0	0	0	0	22.9	25.0
Canola oil	7.9	11.3	40	40	22.9	25
Di-calcium phosphate	6.7	6.7	8.5	9	25.7	25.7
Limestone	5.3	5.3	9	5	11.9	11.9
Titanium dioxide	0	0	5	5	5	5
DL-methionine	2.5	2.4	2.4	2.4	0	0
L-lysine	2.7	2.5	1.6	1.5	0	0
Salt	2.3	2.3	2.3	2.3	2.5	2.5
Mineral premix[1]	0.75	0.75	0.75	0.75	0.75	0.75
Vitamin premix[2]	0.5	0.5	0.5	0.5	0.5	0.5
L-threonine	1	0.95	0.9	0.9	0	0
$NaHCO_3$	1.1	1.1	0.3	0.3	1.9	1.9
$MgSO_4$	0	0	0	0	1.2	1.2
KCl	0	0	0	0	9.0	9.0
Choline chloride	0.5	0.5	0.6	0.6	3.5	3.5

[1] Trace minerals supplied per kilogram of diet: Cu, 16 mg; Fe, 40 mg; I, 1.25 mg; Se, 0.3 mg; Mn, 120 mg; Zn, 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg
[2] Vitamins supplied per kilogram of diet: vitamin A, 12 000 IU; Vitamin D_3, 5 000 IU; vitamin E, 75 mg, vitamin K, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; vitamin B_{12}, 16 µg; biotin, 200 µg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg.
NC = negative control; PC = positive control. NC^{Ø} = negative control in special diet$^{©}$; PC^{Δ} = positive control in special diet$^{©}$

Table 3 Composition (g/kg) of starter (0 - 10 days), grower (10 - 26 days), and special (26 - 31 days) diets for broiler chickens

	Starter diets		Grower diets		Special diets©	
	NC	PC	NC	PC	NC^{Ø}	PC^{Δ}
ME, MJ/kg	12.59	12.59	13.29	13.29	14.0	14.0
Crude protein	226	226	217	217	190	190
Crude fat	45	45	71	70	81	75
Crude fibre	25	25	24	23	30	28
Arginine	14.4	14.4	13.9	14.0	12.0	16.0
Lysine	14.0	14.0	13.3	14.0	11.0	12.0
Methionine	6.0	6.0	5.5	5.5	4.0	4.5
M+C	9.0	9.0	8.5	8.5	7.1	7.5
Threonine	10.0	10.0	8.6	8.7	6.5	6.8
Calcium	10.0	10.0	9.4	9.6	8.5	8.5
Available phosphorus	5.0	5.0	4.5	4.5	4.2	4.2

Calculated nutrient composition; ©determined nutrient composition
NC = negative control; PC = positive control. NC^{Ø} = negative control in special diet©; PC^{Δ} = positive control in special diet©
ME = metabolizable energy

Each cage was equipped with a medium-sized feeder (78 x 12 x 8 cm) and two nipple drinkers. The temperature of the room was set at 33 ºC for the first two days, with a relative humidity of between 49% and 60%. This temperature was gradually reduced to 24 °C by day 19, and maintained at this level for the remaining period. For the first two days, 24 hours of light (20 lux) were provided. This was then reduced to 23 hours for the next six consecutive days, followed by 20 hours of light (10 lux) for the remaining days. Feed was provided ad libitum in the form of crumble for starters and pellets for growers and special diets in phase 2, and the birds had free access to water.

One and two birds were randomly selected on days 10 and 25, respectively, and electrically stunned and killed by cervical dislocation to assess the weights of the pancreas and duodenum. Samples of ileal digesta were also collected on day 25 to assess protein and amino acid digestibility.

In the second phase, birds (6 per replicate) were supplied with special diets, which contained commercial SBM or RFSB as the sole source of protein and amino acids (Tables 2 and 3). These diets were fed continuously for five days, that is, between 26 and 31 days old. As in the first phase, there were five diets, namely NC, NC-Pro, NC-Phy, NC-Enz (combined) and PC. The main objective of this phase was to assess the digestibility of RFSB protein in the presence of the enzymes. Titanium dioxide (TiO_2) was added to grower and phase 2 diets as a marker to enable assessment of ileal nutrient digestibility. The pancreas and duodenum were collected and weighed at 25 and 31 days old. The concentration of TiO_2 in the ileal digesta and diets was determined using the method described by Short *et al.* (1996).

At sampling on days 10 and 25, blood was collected into EDTA-coated tubes to assess blood composition and liver function. The blood was collected from the right jugular vein, shaken gently, and placed on ice. The sample was centrifuged at 3000 x *g* for 10 min to obtain the serum. Serum samples were analysed at Laverty Pathology (Armidale, NSW, Australia) for aspartate aminotransferase (AST) (EC 2.6.1.1) and alanine aminotransferase (ALT) (EC 2.6.1.2). The methods were based on those recommended by the International Federation for Clinical Chemistry (IFCC), which were developed by Shumann *et al.* (2002).

Colorimetric methods were used to evaluate the recovery rate of exogenous protease enzymes in the mixed diets (Keltti, 2014). Evaluation of the recovery rate of exogenous phytase enzyme activities in the mixed diets was carried out according to the procedures described by Yasar & Desen (2014).

Descriptive statistics were used to present the nutrient and anti-nutrient composition of the ingredients and diets. The general linear model (GLM) of Minitab software version 17 (Minitab, 2013) was used to analyse the data from the feeding trial. Differences between mean values were separated by Duncan's multiple range test and were considered significant at P <0.05. This study was approved by the University of New England Animal Ethics Committee (Authority No AEC16-062).

Results

The analysed concentration of TI in the RFSB as a feed ingredient was 16564 TIU/g. The concentrations of crude fibre, CP and ether extract were 62, 405 and 201 g/kg, respectively. The concentration of TI in the PC diet was 1940.0 TIU/kg (Table 4). When the commercial SBM was replaced with RFSB at 25% (75 g/kg diet), the concentration of TI was increased by about fivefold (NC). Available lysine content was slightly reduced while protein solubility was increased.

Table 4 Effects of partial replacement of soybean meal with raw full-fat soybean on diet quality of broiler diets

	RFSB in diets (g/kg)	
	0	**75**
Available lysine (g/kg)	16.2	15.45
Nitrogen solubility (g/kg)	155.3	222.9
Trypsin inhibitor (TIU/g)	1,940.0	10,193.4

RFSB = raw, full-fat soybean

The *in vitro* digestibility of CP in RFSB was improved (P <0.001) significantly owing to supplementation of microbial protease or a combination of protease and phytase (Figure 1). However, phytase on its own did not improve the digestibility of CP in this study.

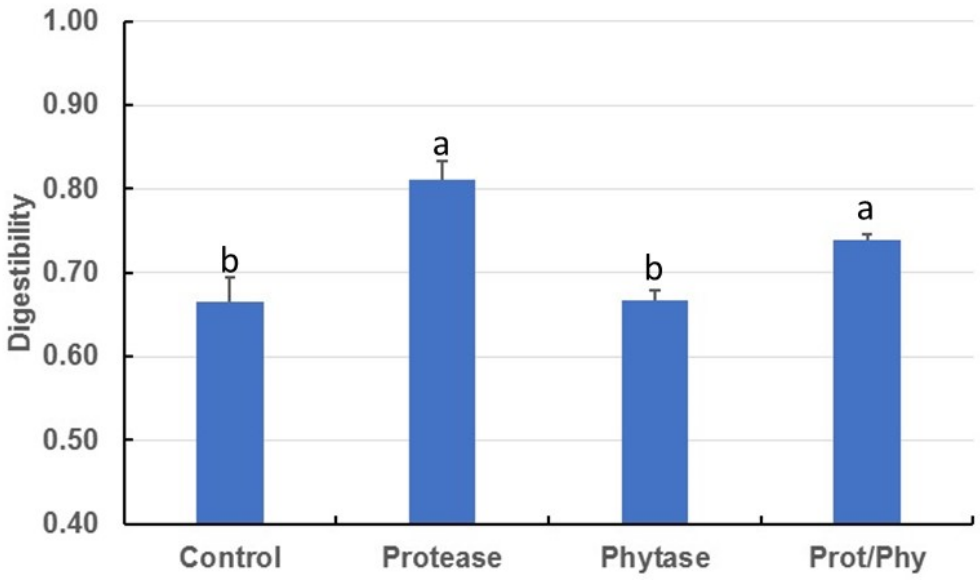

Figure 1 Effects of a two-step enzymatic *in vitro* test on crude protein digestibility of raw soybean samples
[ab]Means with different superscript letters within a column are significantly different. Each test was replicated five times

The results of FI, BWG and FCR of birds fed on the test diets containing RFSB (75 g/kg of diet) and supplemented with microbial enzymes are shown in Table 5. Between hatch and 10 days, chicks in the PC group consumed less (P <0.01) feed and gained less weight than those in the NC and NC-enzyme groups. However, over the entire starter and grower phases (1 - 25 days), the PC group consumed more feed (P <0.05), grew faster (P <0.001) and were slightly more efficient (not significantly) than birds fed the diets

containing RFSB, with or without the enzyme supplements. Supplementation with protease or protease and phytase increased (P <0.05) FI, but only the combination of enzymes increased (P <0.001) BWG between hatch and 25 days, compared with the NC.

Table 5 Effects of supplementing enzymes and raw, full-fat soybean on feed intake, body-weight gains and feed conversion ratio for broiler diets

Protease PROT/kg	Phytase FYT/kg	FI (1 - 10 days)	FI (1 - 25 days)	BWG (1 - 10 days)	BWG (1 - 25 days)	FCR (1 - 10 days)	FCR (1 - 25 days)
0	0	253.8	1453.2^{b}	216.4	958.1^{b}	1.173^{a}	1.517
	2000	261.2	1525.9^{ab}	226.8	1038.0^{ab}	1.151^{ab}	1.471
15000	0	260.4	1462.3^{b}	227.6	984.0^{b}	1.145^{ab}	1.487
	2000	243.9	1642.8^{a}	213.8	1108.1^{a}	1.141^{b}	1.488
Pooled SEM		6.05	21.68	5.19	16.56	0.005	0.014
P-value		0.75	0.001	0.73	0.002	0.082	0.724
Positive control (PC)		192.3	1643.9	165.6	1146.1	1.160	1.437
Main effects							
0		257.5	1489.6	221.6	998.1	1.162	1.494
15000		235.5	1569.2	205.3	1046.1	1.146	1.502
	0	257.1	1457.7	222.0	971.1	1.159	1.502
	2000	235.8	1601.0	204.9	1073.1	1.149	1.494
Contrasts, *P*-values							
PC vs NC		0.00	0.013	0.00	0.004	0.228	0.026
PC vs protease		0.01	0.025	0.001	0.009	0.152	0.282
PC vs phytase		0.003	0.051	0.002	0.036	0.481	0.357
PC vs NC + protease + phytase		0.013	0.983	0.006	0.492	0.285	0.316
Sources of variation							
Protease		0.19	0.03	0.25	0.07	0.01	0.78
Phytase		0.20	0.01	0.23	0.01	0.06	0.76
Protease x phytase		0.09	0.05	0.06	0.38	0.02	0.17

[a-b]Means with different superscript letters within a column are significantly different; SEM: pooled standard error of means
BWG: bodyweight gain; FI: feed intake; FCR: feed conversion ratio
Protease (15000 PROT/kg), phytase (2000 FYT/kg) or a combination of these enzymes was supplemented to the diet of NC group
The diet that was used as a positive control (PC) was prepared without enzymes or RFSB

The gross responses of birds to diets in which SBM or RFSB was the sole source of CP and AAs and to microbial enzyme supplements are shown in Table 6. Regardless of enzyme supplementation, birds allocated to PC consumed more feed (P <0.001), grew faster (P <0.001) and were more efficient (P <0.01) than birds on the NC diets. Although enzyme supplementation of the NC diet tended to improve FI, BWG and FCR, this was not significant (P >0.05).

The relative weight of the pancreas at days 10, 25 and 31 was lower (P <0.05) in birds fed the PC diet than in those offered NC or NC enzyme-supplemented diets (Table 7). Enzyme supplementation to the NC diet tended to reduce the relative weight of the pancreas. This effect was significantly (P <0.001) higher for the combined enzymes at days 10 and 31.

Consumption of diets that contained RFSB and those supplemented with protease and/or phytase had no effect on liver AST and ALT or blood PCV (Table 8). Blood pH and electrolyte values were also unaffected (Table 9).

Regardless of the protein source, there were no interacting effects between microbial protease and phytase supplementation across the two trial feeding phases. Birds fed the PC diet had significantly (P <0.002) higher apparent ileal digestibility (AID) of CP and most AA at day 25 than birds fed the NC diets (Table 10). Supplementation of the NC diet with protease, phytase or both enzymes improved (P <0.002) the AID of the CP, but only marginally improved the digestibility of some of the AA, compared with the NC group.

The trend in ileal digestibility of protein and AA, which was measured at day 31, when the sole source of protein and AAs was SBM or RFSB, was similar to that at day 25, although the values were much lower in the second phase (Table 11). The AID of CP and most AAs was significantly higher (P <0.01) in birds on the PC diet than on the NC diets. The AID of CP and a few AAs was improved when the NC diet was supplemented with protease or a combination of protease and phytase, but rarely with phytase only.

Table 6 Response of broilers to enzyme supplements when the sole source of dietary crude proteins and amino-acids was raw, full-fat soybean or commercial soybean meal (25 - 31 days)

Protease, PROT/kg	Phytase, FYT/kg	FI (26 - 31 days)	BWG (26 - 31 days)	FCR (26 - 31 days)
0	0	405.3	112.5	3.603
	2000	369.1	125.7	2.936
15000	0	426.9	137.9	3.096
	2000	413.9	140.7	2.942
Pooled SEM		14.6	9.6	0.209
P-value		0.57	0.74	0.67
Positive control (PC)		590.8	336.1	1.785
Main effects				
0		387.2	119.1	3.437
15000		420.4	139.3	3.387
	0	416.1	125.2	3.622
	2000	391.5	133.2	3.202
Contrasts, *P*-values				
PC vs NC		0.00	0.00	0.01
PC vs protease		0.016	0.001	0.007
PC vs phytase		0.001	0.001	0.001
PC vs protease + phytase		0.00	0.00	0.018
Sources of variation				
Protease		0.28	0.33	0.91
Phytase		0.42	0.69	0.34
Protease x phytase		0.70	0.79	0.44

[a-b] Means with different superscript letters within a column are significantly different; SEM: pooled standard error of means

BWG: body weight gain; FI: feed intake; FCR: feed conversion ratio

Protease (15000 PROT/kg), phytase (2000 FYT/kg) or a combination these enzymes was supplemented to diet NC

The positive control (PC) diet was prepared without enzyme and RFSB

Table 7 Influence of supplementing enzymes on the relative weight (g/100 g of bodyweight) of pancreas and duodenum when commercial soybean meal was partially replaced with raw full-fat soybean at days 10 and 25 or when raw, full-fat soybean was used as a sole source of crude protein and amino-acids (at days 31)

Protease, PROT/kg	Phytase FYT/kg	10 days		25 days		31 days	
		Pancreas	Duodenum	Pancreas	Duodenum	Pancreas	Duodenum
0	0	0.73	1.86	0.37	1.17	0.34	0.82
	2000	0.62	1.93	0.37	1.06	0.34	0.80
15000	0	0.65	1.94	0.34	1.08	0.38	0.84
	2000	0.61	1.71	0.34	1.02	0.29	0.81
Pooled SEM		0.02	0.05	0.01	0.03	0.01	0.02
P-value		0.07	0.33	0.44	0.40	0.18	0.80
Positive control (PC)		0.44	1.83	0.28	1.11	0.19	0.71
Main effects							
0		0.67	1.90	0.37	1.12	0.34	0.81
15000		0.63	1.83	0.34	1.05	0.34	0.82
	0	0.69[a]	1.90	0.35	1.12	0.36	0.83
	2000	0.61[b]	1.82	0.35	1.04	0.32	0.80
Contrasts, *P*-values							
PC vs NC		0.00	0.83	0.01	0.53	0.001	0.00
PC vs NC + protease		0.00	0.44	0.05	0.69	0.001	0.004
PC vs NC + phytase		0.00	0.39	0.03	0.66	0.001	0.047
PC vs NC + protease + phytase		0.005	0.17	0.04	0.35	0.002	0.023
Sources of variation							
Protease		0.18	0.48	0.11	0.30	0.86	0.68
Phytase		0.03	0.39	0.95	0.21	0.12	0.38
Protease x phytase		0.31	0.14	0.49	0.63	0.11	0.87

[a-b] Means with different superscript letters within a column are significantly different; SEM: pooled standard error of means, protease (0 or 15000 PROT/kg), phytase (0 or 2000 FYT/kg) or a combination of these enzymes was supplemented to NC. The positive control (PC) diet was prepared without any enzyme and RFSB

Table 8 Activities of liver enzymes, Alanine transaminase; Aspartate transaminase, and packed cell volume in birds fed diets containing raw, full-fat soybean with or without microbial enzyme supplements

	10 days		25 days		
	AST, U/L	ALT, U/L	AST, U/L	ALT, U/L	PCV
Negative control (NC)	163	<5	185	<5	30.7
NC + protease	172	<5	198	<5	31.5
NC + phytase	174	<5	244	<5	34.2
NC + protease + phytase	165	<5	199	<5	32.8
Positive control (PC)	167	<5	214	<5	30.7
Pooled SEM	3.31	-	7.64	-	0.74
P-value	0.84	-	0.13	-	0.53

The negative control diet (NC) had raw, full-fat soybean (RFSB) meal as its only protein source and no enzyme supplementation. The PC diet had commercial SBM as its only protein source and no enzyme or RFSB supplementation. Protease (0 or 15000 PROT/kg) and phytase (0 or 2000 FYT/kg) were supplemented in the test diet
AST: aspartate aminotransferase; ALT: alanine aminotransferase; PCV: packed cell volume

Table 9 Effects of raw full-fat soybean and protease supplementations on values of acidity and electrolytes in blood plasma of broilers at days 10 and 25

	10 days				25 days		
	pH	**K^+**	**Na^+**	**Cl^-**	**pH**	**Na^+**	**Cl^-**
Negative control (NC)	7.5	17.9	127.2	97.8	7.3	120.6	94.4
NC + protease	7.5	16.6	133.7	103.5	7.3	128.7	99.3
NC + phytase	7.4	17.7	131.3	98.5	7.4	123.8	94.2
NC + protease + phytase	7.5	17.9	135.0	102.8	7.3	103.3	115.4
Positive control (PC)	7.4	17.0	135.0	101.8	7.3	127.2	104.5
Pooled SEM	0.02	0.32	1.34	1.00	0.03	4.30	2.90
P-value	0.17	0.31	0.11	0.06	0.16	0.12	0.06

The negative control diet had raw full-fat soybean meal (RFSM) as its only protein source and no enzyme supplementation. The positive control diet had commercial SBM as its only protein source and no enzyme or RFSB supplementation. Protease (15000 PROT/kg) and phytase (2000 FYT/kg) were supplemented in the test diet

Discussion

Although the concentration of TI in the test sample of RFSB was 16564 TIU/g, results of an *in vitro* digestibility of CP test were improved significantly because of the test enzymes, particularly combined enzyme supplementation. Similarly, in the feeding trial (0 - 25 days), the FI and BWG of birds on the PC and NC diets, which were supplemented with combined enzymes, were greatly improved, compared with the same measurements on other diets. These results in general are in line with previous reports (Malathi & Devegowda, 2001; Srinath *et al*., 2012; Erdaw *et al*., 2017a; 2018) that better response were found in *in vitro* and *in vivo* experiments when combined microbial enzymes were used rather than individual application. These results suggest synergistic and combined effects of the two enzymes on ANF.

The relative weight of the pancreas at days 10, 25 and 31 was lower in birds fed the PC diet than in those offered the NC or NC enzyme-supplemented diets. These results are in line with a previous report (Erdaw *et al*., 2017c; 2018). Enzyme supplementation of the NC diet tended to reduce the relative weight of the pancreas. This effect was significant for the combined enzymes at days 10 and 31. The weight of the duodenum was reduced significantly due to supplementation of phytase in broiler diets when the sole source of CP and AAs was RFSB. These findings disagree partially with those of other researchers (Mogridge *et al*., 1996; Mayorga *et al*., 2011), who reported that birds fed diets that contained RFSB had heavier pancreas and duodenum, although there was no indication that the diets were supplemented with microbial enzymes. The reduction in weight of duodenum in the current study might be due to the positive contribution of microbial enzymes, particularly phytase, against anti-nutritional factors (ANF) in broiler diets.

Consumption of diets containing RFSB or supplemented with protease and/or phytase had no effect on liver AST and ALT or blood PCV. These results confirm the safety of the RFSB at the tested levels.

Birds fed on the diets with RFSB as the sole source of CP and AAs and supplemented with combined enzymes had lighter weight of pancreas. Similarly, the BWG and feed efficiency of these birds were improved by 11.7% and 24.8% when supplemented with protease and phytase, respectively, compared with birds in the NC group (26 - 31 days). Not only were the combined microbial enzymes effective in the latter phase of this study, but supplementation of microbial protease alone into the NC diet contributed to approximately 5.3% and 22.5%, or 11.4% increase in FI, BWG and feed efficiency, respectively, compared with birds on NC between 26 and 31 days. These results in general agree with previous reports (Ao, 2011; Barletta, 2011; Erdaw *et al.,* 2017a; 2017b; 2017c) that ANF, for example proteinaceous anti-nutrients and stored proteins, can be broken down by supplementing the diets or ingredients with microbial protease or phytase.

The AID of CP at day 25 was greatly improved when the diet was supplemented with combined enzymes in NC followed by microbial protease. Similarly, Erdaw *et al*. (2017d) reported that when broiler diets with ANF were supplemented with microbial protease, the loss of undigested and unabsorbed ileal CP (endogenous losses) was reduced, leading to an increase in the AID and standardized ileal digestibility (SID) of CP.

Table 10 Influence of raw, full-fat soybean and enzyme supplementations on coefficient values of apparent digestibility of CP and AAs at days 25 in diets for broilers

Protease	Phytase	CP	Indispensable amino acids									Dispensable amino acids			
			Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val	Ala	Gly	Pro	Ser
0	0	0.75	0.76	0.75	0.70	0.73	0.78	0.89	0.75	0.66	0.70	0.72	0.64	0.72	0.67
	2000	0.76	0.74	0.75	0.70	0.72	0.79	0.89	0.74	0.67	0.69	0.70	0.64	0.71	0.67
15000	0	0.77	0.76	0.76	0.71	0.74	0.80	0.90	0.76	0.69	0.71	0.72	0.66	0.73	0.68
	2000	0.76	0.76	0.77	0.72	0.75	0.80	0.90	0.76	0.70	0.71	0.73	0.65	0.73	0.69
Pooled SEM		0.00	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01
P-value		0.26	0.69	0.77	0.52	0.50	0.47	0.41	0.61	0.24	0.58	0.46	0.74	0.64	0.46
Positive control (PC)		0.79	0.79	0.80	0.78	0.80	0.83	0.93	0.81	0.71	0.76	0.77	0.68	0.77	0.74
Main effects															
0		0.75	0.75	0.75	0.70	0.72	0.79	0.89	0.74	0.67	0.69	0.71	0.64	0.71	0.67
15000		0.76	0.76	0.76	0.72	0.74	0.80	0.90	0.76	0.69	0.71	0.73	0.66	0.73	0.69
	0	0.76	0.76	0.76	0.71	0.74	0.79	0.90	0.75	0.68	0.70	0.72	0.65	0.72	0.68
	2000	0.76	0.75	0.76	0.71	0.73	0.79	0.89	0.75	0.68	0.70	0.71	0.65	0.72	0.68
Sources of variation															
Protease		0.29	0.37	0.33	0.15	0.22	0.13	0.10	0.21	0.06	0.21	0.19	0.29	0.25	0.13
Phytase		0.83	0.51	0.83	0.93	0.70	0.67	0.82	0.90	0.47	0.92	0.69	0.76	0.85	0.85
Protease x phytase		0.15	0.67	0.75	0.79	0.41	0.91	0.92	0.67	0.94	0.60	0.42	0.95	0.61	0.68
Contrast, *P*-values															
PC vs NC		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PC vs NC + protease		0.01	0.03	0.01	0.00	0.00	0.01	0.00	0.00	0.03	0.00	0.00	0.04	0.01	0.00
PC vs NC + phytase		0.02	0.00	0.01	0.00	0.00	0.02	0.01	0.00	0.03	0.00	0.01	0.02	0.00	0.00
PC vs NC + protease + phytase		0.13	0.04	0.02	0.00	0.00	0.03	0.01	0.01	0.14	0.01	0.02	0.26	0.02	0.00

PC: positive control diet; NC: negative control diet; SEM: standard error of mean. CP = crude protein; Arg = arginine; His = histidine; Le = leucine; Ile = isoleucine; Met = methionine; Lys = lysine; Phe = phenylalanine; Thr = threonine; Val = valine; Ala = alanine; Gly = glycine; Pro= proline; Ser = serine

Table 11 Influence of raw, full-fat soybean and enzyme supplementations on the coefficient values of apparent digestibility of crude protein and amino acids at days 31 on broilers

Protease	Phytase	CP	Indispensable amino acids									Dispensable amino acids			
			Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val	Ala	Gly	Pro	Ser
0	0	0.63	0.53	0.60	0.40	0.44	0.34	0.60	0.47	0.42	0.39	0.43	0.36	0.48	0.40
	2000	0.624	0.56	0.63	0.41	0.45	0.37	0.52	0.49	0.43	0.40	0.44	0.38	0.51	0.41
15000	0	0.639	0.58	0.64	0.46	0.49	0.41	0.50	0.52	0.47	0.45	0.47	0.42	0.53	0.45
	2000	0.632	0.57	0.63	0.44	0.47	0.39	0.55	0.51	0.45	0.43	0.46	0.40	0.51	0.43
Pooled SEM		0.008	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01
P-value		0.94	0.20	0.55	0.19	0.34	0.36	0.35	0.25	0.22	0.25	0.43	0.33	0.35	0.40
Positive control (PC)		0.714	0.66	0.72	0.66	0.67	0.51	0.72	0.68	0.60	0.64	0.65	0.56	0.63	0.64
Main effects															
0		0.63	0.55	0.61	0.41	0.44	0.36	0.56	0.48	0.42	0.40	0.43	0.37	0.49	0.41
15000		0.64	0.58	0.63	0.45	0.48	0.4.0	0.52	0.51	0.46	0.44	0.46	0.41	0.52	0.44
	0	0.64	0.56	0.62	0.43	0.46	0.37	0.55	0.49	0.45	0.42	0.45	0.39	0.51	0.42
	2000	0.63	0.56	0.63	0.43	0.46	0.38	0.53	0.50	0.44	0.42	0.45	0.39	0.51	0.42
Source of variation															
Protease		0.63	0.08	0.34	0.05	0.10	0.11	0.34	0.08	0.06	0.06	0.12	0.12	0.17	0.12
Phytase		0.71	0.63	0.74	0.99	0.91	0.81	0.68	0.88	0.71	0.84	0.86	1.00	0.97	0.92
Protease x phytase		0.99	0.24	0.31	0.44	0.47	0.46	0.14	0.35	0.47	0.49	0.62	0.34	0.24	0.48
Contrast, *P*-values															
PC vs NC		0.007	0.001	0.001	0.001	0.001	0.001	0.007	0.001	0.001	0.001	0.001	0.001	0.001	0.001
PC vs NC + protease		0.001	0.002	0.001	0.001	0.001	0.005	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
PC vs NC + phytase		0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
PC vs NC + protease + phytase		0.022	0.001	0.001	0.001	0.001	0.003	0.005	0.001	0.001	0.001	0.001	0.001	0.001	0.001

PC: positive control diet; NC: negative control diet; SEM: standard error of mean. CP = crude protein; Arg = arginine; His = histidine; Le = leucine; Ile = isoleucine; Met = methionine; Lys = lysine; Phe = phenylalanine; Thr = threonine; Val = valine; Ala = alanine; Gly = glycine; Pro = proline; Ser = serine

The current findings agree partially with those of previous researchers (Guggenbuhl *et al*., 2012; Romero & Plumstead 2013; Cowieson *et al*., 2016), who observed an increase in AID of AAs in poultry and piglets and health benefits in response to inclusion of microbial protease.

When microbial protease, phytase or combined enzymes were supplemented to the NC diet, approximately 8.7%, 2.2% and 6.7% increases of AID were recorded for the total AAs, respectively, at day 31, but these changes were not statistically significant. These results agree with those of other researchers (Ao, 2011; Barletta, 2011; Pettersson & Pontoppidan, 2013; Erdaw *et al.,* 2017a; 2017b; 2017c), who reported that the adverse impact of ANF, particularly TI, was reduced owing to supplementation of microbial enzymes. The improvement in digestibility of CP and AAs in the current study, in which diets contained a high concentration of ANF, including TI, might be because of the supplemented microbial protease and/or phytase. This might be also due to the contribution of the tested microbial feed enzymes, which tend to be potent against some of the ANF, including TI. The test enzymes appear to be more effective as a cocktail.

Conclusion

The results of the current study confirmed the negative impact of RFSB on growth and efficiency of broiler chickens. The test enzymes, particularly protease and when combined with phytase, improved *in vitro* and *in vivo* digestibility of protein. The BWG and FCR of birds up to 25 days old were also improved. However, the *in vivo* digestibility of most of the AA was not significantly improved by enzyme supplementation. The health of the birds was not compromised by RFSB at the level that was tested. Further tests are required to establish the exact mechanisms of the test enzymes. There is also a need to re-develop the test protease to sustain its *in vitro* activity when it is applied *in vivo*.

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Authors' Contributions

MME and PAI were in charge of the experimental design, implementation and writing the manuscript. RAP participated in interpreting and reviewing the results of the study. Only the named authors contributed.

Conflict of Interest Declaration

There are no conflicts of interest

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