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Carbohydrases and phytase with rice bran, effects on amino acid digestibility and energy use in broiler chickens

C. Gallardo[1], J. C. Dadalt[2] and M. A. Trindade Neto[2†]

[1]*Department of Veterinary Sciences and Biological, Universidad Cientifica del Sur, Lima 15067, Peru;* [2]*Department of Animal Science, University of Sao Paulo, CEP: 13635900, Pirassununga, Sao Paulo, Brazil*

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*Protein sources from cereals are used in broiler diets, usually in order to reduce feeding costs. However, their efficient use in poultry diets is limited by the level of fiber whose compounds are resistant to digestion in the small intestine; due to this sugars are not digested by endogenous poultry enzymes. The aim of this study was to determine the effect of multi-carbohydrase (**MC**) and phytase (**Phy**) on the total retention of nutrients, retention of apparent metabolizable energy corrected for nitrogen (AME_N) (trial 1) and apparent and standardized ileal digestibility of amino acids (trial 2) of rice bran (**RB**). A total of 245-day-old male broilers (Cobb 500) was distributed at 21-day-old in a completely randomized design in a 2 × 2 + 1 (0 and 200 mg/kg MC; 0 and 50 mg/kg Phy, and basal diet – **BD**) factorial arrangement of treatments, to give seven replicates and seven birds per replicate. The BD based on corn (trial 1) and cornstarch and casein (trial 2) was used only to determine the coefficients of retention of nutrients and energy, and coefficients of digestibility of amino acids of the RB. The test diets were made by mixing BD and RB 7 : 3 wt/wt basis. There was interaction (P<0.05) between MC × Phy for DM, nitrogen and AMEN, retention and no interaction (P>0.05) for ash, calcium, phosphorous and NDF was observed. Enzymes interacted (P<0.05) on standardized ileal digestibility of arginine, histidine, leucine, methionine, phenylalanine, threonine, valine, aspartic acid, glutamic acid, proline and serine. Dietary combination of MC and Phy resulted in higher (P<0.05) standardized digestibility of arginine, histidine, methionine and threonine relative to single enzyme supplementation or control diet without enzymes. Enzyme isolated inclusions in the diets improved (P<0.05) standardized digestibility of methionine. The supplementation of carbohydrases and Phy in RB will improve the nitrogen, energy and amino acids utilization for broiler chickens.*

Keywords: amino acid digestibility, antinutritional factor, energy, enzyme, nitrogen retention

Implications

The supplementation of exogenous enzymes in broiler chicken diets has proven to be an effective method to improve nutrient retention in plant feedstuffs. The probable cleavage of non-starchy polysaccharides by the inclusion of carbohydrase and possible phytate neutralization by phytase action allowed the accessibility of the enzymes to the substrates. The results imply that the supplementation composed of carbohydrases and phytase may increase the energy retention, nitrogen and amino acid digestibility in broiler chickens fed rice bran.

Introduction

Cereals are the most important feed ingredients in broiler diets, but their efficient use in monogastric diets is limited by anti-nutritional factors such as non-starch polysaccharides (**NSPs**) and phytate. The rice (*Oryzae sativa*) grain consists of a hull caryopsis, and bran below the hull, germ and endosperm (Sera *et al.*, 2005). Rice bran (**RB**) is the by-product obtained after milling of brown rice (Renuka and Arumughan, 2007), and it is considered as an excellent source of nutrients in animal feeds; however, the high phytate and NSP levels limit its nutritive value and availability of the nutrients. The increase in NSP intake may increase the intestinal viscosity, reduce the intestinal transit time and negatively affect the use of nutrients (Kiarie *et al.*, 2016). On the other hand, RB has high total levels of phosphorus (1.5%) as compared to corn (0.28%) and soybean meal (0.65%), (National Research Council, 1994). However, RB is an ingredient that has low rates of phosphorus availability due to its high phytate content.

Enzymes are biological catalysts and are some of the most notable biomolecules due to their extraordinary specificity

† E-mail: messiastn@usp.br

and catalytic power. It has been reported that exogenous enzymes supplementation decreases the anti-nutritional effects of NSP and phytate (Kiarie *et al.*, 2016). For example, if xylanase and phytase are included in the same diet, the former hydrolyzes the NSP, thus providing better access for the latter to reduce the interaction of phytate with protein and starch molecules as well as reducing binding of elemental phosphorus (Dadalt *et al.*, 2017). Previous studies have demonstrated synergic effects of phytase and carbohydrases on nutrients available, energy use and amino acids digestibility in pigs and broiler chickens (Dadalt *et al.*, 2017; Gallardo *et al.*, 2017).

Therefore, inconsistencies in these findings warrant further investigations using multi-carbohydrase (**MC**) complex and phytase (**Phy**). We hypothesized that the simultaneous supplementation of multi-carbohydrase and phytase will improve nutrient and energy utilization in RB fed to broiler chickens. Therefore, the objective of the present study was to determine the effect of MC and Phy on the total retention of nutrients, retention of metabolizable energy corrected for nitrogen ($\mathbf{AME_N}$) and apparent and standardized ileal digestibility of amino acids of RB.

Material and methods

All research methods and procedures were approved by the ethics and animal experimentation committee at the University of São Paulo (project 2843/2012), Brazil, and were followed according to animal welfare.

The methodology section was similar to that already described in Gallardo *et al.* (2017 and 2018).

Rice bran and enzymes

A commercial by-product RB was produced by Hi-Tech Feeds; Pelotas, Rio Grande do Sul, Brazil. The chemical composition of RB used is shown in Table 1.

The enzymes used were a MC blend that provided per kg diet α-galactosidase (0 or 700 U), galactomannanase (2200 U), xylanase (30 000 U) and β-glucanase (22 000 U) (ENDOPOWER BETA, Uniquimica, Sao Paulo, Brazil) and Phy (GENOPHOS, Uniquimica, Sao Paulo, Brazil). Analytical enzyme activities in mixed feeds were guaranteed by Uniquimica. The MC consisted of 40% dehydrated fermentation product from *Aspergillus niger* (PRL 2351) and *Aspergillus oryzae* (ATCC66222). The Phy was a *Saccharomyces cerevisiae*–derived (KCCM 80051), that contained genes of *Escherichia coli* and *Citrobacter braakii* and provided 500 units of phytase activity (FTU) per kg diet. One unit of α-galactosidase was defined as the quantity of the enzyme that liberates 1 μmol p-nitrophenol per minute. One unit of galactomannanase was defined as the quantity of the enzyme that decreases to half the initial viscosity of galactomannan per minute. One unit of xylanase was defined as the amount of the enzyme that generated 1 μmol of the xylose from xylan per minute at pH 5.5 and 50°C, and one β-glucanase unit was defined as the amount of the enzyme that generated 1 μmol of the reducing sugar glucose from β-glucan per minute at pH 4.8 and 50°C. One Phy unit (FTU) was defined as the quantity of enzyme required to liberate 1 mmol inorganic phosphate per minute, at pH range 1.5–6.5, from an excess of 15 mM-sodium phytate at 37°C. The maximum activity was defined at pH 4.5.

Table 1 *Nutrient composition (as-fed basis) of rice bran used in experimental diets for broiler chickens*

Nutrient component	
DM (%)	89.57
CP (%)	13.91
Ash (%)	10.84
Gross energy (MJ/kg)	17.66
Fat (%)	12.34
Total phosphorus (%)	1.53
Phytic phosphorus	1.22
Calcium (%)	0.12
Neutral detergent fiber (%)	20.55
Essential amino acids (%)	
Arginine	1.13
Histidine	0.38
Isoleucine	0.42
Leucine	0.97
Lysine	0.67
Methionine	0.22
Phenylalanine	0.62
Threonine	0.64
Tryptophan	0.23
Valine	0.73
Glycine	0.65
Non-essential amino acids (%)	
Alanine	0.89
Aspartic acid	1.45
Cystine	0.15
Glutamic acid	1.71
Proline	0.62
Serine	0.71
Tyrosine	0.48

DM = dry matter; CP = crude protein.

Animals, experimental design and management

A total of 245 male broiler chickens (Cobb 500, 1-day-old) were allocated in a completely randomized design in a $2 \times 2 + 1$ (0 and 200 mg/kg MC; 0 and 50 mg/kg Phy, and basal diet (**BD**)) factorial arrangement of treatments, to give seven replicates and seven birds per replicate. At 21 days old, the birds were kept in metabolic cages (1.0×0.4 m) in a controlled environment for 21 days. The cages were housed in an environmentally controlled room with 24 h fluorescent lighting. Room temperature was maintained at 32°C during the first week, with a weekly reduction of 3°C until reaching a temperature of 26°C. Each metabolic cage was equipped with a feeder and a nipple drinker for *ad libitum* access to feed and water. Birds were vaccinated in the hatchery against Avian infectious bronchitis, Newcastle disease and infectious bursal disease, and mortality was recorded on daily basis throughout the study.

Table 2 *Composition of diets, as fed basis used in trial 1 and trial 2 in broiler chickens*

	Trial 1 – Total collection diet		Trial 2 – Ileal collection diet	
Ingredients (%)	BD	RB	BD	RB
RB[1]	–	30.00	–	30.00
Corn 7.88 %	85.01	59.51	–	–
Corn starch	–	–	50.27	35.19
Soybean oil	2.00	1.40	1.20	0.84
Choline chloride (60 %)	0.02	0.01	0.02	0.01
Salt	0.25	0.18	0.32	0.22
Calcium carbonate	0.38	0.27	0.35	0.24
Limestone	0.81	0.57	0.69	0.48
Dicalcium phosphate[2]	2.40	1.68	2.37	1.66
Cellulose	5.00	3.50	5.00	3.50
Dextrose	–	–	30.00	21.00
Casein	–	–	5.00	3.50
Chromic oxide	–	–	0.30	0.21
Vitamin-mineral premix[3]	0.5	0.36	0.50	0.35
Kaolin[4]	3.62	2.52	3.99	2.79
Calculated composition				
DM (%)	89.8	–	92.93	–
CP (%)	6.9	–	4.51	–
Calcium (%)	0.92	–	0.86	–
Available phosphorus (%)	0.4	–	0.34	–
Metabolizable energy (MJ/kg)	12.77	–	12.98	–
Sosium (%)	0.22	–	0.22	–
Choline (mg/kg)	2000	–	1900	–
Linoleic acid (%)	2.68	–	0.63	

BD = basal diet; RB = rice bran; DM = dry matter; CP = crude protein.
[1]Test diets were made by mixing BD and RB 7 : 3 wt/wt basis, without or with MC (700 U of α-galactosidase, 2200 U of galactomannanase, 30 000 U of xylanase and 22 000 U of β-glucanase/kg of diet); Phy (provided 500 FTU/kg of diet). MC and Phy replaced kaolin.
[2] Contained 20% Ca, 18.5% P.
[3]Vitamin-mineral premix contained per kilogram of premix: Vitamin A, 1 750 000 IU; Vitamin D_3, 550 000 IU; Vitamin E, 2750 IU; Vitamin K, 400 mg; Vitamin B_1, 500 mg; Vitamin B_2, 1250 mg; Vitamin B_6, 750 mg; Vitamin B_{12}, 3000 mcg; niacin, 8750 mg; pantothenic acid, 3250 mg; folic acid, 200 mg; choline, 82.01 g; Fe, 12.50 g; Mn, 17.50 g; Zn, 12.50 g; Cu, 24.95 g; I, 300 mg; Se, 50 mg; Monensin 25 g; Halquinol (5,7-dichloro-quinolin-8-ol) 7500 mg.
[4]Kaolin = mineral kaolinite (Used as inert ingredient to adjust the formulation of diet).

Diets and experimental period

In each trail was used a BD (Table 2) based on corn (trial 1) and cornstarch and casein (trial 2) to determine the coefficients of retention of nutrients and energy, and coefficients of digestibility of amino acids of the RB. The test diets were made by mixing BD and RB 7 : 3 wt/wt basis.

Broilers were fed a commercial starter corn-soybean meal-based diet for 10 days and subsequently were introduced to experimental diets. The objective of trial 1 was to determine the retention of nutrients and AMEN in RB, using a corn-based diet in birds from day 11 to day 18, considering first 4 days for adaptation. The excreta samples were collected two times daily (0830 and 1730 h) for 3 consecutive days (days 15 to 18). At the end of excreta collection, the samples were thawed at ambient temperature, homogenized and sub-samples packed in plastic containers and stored in freezer until analysis.

For measuring standardized ileal digestibility of amino acids in trial 2 from day 19 to day 21, a 5% casein/corn starch diet was used for the calculation of amino acids flows. The Chromium (III) oxide (Cr_2O_3, 0.3%) was added to all diets in an indigestible marker.

All diets were supplemented with vitamins and minerals to supply minimal requirements for starter broiler (NRC, 1994), but marginally lower in CP and metabolizable energy (Table 2). After 3-day adaptation (Moran, 1984), all birds were weighed and killed by cervical dislocation, and abdominal cavity opened immediately to expose the digestive tract. Ileum terminal segment of 10 cm was sectioned 2 cm proximal to the ileocecal junction to access ileal digesta. The ileal samples were frozen, and stored at –20°C until processed.

Chemical analysis and data processing

Excreta and ileal samples were freeze-dried for 72 h at –40°C (LH 0401, Terroni, Sao Carlos, Brazil). Before analyses, the diets, RB, excreta and ileal digesta samples were finely ground by an analytic mill processing to pass through a 2 mm screen (A11 Basic, IKA, Shanghai, China). Standard Association of Official Analytical Chemists (2005) procedures were used for determination of DM (method 930.15), ash (method 942.05), gross energy, nitrogen (method 990.03), total phosphorus (method 946.06), calcium (method 978.02) and NDF (Goering and Van Soest, 1970). Trial 2 diets and ileal digesta samples were further analyzed for amino acids content (method 994.12). The gross energy content was measured by a bomb calorimeter (IKA C5000, Karnataka, India) using benzoic acid as an internal standard. After processing samples in a muffle furnace for 8 h at 550°C, the samples were analyzed for ash. Before the determination of calcium and phosphorus concentration in diets and excreta sample, and chromium concentration in ileal digesta were digested in concentrated nitric acid and 70% perchloric acid. The calcium content and chromium concentration in the diet and ileal digesta were determined using inductively coupled plasma optical emission spectrometry (Model 710 ICP-OES, Agilent Technologies, Santa Clara, CA, USA). The Fiske-Subbarow reducer solution and acid molybdate were incorporated into the digested samples to determine the concentration of phosphorous by spectrophotometric reading of absorption at 620 nm (Model Cary 60 UV-Vis, Agilent Technologies). The nitrogen contents were analyzed in a nitrogen distiller (Model TE-036/1, Tecnal, Piracicaba, Brazil) using the Kjeldahl method. For amino acids, 100 mg sample was hydrolyzed with 6M HCl at 110°C for 24 h, followed by neutralization with 4 ml of 25% (wt/vol) NaOH and cooled to room temperature. The mixture was then equalized to 50 ml volume with sodium citrate buffer (pH 2.2) and analyzed using an amino acid analyzer (1260 Infinity LCs (Agilent Technologies). Tryptophan was determined by the colorimetric method of Spies (1967) using standard curve of pure tryptophan (Merck, Germany) and detected at 590 nm, with spectrophotometer (DU-640 UV/Vis – Beckman Coulter, Basking

Ridge, New Jersey, USA). Cyst(e)ine and methionine were analyzed as cysteic acid and methionine sulphone by oxidation with performic acid for 16 h at 0°C and neutralization with hydrobromic acid prior to hydrolysis. Cystine was expressed as cystine.

Calculations

The retention of nutrients was calculated as described by Hill and Anderson (1958) using the following equations:

$$\text{Retention of nutrients}\,(\%) = 100 \times [(\text{NI} - \text{NO}_{\text{excreta}})/\text{NI}],$$

where NI is the nutrient intake (g), $\text{NO}_{\text{excreta}}$ is the nutrient output in excreta (g).

The retention of nutrients in RB was determined by the difference method (Fan and Sauer, 1995), with the corn-based diet as the BD, using the following equation:

$$\text{DA} = (\text{DD} - (\text{DB} \times \text{DN}))/\text{DRB}$$

Where DA is the retention of a nutrient (%) in an assay feedstuff (RB), DD is the digestibility of a nutrient (%) in the RB-containing diet, DB is the digestibility of a nutrient (%) in the corn-based diet, DN is the contribution of a nutrient (decimal percentage) from corn to the assay diet and DRB is the contribution of a nutrient (decimal percentage) from RB in the RB-based diet.

The apparent metabolizable energy (**AME**) content for RB was calculated according to the following equation (Woyengo *et al.*, 2010):

$$\begin{aligned}&\text{AME of RB}\,(\text{kcal/kg})\\&= [(\text{Gross energy retention for RB}, \%)\\&\quad \times (\text{Gross energy content in RB}, \text{kcal/kg})]/100\end{aligned}$$

The AMEN content for RB was calculated from AME as described by Hill and Anderson (1958) using the following equation:

$$\text{AME}_{\text{N}}(\text{kcal/kg}) = \text{AME} - (8.22 \times \text{ANR})$$

where ANR = apparent nitrogen retained (g/kg of feed intake).

The apparent ileal digestibility of amino acids was calculated using the following formula (Nyachoti *et al.*, 1997):

$$\text{Apparent ileal digestibility}\,(\%) = 100 - [100 \times (\text{AA}_{\text{digesta}} \times \text{Cr}_2\text{O}_{3\text{diet}})/(\text{AA}_{\text{diet}} \times \text{Cr}_2\text{O}_{3\text{digesta}})$$

where AA_{diet} and $\text{AA}_{\text{digesta}}$ are amino acids content (mg/kg of DM) in the diet and digesta, respectively, and $\text{Cr}_2\text{O}_{3\text{diet}}$ and $\text{Cr}_2\text{O}_{3\text{digesta}}$ are indigestible marker content (mg/kg of DM) in the diet and digesta, respectively.

The apparent ileal digestibility of amino acids was standardized using the average values for basal endogenous amino acids losses calculated using the following formula (Nyachoti *et al.*, 1997):

$$\text{AAEL}(\text{g/kg}) = \text{AA}_{\text{digesta}} \times (\text{Cr}_2\text{O}_{3\text{diet}}/\text{Cr2O}_{3\text{digesta}})$$

where AAEL = average endogenous amino acid loss (g/kg of DM).

The standardized ileal digestibility of amino acids was calculated according to the following equation as described by Opapeju *et al.* (2006):

$$\begin{aligned}&\text{Standardized ileal digestibility}\,(\%)\\&= (\text{apparent ileal digestibility} + (\text{AAEL}/\text{AA}_{\text{diet}})) \times 100\end{aligned}$$

Statistical analysis of results

The GLM procedure of SAS (Statistical Analysis System, 2009, version 9.2) was used to determine the main effects of, and interaction between, MC and Phy. The homogeneity of variances was evaluated by Hartley test and the normality of residuals by the Shapiro-Wilk test (UNIVARIATE procedure). The statistical model used was $X_{ij} = \mu + \alpha_i + \beta_j + (a\beta)_{ij} + e_{ij}$, where μ = overall mean, α_i = MC effect, β_j = Phy effect, $(a\beta)_{ij}$ = interaction between MC and Phy, e_{ij} = error contribution with average 0 and variance σ_2, $i = 1 \ldots a$, and $j = 1 \ldots b$. Significance was accepted at $P < 0.05$. Tukey's LSD was used to detect significant differences between means whenever interaction between MC and Phy was observed.

Results

This study was conducted to evaluate nutritive value of RB fed to broiler chickens without or with a MC and Phy supplementation based on manufacturer recommendation. Exogenous enzyme activities were guaranteed and suggested by GNC Bioferm Inc. (Saskatoon, Saskatchewan, Canada).

Retention of nutrients and apparent metabolizable energy corrected for nitrogen

The effects of MC and Phy on total apparent digestibility of bird performance and nutrients and AMEN retention are shown in Table 3. Enzymes supplementation had significant effects ($P < 0.05$) on final BW, feed conversion ratio and retention of all nutrients and AMEN. The interaction effect ($P < 0.05$) between MC and Phy was observed on retention of DM, nitrogen and AMEN. The combination of MC and Phy corresponded to higher DM retention when compared to non-Phy supplementation. However, Phy supplementation coincided with higher nitrogen retention and AMEN in the presence of MC compared to its supplementation alone or without MC. There was no interaction ($P > 0.05$) between the supplemented enzymes on ash, calcium, phosphorus and NDF retention; however, an individual effect ($P < 0.05$) was observed for Phy on ash, phosphorus and calcium retention, and for MC on NDF retention.

Table 3 *Broiler chickens performances and retention of nutrients and apparent metabolizable energy content in rice bran fed without or with multi-carbohydrase (MC)[1] and phytase (Phy)[2] supplementation – trial 1*

	Phy[2] 0		Phy 50			*P*-value[3]		
Item	MC[1] 0	MC 200	MC 0	MC 200	SEM	MC	Phy	MC x Phy
Initial BW (g)	45	44	45	45	0.18			
Final BW (g)	811[c]	824[b]	821[b]	871[a]	11.21			*
Average daily feed intake (g)	48.38	47.99	47.45	49.09	3.07			0.057
Feed conversion ratio (g/g)	1.25[a]	1.22[b]	1.22[b]	1.18[c]	0.14			**
Mortality (%)	0[b]	0[b]	2.04[a]	0[b]	0.01		***	
DM (%)	75.08[c]	78.22[b]	76.98[c]	80.16[a]	0.39	***	*	*
Nitrogen (%)	73.60[d]	75.97[c]	77.70[b]	79.40[a]	0.42	*	***	*
Ash (%)	29.67[c]	30.08[c]	31.99[b]	32.77[a]	0.26	0.084	*	
Calcium (%)	48.16	48.68	50.49	50.57	0.23		*	
Phosphorus (%)	51.94	53.03	56.27	58.68	0.41		***	
Neutral detergent fiber (%)	31.76	33.60	31.72	33.99	0.23	*		
AME_N (MJ/kg)	8.47[c]	8.87[a]	8.73[b]	8.95[a]	0.06	**	*	**

SEM = standard error of mean; BW = body weight; DM = dry matter; AME_N = apparent metabolizable energy corrected for nitrogen.
$n = 7$.
[1] MC, multi-carbohydrase level (0 and 200 mg/kg of diet), (200 mg/kg of MC contains 700 U of α-galactosidase, 2200 U of galactomannanase, 30 000 U of xylanase and 22 000 U of β-glucanase/kg of diet).
[2] Phy, Phytase level (0 and 50 mg/kg of diet).
[3] *P*-value > 0.05; *, ** and ***, *P*-values ≤ 0.05, 0.01 and 0.001, respectively.
[a,b,c,d] Means assigned different letters within a factor of analysis (MC and Phy and their interactions) are significantly different, $P < 0.05$.

Apparent and standardized ileal amino acid digestibility

In Tables 4 and 5 the results of the apparent and standardized ileal digestibility of amino acids in broiler chickens fed without or with exogenous enzymes, respectively, are shown. Single or combined enzyme supplementation in the test diet increased the apparent and standardized ileal digestibility of all amino acids, except ($P > 0.05$) for apparent ileal digestibility of proline. The percentage values of apparent and standardized ileal digestibility of the 18 amino acids, according experimental diets were: non-enzymatic diet, 75.09% and 79.50%; MC, 76.93% and 81.44%; Phy, 77.38% and 82.25; and MC + Phy 78.92% and 84.24%.

Interaction ($P < 0.05$) between MC and Phy was observed on apparent digestibility for arginine, leucine, phenylalanine, tryptophan, threonine, valine, aspartic acid, glutamic acid and serine. The same effect of interaction ($P < 0.05$) was observed on standardized digestibility of arginine, histidine, leucine, methionine, phenylalanine, tryptophan, threonine, valine, aspartic acid, glutamic acid, proline and serine. However, there was no interaction ($P > 0.05$) of MC and Phy on apparent digestibility of histidine, isoleucine, lysine, methionine, alanine, cystine, glycine, proline and tyrosine, and standardized digestibility of isoleucine, leucine, lysine, alanine, cystine, glycine and tyrosine.

Discussion

Retention of nutrients and apparent metabolizable energy corrected for nitrogen

The NSP and phytate are considered anti-nutrient components in broiler feed (Gallardo *et al.*, 2017), and the predominant components in rice NSP are arabinose and xylose (Annison *et al.*, 1995). The arabinose : xylose ratio in rice NSP was 1 : 23 compared to 0 : 58 in wheat NSP, according to Annison *et al.* (1995) who reported that this generally confers high viscosity on the digesta. High viscosity in intestinal content causes reduction in enzyme activities and nutrient absorption (Bao *et al.*, 2013). On the other hand, RB has high total levels of phosphorus (1.5%), compared to corn (0.28%) and soybean meal (0.65%), (NRC, 1994). However, RB is an ingredient that has low rates of phosphorus availability due to its high phytate content.

Several studies (Slominski, 2011; Dadalt *et al.*, 2017; Gallardo *et al.*, 2017) have shown the positive effects provided by enzyme supplementation in poultry diets on nutrients and energy retention. According to Slominski (2011), a dietary mix of enzymes supplemented to promote multiactivities may be more effective than individual supplementation of enzyme. The combined addition of exogenous enzymes may result in additive, subadditive or synergistic effects on nutrient utilization and animal performance (Cowieson *et al.*, 2017; Dadalt *et al.*, 2017).

Thus, increased nutrient and energy retention may be associated with total or partial degradation of these anti-nutrient components. Carbohydrases destroy the cell wall, releasing nutrients and improving nutrient retention in broilers (Gallardo *et al.*, 2017). These enzymes also reduce viscosity of the digesta (Kiarie *et al.*, 2016), and indirectly facilitate the contact between endogenous and exogenous enzymes with the chemical compounds, such as phytase and phytate (Woyengo and Nyachoti, 2011). The increased retention of DM, nitrogen and AMEN in RB in the present study may be directly associated to the improvement in protein, fat, ash, starch and fiber availability due the interaction between MC and Phy.

Table 4 *Apparent ileal digestibility of amino acids in rice bran fed without or with multi-carbohydrase (MC)[1] and phytase (Phy)[2] supplementation to broiler chickens (21 days) – trial 2*

	Phy[2] 0		Phy 50			*P*-value[3]		
Item	MC[1] 0	MC 200	MC 0	MC 200	SEM	MC	Phy	MC × Phy
Essential amino acids (%)								
Arginine	83.96[d]	85.97[c]	86.99[b]	88.04[a]	0.34	**	*	*
Histidine	72.96	75.01	74.23	76.27	0.30	**	0.061	
Isoleucine	75.70[b]	76.90[b]	78.34[a]	79.57[a]	0.28		**	
Leucine	75.26[c]	76.61[b]	78.08[a]	78.98[a]	0.35		**	**
Lysine	73.30	73.69	75.67	75.49	0.33		*	
Methionine	81.00	83.65	84.14	86.49	0.38	**	*	
Phenylalanine	65.40[c]	67.78[b]	66.63[bc]	68.97[a]	0.32	*	0.072	**
Threonine	76.47[c]	78.45[b]	78.50[b]	80.52[a]	0.31	***	***	***
Tryptophan	75.02[d]	76.27[c]	79.82[b]	81.12[a]	0.35		***	***
Valine	71.36[c]	72.62[b]	72.15[b]	74.73[a]	0.26	**	**	**
Glycine	84.62	86.98	88.22	89.86	0.39	0.084	**	
Non-essential amino acids (%)								
Alanine	66.83	68.96	68.10	69.91	0.34	*	*	
Aspartic acid	75.27	76.74[d]	78.24[b]	79.81[a]	0.32	0.065	**	*
Cystine	67.19	68.56	68.96	69.89	0.27	*	*	
Glutamic acid	80.17[d]	83.65[b]	81.86[c]	85.48[a]	0.39	***	0.082	**
Proline	80.71	81.64	81.35	81.93	0.31			
Serine	76.33[c]	77.63[bc]	78.55[b]	80.12[a]	0.27		**	*
Tyrosine	71.08	71.83	73.05	73.42	0.27	0.069	*	

SEM = standard error of mean.
The endogenous ileal amino acids losses (g/kg of DM) using the casein diet were as follows: arginine, 0.10; histidine, 0.42; isoleucine, 0.14; leucine, 0.27; lysine, 0.21; metionine, 0.04; phenylalanine, 0.09; threonine, 0.47; tryptophan, 0.38; valine, 0.28; alanine, 0.22; aspartic acid, 0.38; cystine, 0.04; glutamic acid, 0.35; glycine, 0.03; proline, 0.21; serine, 0.42 and tyrosine, 0.20 g/kg.
$n = 7$.
[1] MC, multi-carbohydrase level (0 and 200 mg/kg of diet), (200 mg/kg of MC contains 700 U of α-galactosidase, 2200 U of galactomannanase, 30 000 U of xylanase and 22 000 U of β-glucanase/kg of diet).
[2] Phy, Phytase level (0 and 50 mg/kg of diet).
[3] *P*-value > 0.05; *, ** and ***, *P*-values ≤ 0.05, 0.01 and 0.001, respectively.
[a,b,c,d] Means assigned different letters within a factor of analysis (MC and Phy and their interactions) are significantly different, $P < 0.05$.

The NSPs also have the ability to bind to multivalent cations, which associate with phytate in ingredients and digesta (Dadalt *et al.*, 2017). Carbohydrases can improve Phy efficacy by eliminating phytate chelating effects in NSP (Slominski, 2011; Kiarie *et al.*, 2016). The increased retention of nitrogen by birds from the dietary supplementation of MC and Phy compared to the results of control birds suggests an increase in protein hydrolysis. Therefore, this digestive process could increase the proportion of soluble protein of low molecular weight available for absorption and retention by the chickens (Freitas *et al.*, 2011). Gallardo *et al.* (2017) observed similar responses in growing chickens when evaluating the effects of MC and Phy supplementation on nitrogen retention and amino acid digestibility of canola meal.

In addition, it is believed that the addition of phytase also favors the availability of energy-reducing mucin production due to the higher cellular turnover triggered by phytate in the diet (Bao *et al.*, 2013; Cowieson *et al.*, 2017), thus leading to greater dietary energy use. *In vitro* studies indicated positive effects when phytase supplementation provided hydrolysis of the phytate-protein complex, and the inhibitory action of phytate on activity of the digestive enzymes was reduced (Rodehutscord *et al.*, 1998). In previous studies, a higher use of nitrogen compounds with Phy in diets for broiler chickens was reported when they found higher CP digestibility (Dersjant-Li *et al.*, 2015), nitrogen retention and amino acid digestibility (Cowieson *et al.*, 2017; Gallardo *et al.*, 2017). On the other hand, Phy can also prevent the formation of binary protein-phytate complexes through previous hydrolysis and reduction of endogenous protein flow and nitrogen losses (Cowieson *et al.*, 2017), increasing the nitrogen retention.

The isolated effect of Phy supplementation was characterized by increased retention of ash and calcium. Dietary supplementation of Phy was associated with increased minerals' availability due to hydrolysis of phytate-bound minerals (Dadalt *et al.*, 2017). The significant effect of single enzyme supplementation on phosphorus retention suggests a possible additive effect when MC and Phy were combined, although there was significant interaction in mineral retention. This response can be justified due to the high total amount of phosphorus in RB, and higher part is complexed with phytate, decreasing the retention of phosphorus, which was also observed in the non-enzymatic diets. Thereby, there is greater need for Phy to hydrolyze the phytate. Thus, MC inclusion and probable phytate neutralization by Phy

Table 5 *Standardized ileal digestibility of amino acids in rice bran fed without or with multi-carbohydrase (MC)[1] and phytase (Phy)[2] supplementation to broiler chickens (21 days) - trial 2*

	Phy[2] 0		Phy 50			*P*-value[3]		
Item	MC[1] 0	MC 200	MC 0	MC 200	SEM	MC	Phy	MC × Phy
Essential amino acids (%)								
Arginine	88.06[b]	88.97[b]	92.13[a]	92.86[a]	0.35	0.079	***	*
Histidine	79.26[b]	80.95[b]	81.03[b]	83.92[a]	0.33	*	**	*
Isoleucine	82.90	84.34	84.40	86.37	0.28	**	**	
Leucine	79.36[c]	79.72[c]	81.77[b]	84.36[a]	0.39	0.062	***	***
Lysine	78.30	80.48	82.19	83.59	0.34	0.054	**	
Methionine	85.90[c]	87.88[b]	87.08[b]	89.45[a]	0.37	**	*	*
Phenylalanine	71.68[c]	74.91[b]	73.09[b]	77.45[a]	0.32	**	0.052	*
Threonine	81.16[d]	85.52[b]	83.46[c]	87.79[a]	0.30	***	0.051	*
Tryptophan	80.03[c]	80.19[c]	83.04[b]	85.11[a]	0.35		**	*
Valine	76.36[c]	77.62[c]	78.15[b]	80.36[a]	0.29	**	*	**
Glycine	84.90	88.05	86.54	90.19	0.38	***	*	
Non-essential amino acids (%)								
Alanine	70.02	72.24	72.37	75.94	0.35	***	***	
Aspartic acid	78.36[d]	80.72[c]	82.77[b]	84.86[a]	0.32	0.071	***	*
Cystine	72.16	73.47	76.63	77.76	0.30		**	
Glutamic acid	84.64[c]	87.47[b]	88.55[b]	90.12[a]	0.39	**	**	**
Proline	80.72[c]	84.94[b]	85.29[b]	88.20[a]	0.31	**	*	*
Serine	80.30[c]	81.48[c]	84.19[b]	86.29[a]	0.29	*	***	**
Tyrosine	76.97	76.98	77.83	77.65	0.27		0.084	

SEM = standard error of mean.
$n = 7$.
[1] MC, multi-carbohydrase level (0 and 200 mg/kg of diet), (200 mg/kg of MC contains 700 U of α-galactosidase, 2200 U of galactomannanase, 30 000 U of xylanase and 22 000 U of β-glucanase/kg of diet).
[2] Phy, Phytase level (0 and 50 mg/kg of diet).
[3] *P*-value > 0.05; *, ** and ***, *P*-values ≤ 0.05, 0.01 and 0.001, respectively.
[a,b,c,d] Means assigned different letters within a factor of analysis (MC and Phy and their interactions) are significantly different, $P < 0.05$.

supplementation improved phosphorus retention. Moreover, improvements on calcium retention were expected because Phy liberates calcium from the calcium-phytate complex. Therefore, as the availability of phosphorus increases, the availability of calcium also increases because both are part of the same complex. The complexing power of phytate can be reduced with dietary Phy, and more free calcium will be available to the bird (Selle *et al*., 2009).

The retention of NDF was affected only by individual MC supplementation; thus, positive effects from MC addition on fiber retention may be associated with the specificity of enzymes within the MC complex. Therefore, this result suggests that carbohydrase supplementation reduces the negative effects of fiber, improving the retention of NDF in RB.

Apparent and standardized ileal amino acid digestibility

As described previously, the average apparent and standardized ileal digestibility for 18 amino acids was higher in birds fed enzyme diets compared to those fed non-enzymatic diets. The effects of the isolated Phy and the combination with MC occurred in the apparent total tract digestibility corresponded to the digestibility of amino acids. Single MC or Phy supplementation influenced the retention of nitrogen and digestibility of amino acids; however, when combined, there was a significant increase on digestibility of amino acids. This observation may be associated with the probable hydrolysis of NSP due to inclusion of MC, and probable phytate neutralization by Phy supplementation. Amino acids digestibility also followed a pattern similar to nitrogen retention. Thus the increase of apparent and standardized ileal digestibility of amino acids induced by MC and Phy supplementation was mainly due to the increase in the retention of nitrogen, available protein and possibly also to the decrease of nitrogen excretion.

The anti-nutritive properties of phytate include amino acids' complexation, inhibition of endogenous enzymes and increase in endogenous secretions of amino acids, which affects their digestibility (Bao *et al*., 2013). On the other hand, Li *et al*. (1996) observed that NSP-degrading enzyme can hydrolyze or dissolve this compound in the cell wall, reducing or eliminating gel packets by increasing amino acid digestion in the small intestine. However, as previously described, the action of MC seems to be more effective at breaking down the fiber components than decreasing the viscosity of digesta. Increases in standardized ileal digestibility of amino acids from MC + Phy supplementation exceeded the sum of individual increases from inclusion of MC or Phy on apparent digestibility for arginine, threonine, tryptophan, valine, aspartic acid, glutamic acid and serine, and standardized digestibility of arginine, histidine, leucine, methionine, threonine, tryptophan, valine, aspartic acid, glutamic acid

and serine. These findings are in agreement with that reported by Ravindran *et al.* (1999), when observed similar synergistic responses using diet with Phy and xylanase supplementation to determine the digestibility of amino acids in wheat-casein diets. While Rutherfurd *et al.* (2002) reported that microbial phytase supplementation in the RB diet improved amino acid digestibility by 3%–12%.

Mucin secretion is associated with threonine, serine and proline, and these concentrations of amino acids are relatively high in broilers (Cowieson *et al.*, 2017). This flow of amino acids through mucin signifies a peptide nucleus of several amino acids (Onyango *et al.*, 2009), probably due to the increased demand for mucosal protection (Bao *et al.*, 2013). According to the authors, this is expensive for birds and inevitably leads to increased loss of endogenous amino acids. Thus, Cowieson *et al.* (2014) also reported that dietary phytate increased threonine, serine and proline losses in chickens and suggested a probable increase in mucin production and excretion.

A large part of the benefit of Phy was associated with reduction of endogenous amino acid losses, as reported by Selle *et al.* (2009), rather than increased digestibility of dietary amino acids, as reported by Gallardo *et al.* (2018) and Liu and Ru (2010). The authors considered higher prevalence of those amino acids that have great turnover: cystine, threonine, proline and glycine. Onyango *et al.* (2009) identified an increased amino acid loss that included arginine and glycine, along with threonine, serine and proline, when this loss was expressed as the proportion of endogenous CP.

Based on current results, it is expected that enzymes can release proteins and amino acids encapsulated in the cell wall or bound to phytate in RB, which may increase their digestibility.

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C. Gallardo 0000-0002-8070-8767
M. A. Trindade Neto 0000-0001-6481-9566

Declaration of interest

The authors have no conflict of interest to report.

Ethics statement

All research methods and procedures were approved by the ethics and animal experimentation committee at the University of São Paulo (project 2843/2012), Brazil, and were followed according to animal welfare.

Software and data repository resources

This study was a doctoral thesis; the data were deposited in online archives of University of Sao Paulo: http://www.teses.usp.br/teses/disponiveis/10/10135/tde-20022017-084441/en.php

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