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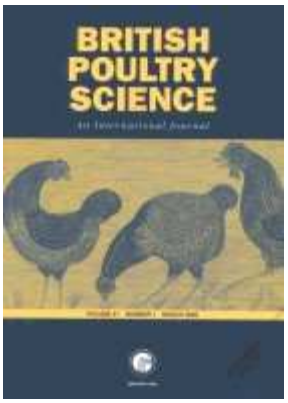

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The effects of supplementary bacterial phytase on dietary true metabolisable energy, nutrient digestibility and endogenous losses in precision fed turkeys

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Running title: Phytase for turkeys

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Abstract 1. A total of 40 female BUT9 turkeys were used in a precision-feeding assay to investigate the effect of dietary phytase on true metabolisable energy corrected for N retention (TME_N), true nutrient digestibility (TND), and the excretion of endogenous mucin, measured as sialic acid (SA).

2. Five treatments were used in this study: control (C), C + 250 phytase units (FTU) per kg feed, C + 500 FTU, C + 2500 FTU, and glucose only for endogenous losses estimation. Diets were formulated to be nutritionally adequate with the exception that the P content was relatively low (3.6 g/kg non-phytate P).

3. Inclusion of phytase increased TND in a quadratic manner with the optimum being achieved at approximately 500 FTU, at which TND was 37 % greater than in the control. The concentration of SA in the excreta decreased linearly with increased phytase supplementation. Dietary TME_N, true dry matter digestibility (TDMD) and true mineral digestibility coefficients were not significantly affected by phytase supplementation.

4. Phytase inclusion increased digestibility coefficients for indispensable, dispensable and total amino acids in a linear manner. The scale of the response to phytase was greatest with threonine and least with lysine digestibility, suggesting a specific mechanism of action that benefits gut health.

5. The strong negative relationship between secretion of SA and threonine digestibility suggests that a large part of the threonine benefit may be from reduced mucin synthesis. This supports the hypothesis that dietary phytase may play a role in improving the health status of the intestine and, as a result, reduces the maintenance energy requirements of turkeys.

INTRODUCTION

The majority of P in plants, including maize and soybean meal, is contained in phytic acid or its salts, collectively known as phytates (Nelson *et al.*, 1971; Reddy *et al.*, 1982). Phytate is

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viewed as an anti-nutrient since it chelates minerals and reacts with dietary protein, thus reducing the availability of many nutrients (Selle and Ravindran, 2007). Poultry do not produce meaningful quantities of endogenous phytase, the enzyme that can hydrolyse the ester bonds between the phosphate groups and the inositol ring in phytates. As a result, supplementation of poultry diets with commercially produced phytases is routine. The beneficial effect of this enzyme is understood to be due to the direct hydrolytic effects on phytate and the subsequent improvement in the availability of minerals, amino acids and energy, concomitant with reduced endogenous losses from the gastrointestinal tract (GIT) (Selle and Ravindran, 2007; Cowieson and Ravindran, 2007; Pirgozliev *et al.,* 2009*a*).

The majority of studies on phytase supplementation of feed have been conducted with broiler chickens, with relatively few having been conducted with turkeys, possibly as a result of an assumption that both species will have a similar response. However, Pirgozliev *et al*. (2007) demonstrated that turkeys were more responsive to phytase than chickens and, as a result, cast doubt on the aforementioned assumption. Phytase activity, and therefore phytate hydrolysis, in the intestines depends on intestinal pH, endogenous phytase activity and transit time, parameters that may differ between the two species (Maenz and Classen, 1998; Kerr *et al*., 2000; Simon and Igbasan, 2002). It therefore seems evident that application of broiler data to turkeys may be ill-founded. There is a lack of information that relates exogenous phytase activity with metabolisable energy, nutrient digestibility and endogenous losses when maize-soybean meal diets are fed to young turkeys.

The objective of this experiment was to address this issue and to further investigate the effects of phytase dose on excretion of intestinal mucin, as measured by the marker sialic acid (SA) in precision fed female turkeys.

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MATERIALS AND METHODS

Diets

An *Escherichia coli*-derived phytase (Quantum™, EC 3.1.3.26; AB Vista Feed Ingredients, Marlborough, UK) was used in this experiment. Five treatments were used in this study: a control (C), C + 250 phytase units (FTU) per kg feed (C250), C + 500 FTU/kg (C500), C + 2500 FTU/kg (C2500) and glucose (G) only for endogenous losses estimation. Diets were formulated to be nutritionally adequate with the exception of P, which was present at a relatively low level (3.6 vs 7.0 g/kg diet; Table 1). The enzyme was added to the diets in powder form and all diets were fed in meal form.

Table 1 near here

Bird husbandry and excreta collection

A precision feeding experiment (McNab and Blair, 1988) was conducted to compare the effect of three dietary concentrations of phytase on the true digestibility of dietary nutrients, TME_N and SA excretion. A total of 40 female BUT9 turkeys were allocated to each of the 5 treatments used in this study (C, C250, C500, C2500 and G), each treatment being replicated 8 times in a randomised block design. Initially, the experimental birds were reared on a standard commercial wheat-based broiler diet from 1-d-old until they reached 45 d of age. The birds were then placed on a raised slatted floor pen without access to feed, litter or excreta. Water was supplied *ad libitum* throughout the study *via* a suspended nipple drinker line. To alleviate the stress of feed deprivation, the birds were given 50 ml of glucose solution (600g/l) after 24 h. After a further 24 h, the birds were given 50 g of the test diets and placed in individual cages (0.5m x 0.8m), designed for excreta collection, at a constant house temperature of 20°C and 23 h of light per d. The turkeys used for endogenous loss estimation were fed 50 ml glucose solution instead of the experimental diets. Excreta were quantitatively collected for 48 h after feeding.

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The study was approved Animal Experimental Committee of SAC.

Laboratory analysis

After collection, excreta were freeze-dried, weighed and milled to pass through a 0.75-mm mesh. The gross energy (GE) of feed and excreta was determined using an adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL, 61265, USA). The nitrogen content of the excreta was determined by the method of Sweeney (1989) using an FP-200 nitrogen analyser (LECO®, St. Joseph, MI, USA). The TME_N of each of the experimental diets was then calculated as described by McNab and Blair (1988). Amino acid content in feed and excreta was determined by HPLC following oxygen-free hydrochloric acid digestion (Jones *et al.*, 1981). The system comprised a Dionex ASI-100 autosampler fitted with a Dionex P580 pump and a Dionex RF-2000 detector (Sunnyrale, California, USA). The flow rate used was 1 ml/min and the column used was a Spherisorb ODS2 (150 x 4.6mm fitted with a Waters guard cartridge). Since the method of hydrolysis destroys methionine, cystine and tryptophan, data for these amino acids are not reported. The coefficient of metabolisability of glycine is not presented because of confounding effect of glycine yield from acid hydrolysis of uric acid in excreta (Soares *et al*., 1971).

The mineral contents of feed and excreta were determined by inductively coupled plasma emmission spectrometry (Optima 4300 DV Dual View ICP-OE spectrometer, Perkin Elmer, Beaconsfield, UK), (Tanner *et al*., 2002). The concentration of excreta sialic acid was determined by the periodate-resorcinol method described by Jourdian *et al*. (1971).

The true digestibility coefficients for DM, N, amino acids and minerals were determined as follow:

([*intake* + (*endogenous loss*) - *excreted*]/ *intake*)

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where *intake* is the intake of DM, N, amino acid or minerals by precision fed birds, *endogenous loss* is the DM, N, amino acid or mineral excreted by glucose fed birds, and *excreted* is the DM, N, amino acid or mineral excreted by bird fed experimental diets.

Statistical analysis

The observational unit was a cage with one bird. Statistical analyses were performed by GenStat (GenStat, 11th edition, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). The data were analysed by ANOVA with a random term block and a fixed term treatment. Polynomial contrasts were performed to test for linear and quadratic relationships between phytase dose and the variable in question. Correlation coefficients were also generated to test for a possible relationship between the different variates. In all instances, differences were reported as significant at $P \leq 0.05$ and trends were noted when the P value was 0.05 - 0.10.

RESULTS

All birds remained healthy throughout the study period. There were no effects of diet on dietary TME_N, TDMD of SA excretion over the 48-h collection period (Table 2). The significant quadratic response of TND to phytase supplementation suggested that the optimum phytase addition for nitrogen retention was in the range of 500 FTU/kg feed, at which, TND was 37.3% higher than the control. SA concentration in the faeces was reduced in a significant linear fashion with increasing phytase dosage.

Despite the relatively large numerical increments in Ca and P digestibility with increasing phytase dosage, these differences were not significant (Table 3). The digestibility of all remaining minerals was unaffected by phytase dosage.

Tables 2 & 3 near here

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Exogenous phytase supplementation significantly improved the coefficients of digestibility of most of the measured dietary amino acids (Table 4) and in a dose dependent, linear manner. Threonine was the most responsive amino acid and histidine the least responsive.

Tables 4 & 5 near here

Total SA excretion related negatively to most of the determined parameters (Table 5), the most pronounced being that with TME_N (r = -0.896; P <0.001) and true dietary threonine digestibility coefficient (r = -0.909; P <0.001).

DISCUSSION

The fact that TND was subject to a quadratic dose relationship with phytase dose, the optimum being in the region of 500 FTU, suggests that nitrogen digestibility responds very rapidly to the first few units of phytase then reaches a plateau and may even decrease with further increments in phytase. Whilst the initial beneficial effect was as expected, the decline with higher doses is novel and may be specific to turkeys. The fact that the relationship between dose and response was quadratic with TND but linear with all amino acids suggests that the apparent loss in N digestibility beyond 500 FTU is a result of increased losses of non protein nitrogen. This may be a consequence of the precision feeding procedure which limits total nutrient intake and perhaps provides the animal with nutrients out of synchronisation with needs. Indeed, Härtel (1986) demonstrated that there is a lack of correlation between data obtained by precision compared to *ad libitum* fed birds. In addition, the results may be confounded by the use of excreta instead of ileal samples for amino acid determination. It has been demonstrated that amino acid metabolism by microflora in the lower gut of chickens may be substantial and that amino acid digestibility measured in the terminal ileum would differ from that measured in the excreta (Ravindran *et al*., 1999).

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Phytase supplementation more noticeably improved the digestibility of threonine, which is prevalent in endogenous secretion, than of lysine, which is primarily of dietary origin. Threonine comprises about two-thirds of the amino acid composition of mucin, and over half of the absorbed dietary threonine is used by the enterocytes (Allen, 1989). This suggests that phytase supplementation results in less mucin being excreted as has been reported earlier (Cowieson *et al*., 2004).

Whilst the SA content of the excreta was significantly reduced with increased phytase dosage, total SA losses were not. It should be noted, however, that the percentage reductions in these parameters were actually greater for total losses, suggesting that lack of significance may have been achieved with either greater precision or replication. Regardless, the general trends were in agreement with the literature. Threonine digestibility was inversely related to total SA losses and SA concentration in the excreta. In situations associated with increased endogenous excretions from the gastrointestinal tract, *e.g.* relatively high dietary phytate, threonine availability may limit intestinal mucin synthesis and consequently reduce gut barrier functionality. Alternatively, increased mucin secretion will increase threonine losses and hence reduce its metabolisability. These data therefore confirm the hypothesis that phytase application reduces mucin losses in a dose dependent manner, and, as a result, this is accompanied by a dose dependent increment in threonine digestibility (Cowieson *et al*., 2004, 2006; Pirgozliev *et al*., 2007, 2008).

The positive effect of dietary phytase on the utilisation of N and amino acids may be a result of several mechanisms. The stepwise manner of dephosphorylation of dietary phytate will lead to production of various *myo*-inositol isomers and phosphates (Venekamp *et al*., 1995; Greiner *et al*., 2000). The conditions of the gastrointestinal tract (time, pH, protease exposure) of poultry are not ideal for phytase to function optimally, so it is unlikely that

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commercial dosages of phytase will be able to dephosphorylate dietary phytate completely. Typically, 500 FTU of an Aspergillus phytase will result in only 40% IP6 destruction (Ravindran *et al*., 2001). However, with increased phytase dosage, *e.g*. 2500 FTU/kg feed, there will likely significantly less intact IP6, with relatively high number of lower ester forms (Ravindran *et al.*, 2001; Zyla *et al*., 2004; Pirgozliev *et al.*, 2008). It has been already shown that the extent of interactions among N, amino acids and *my*o-inositol isomers will depend on the degree of phosphorylation of the inositol ring (Knuckles *et al*., 1989; Harland and Narula, 1999). Lower molecular weight *myo*-inositol isomers such as mono- and di-phosphates have limited capacity to form chelates and to inhibit the digestibility of protein (Knuckles *et al*., 1989; Harland and Narula, 1999). It has been also reported that removal of phosphate residues from phytate results in a reduced impairment of intestinal uptake of essential dietary minerals, *e.g.* P (the topic reviewed by Greiner and Konietzny, 2006). Presumably, some of the beneficial effects noted in the current study associated with dietary phytase supplementation were due to dephosphorylation of dietary phytate into lower molecular weight *myo*-inositol isomers and a reduced capacity to interfere with amino acid utilization (Ravindran *et al*., 2001; Cowieson *et al*., 2006).

Dietary phytase has quite consistently increased AME of broiler diets based on wheat and/or sorghum (Selle and Ravindran, 2007), so the lack of a significant difference in dietary TME_N in this study was not expected. However, the differences in the morphological and physiological development of the chickens gut compared to those of turkeys at the same age (Sklan *et al*., 1982), may explain the fact that the changes in dietary metabolisable energy in this study did not exactly follow the same pattern noted with chickens. It has been also suggested that that metabolisable energy measures are not sensitive enough for evaluation of

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energetic effect of phytases since they do not take intake into account and *post* absorptive effects, both of which contribute to net energy (Pirgozliev *et al*., 2009*b*).

Including phytase in the diet did not result in significant increments in mineral digestibility coefficients in this study, although it has been often reported in work with chickens (Selle and Ravindran, 2007). However, Rodehutscord and Dieckmann (2005) have demonstrated that different poultry species utilise dietary minerals with varying degrees of efficiency. For example, utilisation of plant P was 58% in broilers and only 39% in turkeys. The lack of sufficient mineral supply in the experimental diets in this study may therefore partly explain the lack of response to phytase. Research by Létourneau-Montminy *et al*. (2008) also suggested that differences in level of production, *e.g.* growth (associated with amino acid digestibility) and bone mineralisation (associated primarily with mineral digestibility), may require different mineral balance and also different supplemental phytase activities.

It may be concluded that young turkeys can tolerate phytase activities much higher than 1000 FTU and have been shown to benefit from such doses. The results from this study show that dietary phytase primarily influences the digestibility of dietary nitrogen and amino acids, with the effect on threonine being most noticeable. It is unclear why nitrogen digestibility peaks at 500 FTU/kg then falls, whilst amino acid digestibility continued to improve, but this is possibly related to an imbalance in amino acids leading to increased uric acid losses. The linkage between threonine digestibility and mucin losses, as measure by SA losses, further emphasises the importance of this amino acids in mucin synthesis. Clearly phytate and phytase play a role in determining mucin losses and, as a result, the threonine requirements of the animal. However, to understand the mode of action of dietary phytases further research on *myo*-inositol isomers of lower molecular weight needs to be completed.

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Table 1. *Ingredient composition (g/kg 'as fed') of the control experimental diet*

Feedingstuff	(g/kg)		
Maize	475.7		
Soya bean meal (48)	444.5		
Vegetable oil	30.0		
Limestone	25.0		
Mono DCP	10.0		
Salt	3.5		
Lysine HCl	2.0		
Methionine	4.3		
Vitamin mineral premix	5.0		
Total	1000		
Calculated analysis (as fed basis)			
ME (MJ/kg)	12.19		
Sodium g/kg	0.16		
Total P (g/kg)	6.40		
Non-phytate P (g/kg)	3.60		
Analysed values (DM)[2]			
GE (MJ/kg)[3]	19.40		
CP (N x 6.25) g/kg	288		
DM (g/kg)	881		
Amino acid composition (g/kg DM)		*Mineral composition (g/kg DM)*	
Alanine	14.40		
Arginine	17.77	Calcium	15.2
Aspartate	34.28	Potassium	13.40
Glutamate	58.25	Magnesium	2.15
Histidine	6.69	Manganese	0.12
Isoleucine	15.29	Sodium	1.45
Leucine	26.08	Phosphorus	7.17
Lysine	19.92	Sulfur	3.52
Phenylalanine	15.07	Zinc	0.09
Serine	10.06		
Threonine	9.74		
Tyrosine	6.37		
Valine	15.38		

[1]The vitamin and mineral premix contained vitamins and trace elements to meet the requirements specified by NRC (1984). All the experimental diets were designed to be low in P. The premix provided (units/kg diet): retinol 3600 µg, cholecalciferol 125 µg, α-tocopherol 34 mg, menadione 3 mg, thiamine 2 mg, riboflavin 7 mg, pyridoxine 5 mg, cobalamin 15 µg, nicotinic acid 50 mg, pantotenic acid 15 mg, folic acid 1 mg, biotin 200 µg, iron 80 mg, copper 10 mg, manganese 100 mg, cobalt 0.5 mg, zinc 80 mg, iodine 1 mg, selenium 0.2 mg and molybdenium 0.5 mg.
[2]Analyses were performed in duplicate.
[3]Gross energy.

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Table 2. *The effect of phytase on true metabolisable energy corrected for nitrogen retention (TME_N), true dry matter (TDMD) and true nitrogen (TND) digestibility coefficients, and sialic acid (SA) excretion when precision fed to 47-d-old female turkeys*

Parameters/ Treatments	C[1]	C+250[2]	C+500[2]	C+2500[2]	LSD[3]	P[4]	L[5]	Q[6]
TME_N (MJ/kg DM)	15.97	16.22	16.37	16.33	1.057	NS	NS	NS
TDMD	0.788	0.806	0.813	0.815	0.0652	NS	NS	NS
TND	.590	0.615	0.810	0.668	0.1127	<0.05	<0.05	<0.05
SA (µg/g excreta)	2.110	2.253	1.913	1.859	0.2983	<0.05	<0.05	NS
SA (µg excreted/48 h)	38.6	38.7	31.6	31.2	11.12	NS	0.099	NS

[1]Control maize-based diet was low (3.6 g/kg non-phytate P) in available phosphorus.
[2]Phytase activity (units/kg) in diet.
[3]Least significant differences between means.
[4]Significance between treatments determined by ANOVA.
[5]Linear effects of added dietary phytase activities.
[6]Quadratic effects of added dietary phytase activities.
[4, 5, 6] Results are statistically significant when $P \leq 0.05$ (the P values around 0.1 are described as showing a tendency).

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Table 3. *The effect of phytase on true digestibility of dietary minerals when precision fed to 47-d-old female turkeys*

Minerals/ Treatments	C[1]	C+250[2]	C+500[2]	C+2500[2]	LSD[3]	P[4]	L[5]	Q[6]
Ca	0.651	0.656	0.646	0.738	0.0981	NS	0.107	NS
K	0.526	0.529	0.573	0.526	0.2127	NS	NS	NS
Mn	0.470	0.415	0.478	0.490	0.1529	NS	NS	NS
Mg	0.458	0.533	0.593	0.580	0.2137	NS	NS	NS
Na	0.640	0.699	0.748	0.703	0.2557	NS	NS	NS
P	0.429	0.640	0.600	0.558	0.1990	NS	NS	0.074
S	0.731	0.753	0.780	0.731	0.1399	NS	NS	NS
Zn	0.502	0.463	0.485	0.587	0.2711	NS	NS	NS

[1]Control maize-based diet was low (3.6 g/kg non-phytate P) in available phosphorus.
[2]Phytase activity (units/kg) in diet.
[3]Least significant differences between means.
[4]Significance between treatments determined by ANOVA.
[5]Linear effects of added dietary phytase activities.
[6]Quadratic effects of added dietary phytase activities.
[4, 5, 6] Results are statistically significant when $P \leq 0.05$ (the P values around 0.1 are described as showing a tendency).

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Table 4. *The effect of phytase on the true digestibility coefficients of dietary amino acids when precision fed to 47-d-old female turkeys*

Amino acids/ Treatments	C[1]	C+250[2]	C+500[2]	C+2500[2]	LSD[3]	P[4]	L[5]	Q[6]
Indispensable								
Arginine	0.935	0.935	0.962	0.959	0.0216	<0.05	<0.05	NS
Histidine	0.928	0.922	0.956	0.949	0.0218	<0.05	<0.05	NS
Isoleucine	0.891	0.893	0.917	0.923	0.0284	0.069	<0.05	NS
Leucine	0.902	0.905	0.928	0.931	0.0242	<0.05	<0.05	NS
Lysine	0.934	0.924	0.958	0.961	0.0234	<0.05	<0.05	NS
Phenylalanine	0.898	0.906	0.927	0.931	0.0241	<0.05	<0.05	NS
Threonine	0.831	0.813	0.870	0.883	0.0508	<0.05	<0.05	NS
Valine	0.868	0.868	0.897	0.904	0.0372	0.105	<0.05	NS
Dispensable								
Alanine	0.864	0.862	0.895	0.900	0.0382	0.097	<0.05	NS
Aspartic acid	0.909	0.901	0.922	0.940	0.0275	<0.05	<0.05	NS
Glutamic acid	0.930	0.927	0.943	0.954	0.0183	<0.05	<0.05	NS
Serine	0.874	0.858	0.908	0.907	0.0467	0.092	<0.05	NS
Tyrosine	0.869	0.869	0.924	0.915	0.0450	<0.05	<0.05	NS
Average indispensable	0.897	0.894	0.926	0.929	0.0287	<0.05	<0.05	NS
Average dispensable	0.889	0.883	0.918	0.923	0.0334	0.05	<0.05	NS
Average total amino acids	0.895	0.891	0.923	0.927	0.0302	<0.05	<0.05	NS

[1]Control maize-based diet was low (3.6 g/kg non-phytate P) in available phosphorus.
[2]Phytase activity (units/kg) in diet.
[3]Least significant differences between means.
[4]Significance between treatments determined by ANOVA.
[5]Linear effects of added dietary phytase activities.
[6]Quadratic effects of added dietary phytase activities.
[4, 5, 6] Results are statistically significant when $P \leq 0.05$ (the P values around 0.1 are described as showing a tendency).

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Table 5. *Correlation matrix for the dietary metabolisable energy, nitrogen, dry matter, mineral and amino acids response criteria of female turkeys to dietary phytase supplementation*

	TME_N (DM)	TDMD	TND	TDThre	TDDisp	TDIndisp	TDTotal	TDS	TDP	TDCa	SATotal	SAConc
TME_N (DM)	1.000											
TDMD	0.983	1.000										
TND	0.858	0.933	1.000									
TDThre	0.873	0.810	0.680	1.000								
TDDisp	0.878	0.815	0.664	0.987	1.000							
TDIndisp	0.867	0.801	0.675	0.990	0.996	1.000						
TDTotal	0.872	0.807	0.672	0.990	0.999	1.000	1.000					
TDS	0.914	0.942	0.896	0.760	0.752	0.736	0.742	1.000				
TDP	0.772	0.833	0.807	0.401	0.416	0.424	0.422	0.725	1.000			
TDCa	0.807	0.808	0.647	0.719	0.708	0.700	0.703	0.752	0.614	1.000		
SATotal	-0.896	-0.866	-0.792	-0.909	-0.877	-0.877	-0.878	-0.843	-0.612	-0.797	1.000	
SAConc	-0.472	-0.412	-0.421	-0.717	-0.659	-0.664	-0.663	-0.444	-0.076	-0.519	0.786	1.000

d.f. = 10. Correlation coefficients greater than 0.576 and 0.708 are statistically significant at 5% (P <0.05) and 1% level (P <0.001), respectively.

Key: TME_N (DM), TDMD and TND are the determined dietary true metabolisable energy corrected for nitrogen retention, true dry matter and true nitrogen digestibility coefficients.

TDThre, TDDisp, TDIndisp and TDTotal are the determined true digestibility coefficients of dietary threonine, dispensable, indispensable and total amino acids.

TDS, TDP and TDCa are the determined true digestibility coefficients of dietary sulfur, phosphorus and calcium.

SATotal is the total amount of sialic acid excreted during the 48 hour experimental period. SAConc is the concentration of the excreted sialic acid.

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