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Improvements in growth performance, bone mineral status and nutrient digestibility in pigs following the dietary inclusion of phytase are accompanied by modifications in intestinal nutrient transporter gene expression

Stafford Vigors[1], Torres Sweeney[2], Cormac J. O'Shea[1], John A. Browne[2] and John V. O'Doherty[1]*

[1]*School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Republic of Ireland*
[2]*School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Republic of Ireland*

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Abstract

Phytase (PHY) improves growth performance, nutrient digestibility and bone structure in pigs; however, little is known about its effects on intestinal nutrient transporter gene expression. In the present study, a 44 d experiment was carried out using forty-eight pigs (11·76 (SEM 0·75) kg) assigned to one of three dietary treatment groups to measure growth performance, coefficient of apparent ileal digestibility (CAID), coefficient of apparent total tract nutrient digestibility (CATTD) and intestinal nutrient transporter gene expression. Dietary treatments during the experimental period were as follows: (1) a high-P (HP) diet containing 3·4 g/kg available P and 7·0 g/kg Ca; (2) a low-P (LP) diet containing 1·9 g/kg available P and 5·9 g/kg Ca; (3) a PHY diet containing LP diet ingredients + 1000 phytase units (FTU)/kg of PHY. The PHY diet increased the average daily gain ($P<0·05$) and final body weight ($P<0·01$) and decreased the feed conversion ratio ($P<0·05$) compared with the LP diet. Pigs fed the PHY diet had a higher CAID of gross energy compared with those fed the HP and LP diets ($P<0·001$). Pigs fed the PHY diet had increased CAID of P ($P<0·01$) and CATTD of Ca and P ($P<0·001$) compared with those fed the LP diet. The PHY diet increased the gene expression of the peptide transporter 1 (*PEPT1/SLC15A1*) ($P<0·05$) in the ileum compared with the LP diet. The LP diet decreased the gene expression of the sodium–glucose-linked transporter 1 (*SGLT1/SLC5A1*) and *GLUT2/SLC2A2* ($P<0·05$) and increased the expression of membrane Ca channel (*TRPV6*) and calbindin compared with the HP diet ($P<0·001$). In conclusion, feeding a diet supplemented with PHY improves growth performance and nutrient digestibility as well as increases the gene expression of the peptide transporter *PEPT1*.

Key words: **Phytase: Phosphorus: Gene expression: Nutrient transporters: Bones: Minerals**

P is poorly utilised by pigs because approximately two-thirds of P in feedstuffs of plant origin is present as phytate, which is largely unavailable for hydrolysis in the digestive tract[1]. The phytate molecule is also capable of binding to other nutrients including starch and proteins, thus preventing their absorption[2]. The enzyme phytase (PHY) has been shown to improve P availability and subsequently improve growth, feed efficiency, nutrient utilisation and bone mineralisation to levels comparable to those induced by a diet supplemented with inorganic P, but to reduce nutrient excretion levels[3].

Studies have shown that the dietary inclusion of PHY has an effect on the availability of a number of nutrients[4]. The inclusion of PHY improves the digestibility of amino acids, starch, Ca, gross energy (GE), Fe, fat and Zn[5–9]. The action of PHY is likely to increase the availability of free nutrients including amino acids, peptides, fatty acids, glucose, galactose and fructose in the digesta as the phytate molecule undergoes hydrolysis and the bound starch, protein and fat are released. Although it is apparent that nutrient digestibility improves with the dietary inclusion of exogenous PHY, the physiological response of intestinal nutrient transporters to PHY is not well characterised.

Intestinal enterocytes are constantly exposed to fluctuations in dietary nutrients and therefore have to respond to fluctuations in luminal nutrients[10]. Fluctuations in nutrient availability may affect the gene expression of nutrient transporters through a mechanism of nutrient sensing[11]. Nutrient transporters, expressed on the apical membrane of intestinal

Abbreviations: CAID, coefficient of apparent ileal digestibility; CATTD, coefficient of apparent total tract digestibility; *FABP2*, fatty acid-binding protein 2; GE, gross energy; HP, high-phosphorus; LP, low-phosphorus; *PEPT1*, peptide transporter 1; PHY, phytase; *PMCA1*, plasma membrane Ca^{2+} ATPase; *SGLT1*, sodium–glucose-linked transporter 1.

* **Corresponding author:** J. V. O'Doherty, email john.vodoherty@ucd.ie

absorptive cells, are directly exposed to an environment that changes significantly with diet, and consequently their expression is adaptively regulated by dietary substrates[12].

The hypothesis that improvements in growth performance, skeletal bone mineralisation and nutrient digestibility following supplementation of a low-P (LP) diet with PHY are accompanied by changes in the gene expression of intestinal nutrient transporters involved in peptide, mineral, carbohydrate and fatty acid transport was tested in the present study.

Materials and methods

All procedures used in the present experiment were conducted under experimental licence from the Irish Department of Health in accordance with the Cruelty to Animals Act 1876 and the European Communities (Amendment of the Cruelty to Animals Act 1876) Regulations, 1994.

Experimental design and diets

An experiment with a completely randomised design was carried out to investigate the effects of three dietary treatments on growth performance, coefficient of apparent ileal digestibility (CAID), coefficient of apparent total tract digestibility (CATTD), bone mineral accretion and intestinal nutrient transporter gene expression in growing pigs (43 kg body weight). The experimental period was divided into two components delineated by a diet change to match the requirements of pigs as they progressed from a phase when fed a nutritionally rich diet after weaning (days 0–23) to a phase when fed a lower-specification diet (days 23–44)[13]. The composition and chemical analysis of the experimental diets are summarised in Table 1. Dietary treatments during period 1 (days 0–23) were as follows: (1) a high-P (HP) diet containing 5·9 g/kg total P, 3·4 g/kg available P and 7 g/kg Ca; (2) a LP diet containing 4·9 g/kg total P, 1·9 g/kg available P and 5·8 g/kg Ca; (3) a PHY diet containing LP diet ingredients + 1020 phytase units (FTU)/kg of PHY (Ronozyme®, DSM Nutritional Products Limited). Dietary treatments during period 2 (days 23–44) were as follows: (1) a HP diet containing 5·9 g/kg total P, 3 g/kg available P and 6·7 g/kg Ca; (2) a LP diet containing 4·1 g/kg total P, 1·7 g/kg available P and 4·7 g/kg Ca; (3) a PHY diet containing LP diet ingredients + 1030 FTU/kg of PHY. The HP diet was designed to match the standards set out by the NRC[14] for Ca and P levels, while the LP diet was designed to contain both Ca and P levels 16% lower than those outlined by the NRC during period 1 and 30% lower during period 2[14]. All diets were designed to contain similar levels of standard ileal digestible lysine (13·3 and 11·1 g/kg during periods 1 and 2, respectively) and digestible energy (14·7 and 14·3 MJ/kg during periods 1 and 2, respectively). All diets were offered in meal form.

Table 1. Composition and chemical analysis of the experimental diets (as-fed basis; g/kg unless otherwise stated)

Dietary treatments	HP (days 0–23)	LP (days 0–23)	PHY (days 0–23)	HP (days 23–45)	LP (days 23–45)	PHY (days 23–45)
Ingredients						
Wheat	400·0	400·0	400·0	404·0	406·0	406·0
Barley	194·0	203·5	203·5	307·5	318·5	318·5
Soyabean meal	180·0	180·0	180·0	210·0	210·0	210·0
Full-fat soya	150·0	150·0	150·0	0	0	0
Soya oil	45·0	40·0	40·0	45·0	40·0	40·0
Limestone	4·5	6·0	6·0	6·0	8·0	8·0
Salt	3·0	3·0	3·0	3·0	3·0	3·0
Dicalcium phosphate	11·2	5·0	5·0	12·0	2·0	2·0
Vitamin and mineral premix*	3·0	3·0	3·0	3·0	3·0	3·0
Lys HCl	5·0	5·0	5·0	5·0	5·0	5·0
L-Thr	2·3	2·3	2·3	2·3	2·3	2·3
DL-Met	2·2	2·2	2·2	2·2	2·2	2·2
Celite	0·3	0·3	0·3	0·3	0·3	0·3
Analysis						
DM	882·9	882·9	882·9	890·1	888·1	888·1
Crude protein (N × 6·25)	202·3	199·9	199·9	173·8	185·8	185·8
Neutral-detergent fibre	144·3	103·7	103·7	128·6	126·3	126·3
Ash	45·8	43·9	43·9	44·4	37·3	37·3
Gross energy (MJ/kg)	17·0	17·0	17·0	16·9	16·8	16·8
Digestible energy (MJ/kg)	14·7	14·8	14·7	14·3	14·3	14·3
Lys†	13·2	13·3	13·3	11·1	11·1	11·1
Met and Cys†	7·1	7·2	7·2	7·1	7	7
Thr†	8·6	8·6	8·6	7·2	7·2	7·2
Trp†	2·4	2·4	2·4	2·0	2·0	2·0
Ca (g/kg)	7·0	5·9	5·9	6·7	4·7	4·7
P (g/kg)	5·9	4·9	4·9	5·9	4·1	4·1
Available P (g/kg)†	3·4	1·9	1·9	3·0	1·7	1·7
Phytase activity (FTU/kg)	0	0	1020	0	0	1030

HP, high P; LP, low P; PHY, phytase; FTU, phytase units.
* The premix provided vitamins and minerals (per kg diet) as follows: Cu 25; Zn 100; Se 0·3; Mn 25; I 0·2; retinol 0·3; cholecalciferol 0·05; α-tocopherol 40.
† Calculated for the tabulated nutritional composition[48].

Experimental protocol

A total of forty-eight pigs (Meatline boars × (Large White × Landrace) sows) with an average initial body weight of 11·76 (SD 0·75) kg were used in a 44 d weaner performance study. Pigs were penned in mixed-sex groups of two (eight replicates per treatment) on fully slatted floors (1·68 m × 1·22 m). Feed and water were available *ad libitum* with precaution taken to avoid feed wastage. Feed was kept in the feeders until the time the pigs were weighed, and then the feed was weighed again to calculate the feed conversion ratio. Pigs were weighed weekly. The ambient environmental temperature within the houses was thermostatically controlled. The temperature was set at 28°C during the 1st week and was reduced by 2°C per week to 22°C. Multiple fresh faecal samples were collected daily from all pens on days 10–15 during period 1 and on days 25–30 during period 2 and stored in sterile containers (Sarstedt). During the experiment, feed samples were collected at the time of feeding and stored until chemical analysis. Celite (300 mg/kg) was added to the feed during manufacture to measure the CAID and CATTD using the acid-insoluble ash technique[15]. On day 44, the male pig from each pen was slaughtered. Pigs were killed by lethal injection of Euthatal (pentobarbitone sodium BP; Merial Animal Limited) at a rate of 1 ml/1·4 kg body weight. After slaughter, the right front foot of twenty-four pigs was cleaned of all skin, muscle and connective tissue to remove the third and fourth metacarpals. Following removal, the metacarpals were again cleaned of any remaining flesh. These bones were subsequently used for the assessment of bone ash, P, and Ca levels and bone density. All metacarpals collected were individually stored at −20°C to prevent desiccation until analysis.

Following a 3 h fast and slaughter, the entire digestive tract was removed by blunt dissection. Following tract removal, digesta samples were recovered aseptically from the ileum as sections of approximately 30 cm in length from the ileocaecal valve to measure the CAID of nutrients (N, DM and GE). Tissue samples from the jejunum (60 cm from the stomach) and ileum (8 cm from the ileocaecal valve) were collected and emptied by dissecting along the mesentery and rinsing using sterile PBS (Oxoid) as described previously[16,17]. Sections measuring 1 cm^2, which had been stripped of the overlying smooth muscle, were cut from the tissue samples and stored in RNAlater solution (Applied Biosystems) overnight at 4°C. RNAlater was removed later, and tissue samples were stored at −70°C until RNA extraction.

Laboratory analysis of samples

Feed and faecal samples were analysed for N, DM, organic matter, ash, GE and neutral-detergent fibre. Following collection, faecal samples were dried at 100°C for 48 h. Feed and dried faecal samples were milled through a 1 mm screen (Christy and Norris Hammer Mill). Diet, faecal and digesta samples were analysed for DM (method 934.01) and crude ash (method 942.05) according to the AOAC[18]. The DM content was determined after drying for 24 h at 100°C. The crude ash content was determined after ignition of a weighed sample in a muffle furnace (Nabertherm) at 550°C for 6 h. The ash was then digested in aqua regia ($HCl–HNO_3$ mixture). In the first round of digestion, ash was digested using 20 % aqua regia (4:1 HCl and nitric acid (v/v)) and subsequently digested using 25 % aqua regia. This solution was used for the determination of P and Ca concentrations. The concentration of Ca in feed, digesta and faecal samples was determined using an atomic absorption spectrophotometer (Varian 50; Varian, Inc.) using the method of Ramakrishna & Robinson[19]. The concentration of P was determined spectrophotometrically (PU 8600 UV/visible spectrophotometer, Pye Unicam, Philips) using the method of Cavell[20]. The GE was determined using an adiabatic bomb calorimeter (Parr Instruments) as described by O'Shea *et al.*[21]. The neutral-detergent fibre fraction was analysed using the Fibertec Extraction Unit (Fibertec, Tecator) as described by Van Soest *et al.*[22]. The concentration of N in diet, faecal and digesta samples was determined using a LECO FP 528 (Leco Instruments (U.K.) Limited) as described by O'Shea *et al.*[21]. The concentration of acid-insoluble ash was determined according to the method of McCarthy *et al.*[23]. Feed samples were analysed for PHY activity according to the method reported by Brady *et al.*[24]. PHY activity is expressed as FTU per unit of feed and is defined as the quantity of enzyme that liberates 1 μmol of inorganic P per min from a 1·5 mmol/l solution of sodium phytate at pH 5·5 and 37°C[25].

Bone analysis

Bone samples were analysed for DM, density, ash, Ca and P. Bone density was calculated using a balance (Scout Pro balance 200 g × 0·01 g, Ohaus Limited). The samples were first weighed in air and then weighed by submerging in distilled water using the integral weigh-below hook facility. Bone volume was calculated by subtracting the wet weight from the dry weight, and bone density was determined by dividing the dry weight by the volume[26]. The samples were placed in an oven at 100°C for 16 h to determine DM weight. The samples were then ashed at 650°C in a muffle furnace, and the ash was digested in aqua regia ($HCl–HNO_3$ mixture) and analysed for Ca and P as described above for faecal and digesta samples.

RNA extraction and real-time RT-PCR

Total RNA was extracted from ileal and jejunal samples (25 mg) using TRIzol Reagent (Sigma-Aldrich) according to the manufacturer's instructions. The crude RNA extract was further purified using the GenElute Mammalian Total RNA Miniprep Kit (RTN70, Sigma-Aldrich) according to the manufacturer's instructions. A DNase removal step was included (DNASE7-E70, Sigma-Aldrich). The total RNA was quantified using a NanoDrop-ND1000 spectrophotometer (Thermo Fisher Scientific, Inc.). The purity of RNA was assessed by determining the ratio of the absorbance at 260 nm to that at 280 nm. All total RNA samples had 260:280 nm ratios above 1·8. In addition, the integrity of RNA was verified using the Agilent RNA 6000 NanoChip Bioanalyzer Kit (Agilent Technologies). All samples

had a RNA integrity number above 8 (average 8·3 (SE 0·59)). Total RNA (1 μg) was reverse-transcribed using a commercially available complementary DNA synthesis kit (First Strand cDNA Synthesis Kit, Fermentas) using oligo-deoxy-thymine (dT) primers in a final reaction volume of 20 μl according to the manufacturer's instructions, and minus-RT and no-template controls were included. The final reverse transcription product was adjusted to a volume of 120 μl using nuclease-free water. The mRNA expression profiles of selected candidate genes were analysed by quantitative real-time PCR using the ABI Prism 7500 FAST Sequence Detection System (Applied Biosystems). PCR amplification was performed in a total volume of 20 μl containing 10 μl of master mix (SYBR PCR Master Mix, Applied Biosystems), 1·0 μl of forward and reverse primers (300 pM final), 6·5 μl of RNAse-free water, and 2·5 μl of template complementary DNA (5·0 ng of RNA equivalents). The two-step PCR programme was as follows: 95°C for 10 min for one cycle, followed by 95°C for 15 s and 60°C for 1 min for forty cycles. All reactions were performed in duplicate. Primers were designed for each gene of interest (Primer Express Software version 2.0, Applied Biosystems), and the specificity of all primers was confirmed by melting curve analysis. Primer efficiency was determined using a serial dilution of *Sus scrofa*-derived complementary DNA (1:4 dilution series over seven points). Primers for all the selected nutrient transporters (peptide transporter 1 (*PEPT1/SLC15A1*); sodium–glucose-linked transporter 1 (*SGLT1/SLC5A1*); *GLUT2/SLC2A2*, *GLUT5/SLC2A5*, *GLUT7/SLC2A7*, and *GLUT8/SLC2A8*; fatty acid-binding protein 2 (*FABP2*); Fe-regulated transporter (*SLC40A1*); cluster of differentiation 36/fatty acid translocase (*CD36/FAT*); membrane Ca channel (*TRPV6*); Ca-binding protein (calbindin); plasma membrane Ca^{2+} ATPase (*PMCA1*) and vitamin D receptor (*VDR*)) are given in Table 2. The optimal number of reference targets for this sample set was identified using the geNorm application within the qbasePLUS software package[27] (Biogazelle) and confirmed for the present study (geNorm $V < 0{\cdot}15$). The normalisation factor

Table 2. Porcine specific primers used for real-time PCR

Genes	Accession no.	Primer sequence (5′–3′)	Melting temperature (°C)	Product length (bp)	Efficiency (%)
GAPDH	AF017079.1	F: CAGCAATGCCTCCTGTACCA	59·4	72	100
		R: ACGATGCCGAAGTTGTCATG	57·3		
HMBS	NM_001097412.1	F: CTGAACAAAGGTGCCAAGAACA	58·4	74	104
		R: GCCCCGCAGACCAGTTAGT	61		
PEPT1	NM_214347.1	F: GGATAGCCTGTACCCCAAGCT	61·8	73	98
		R: CATCCTCCACGTGCTTCTTGA	59·8		
SGLT1	NM_001164021.1	F: GGCTGGACGAAGTATGGTGT	59·4	153	90
		R: ACAACCACCCAAATCAGAGC	57·3		
GLUT2	AF054835.1	F: CCAGGCCCCATCCCCTGGTT	65·5	96	101
		R: GCGGGTCCAGTTGCTGAATGC	63·7		
GLUT5	EU_012359	F: CCCAGGAGCCGGTCAAG	60	60	107
		R: TCAGCGTCGCCAAAGCA	55·2		
GLUT7	XM_003127552.3	F: ACATCGCCGGACATTCCATA	57·3	75	110
		R: GCGAGGACTGCAGGAAGATC	61·4		
GLUT8	XM_003480608.1	F: AGCGCCTTTGGCACCTACTT	59·4	62	110
		R: TGCACGTGCGAGGAGTTG	58·2		
FABP2	NM_001031780.1	F: TCGGGATGAAATGGTCCAGACT	62·4	102	106
		R: TGTGTTCTGGGCTGTGCTCCA	61·8		
CD36	NM_001044622.1	F: GGAGAAAAGATCACTACCATCATGAG	61·6	78	98
		R: CTCCTGAAGTGCAATGTACTGACA	61		
VDR	NM_001097414.1	F: CCTTCACCATGGACGACATG	59·4	72	92
		R: TGGCCACGTCGCTGACTT	58·2		
TRPV6	XM_003134594	F: TCCAGACAGAGGACCCTAACAAG	62·4	82	104
		R: GTGAGAAACAGCTCAAAGGTGCTA	61		
Calbindin	NM_214140.2	F: CGCAACAGTCCCATTTAAGGA	57·9	72	90
		R: TCAGCAGAGACATGGGTGGTT	59·8		
PMCA1	NM_214352.3	F: GGGCGGGCAGGTCATT	56·9	86	94
		R: CCGCCGGGAGAAGATCA	57·6		
SLC40A1	XM_003483701	F: GGAGCATCAGCTGTAACTGGAA	60·3	75	109
		R: CCGAACCAGGCCACATTTT	56·7		
SLC1A4	XM_003125088	F: ACCCTCGCCGACTTTTAGTCT	59·8	76	99
		R: GCCTGTGCCGAGAAGTAATCC	61·8		
SLC6A19	XM_003359855	F: GCCACCGTGGTCTACTCCAT	61·4	129	95
		R: GAAGTTCTCCTGCGTCACGTT	59·8		
SLC7A11	XM_003360551	F: CGGCTCCTGGGAAATTTCTC	59·4	72	95
		R: ACCATTCATGGAGCCAAAGC	57·3		
SLC7A1	NM_001012613	F: TCTCATCCTAACGGGACTTTTAACTC	61·6	85	110
		R: GACCAGAACGTTGATACACGTGAA	61		
SLC34A2	XM_003128893	F: GAAAGGCACAGAGACCCACAA	59·8	71	95
		R: AATGGGACGGCTGGAGTTC	58·8		
SLC17A4	XM_001925551	F: TTTTCAATTTCCACCCAACAAAT	58·2	76	105
		R: GGGTGGGCAGAGCTGTGT	56·0		

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *HMBS*, hydroxymethylbilane synthase; *PEPT*, peptide transporter; *SGLT*, sodium–glucose-linked transporter; *FABP*, fatty acid-binding protein; *CD*, cluster of differentiation; *VDR*, vitamin D receptor; *TRPV6*, membrane Ca channel; *PMCA1*, plasma membrane Ca^{2+} ATPase; *SLC*, solute carrier.

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was calculated as the geometric mean of the reference targets glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and hydroxymethylbilane synthase (*HMBS*). Calibrated normalised relative quantities of gene expression for each analysed sample were generated using the qbasePLUS package (Biogazelle) and incorporated efficiency correction.

Statistical analysis

Data were analysed as a completely randomised block design using the general linear model procedure of SAS (SAS Institute, Inc.)[28]. The pen served as the experimental unit for performance and CATTD. For all the other parameters, the individual pig served as the experimental unit. Data were checked for normality using the PROC UNIVARIATE function of SAS (SAS Institute, Inc.). All data presented in the tables are expressed as least-squares means with their standard errors. Means were separated using the Tukey–Kramer method. P values $<0{\cdot}05$ were considered to be statistically significant.

Results

Growth performance

The effect of PHY and P levels on the growth performance of pigs is summarised in Table 3. At the end of the experiment, pigs fed the LP diet had lower overall average daily gain ($P<0{\cdot}05$) and final body weight ($P<0{\cdot}01$) and an increased feed conversion ratio ($P<0{\cdot}05$) compared with those fed the HP and PHY diets.

Apparent ileal digestibility

The effect of PHY and P levels on the CAID of pigs is summarised in Table 4. Pigs fed the PHY diet had higher CAID of GE ($P<0{\cdot}001$) and N ($P<0{\cdot}05$) compared with those fed the HP diet. Pigs fed the PHY diet had higher CAID of P ($P<0{\cdot}01$), ash ($P<0{\cdot}05$) and GE ($P<0{\cdot}001$) compared with those fed the LP diet.

Table 3. Effect of phosphorus and phytase (PHY) levels on the growth performance of pigs during period 1 (days 0–23) and period 2 (days 23–44)

(Least-squares means with their standard errors)

Dietary treatments*	HP	LP	PHY	SEM	*P*
Period 1(days 0–23)					
Initial BW (kg)	11·76	11·76	11·75	0·75	0·894
Final BW (kg)	25·81	25·13	26·73	0·27	0·505
ADFI (kg/d)	0·89	0·93	0·91	0·03	0·594
ADG (kg/d)	0·61	0·58	0·65	0·02	0·495
FCR (kg/kg)	1·49	1·65	1·43	0·07	0·251
Period 2 (days 23–44)					
Final BW (kg)	41·28[a]	37·06[b]	43·16[a]	1·02	0·024
ADFI (kg/d)	1·47	1·49	1·51	0·08	0·594
ADG (kg/d)	0·74[a]	0·57[b]	0·78[a]	0·02	0·001
FCR (kg/kg)	1·99[a]	2·63[b]	1·92[a]	0·13	0·025
Entire period (days 0–44)					
ADFI (kg/d)	1·16	1·19	1·19	0·04	0·805
ADG (kg/d)	0·67[a]	0·58[b]	0·71[a]	0·02	0·026
FCR (kg/kg)	1·73[a]	2·14[b]	1·67[a]	0·09	0·043

HP, high P; LP, low P; BW, body weight; ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.

[a,b] Least-squares mean values within a row with unlike superscript letters were significantly different ($P<0{\cdot}05$).

* A total of eight replicates were used per treatment.

Total tract digestibility

The effect of PHY and P levels on the CATTD of pigs is summarised in Table 4. Pigs fed the PHY diet had higher CATTD of Ca, P and ash compared with those fed the HP and LP diets ($P<0{\cdot}001$) during periods 1 and 2. Pigs fed the LP diet had a lower CATTD of P compared with those fed the HP diet ($P<0{\cdot}001$) during periods 1 and 2.

Bone parameters

The effect of PHY and P levels on the bone parameters of pigs is summarised in Table 5. Pigs fed the LP diet had decreased bone ash content ($P<0{\cdot}001$), bone P content ($P<0{\cdot}01$) and bone density ($P<0{\cdot}01$) compared with those fed the HP and PHY diets.

Jejunal nutrient transporter gene expression

The effect of PHY and P levels on the jejunal nutrient transporter gene expression of pigs is summarised in Table 6. Pigs fed the PHY diet exhibited a numerical increase in the gene expression of the Ca transporter *TRPV6* ($P<0{\cdot}10$) and the P transporter *SLC34A2* ($P<0{\cdot}10$) compared with those fed the HP diet. Pigs fed the HP diet exhibited an increased gene expression of the amino acid transporter *SLC7A11* compared with those fed the PHY diet ($P<0{\cdot}05$). Pigs fed the HP diet exhibited a numerical increase in the gene expression of the amino acid transporter *SLC7A1* compared with those fed the LP diet ($P<0{\cdot}10$).

Ileal nutrient transporter gene expression

The effect of PHY and P levels on the ileal nutrient transporter gene expression of pigs is summarised in Table 7. Pigs fed the PHY diet exhibited an increased gene expression of *PEPT1* compared with those fed the LP diet ($P<0{\cdot}05$). Pigs fed the PHY diet exhibited a numerical increase in the gene expression of *FABP2* ($P<0{\cdot}10$) compared with those fed the LP diet. Pigs fed the LP diet exhibited a lower gene expression of *GLUT2* and *SGLT1* compared with those fed the HP diet ($P<0{\cdot}05$). Pigs fed the LP diet exhibited an increase in the gene expression of the Ca transporters *TRPV6*, calbindin and *PMCA1* compared with those fed the HP and PHY diets ($P<0{\cdot}001$).

Discussion

PHY is used extensively to improve P digestibility in pigs, by increasing the availability of phytate-bound P and improving growth performance and bone mineralisation. PHY supplementation has also been shown to improve the digestibility of a number of nutrients[4]. We hypothesised that improvements

Table 4. Effect of phosphorus and phytase (PHY) levels on the coefficient of apparent total tract digestibility (CATTD) (periods 1 and 2) and the coefficient of apparent ileal digestibility (CAID) of pigs (after slaughter (day 43))

(Least-squares means with their standard errors)

Dietary treatments*	HP	LP	PHY	SEM	*P*
CAID (%)					
DM	72·06[a]	73·50[a,b]	77·06[b]	0·52	0·001
Ash	34·79[a,b]	30·67[a]	43·24[b]	4·61	0·043
P	36·69[a,b]	22·37[a]	47·39[b]	5·31	0·006
Gross energy	69·95[a]	71·47[a]	74·77[b]	0·71	0·001
N	66·95[a]	70·04[a,b]	71·58[b]	1·43	0·001
CATTD (days 0–23) (%)					
DM	81·35	82·3	82·16	1·43	0·292
Ash	40·59[a]	42·46[a]	57·96[b]	2·13	0·001
P	32·89[a]	21·84[b]	54·52[c]	3·23	0·001
Ca	46·41[a]	48·13[a]	75·41[b]	3·98	0·001
Gross energy	75·72	78·86	79·79	1·86	0·331
N	76·14	78·54	76·8	2·05	0·335
Neutral-detergent fibre	13·66	12·22	17·21	2·48	0·246
CATTD (days 23–44) (%)					
DM	82·68	82·66	84·44	0·82	0·258
Ash	43·21[a]	40·37[a]	58·79[b]	3·41	0·002
P	38·94[a]	22·02[b]	55·8[c]	4·31	0·001
Ca	48·77[a]	45·41[a]	76·64[b]	4·30	0·001
Gross energy	79·74	79·53	80·76	7·06	0·662
N	78·11	79·95	80·18	1·02	0·443
Neutral-detergent fibre	15·49	13·66	19·18	2·62	0·343

HP, high P; LP, low P.

[a,b,c] Least-squares mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* CATTD was calculated based on data obtained from two pigs in each of the eight pens, while ileal digestibility was calculated based on data obtained from eight individual pigs after slaughter.

in growth performance, skeletal bone mineralisation and nutrient digestibility following supplementation of a LP diet with PHY are accompanied by changes in the gene expression of intestinal nutrient transporters involved in peptide, mineral, carbohydrate and fatty acid transport.

The primary aim of the present study was to investigate the effects of PHY on nutrient and mineral digestibility, growth performance and bone mineralisation and the secondary aim was to investigate its effect on intestinal nutrient transporter gene expression in pigs. The experimental period was divided into two components delineated by a diet change to match the requirements of pigs as they progressed from a phase when fed a nutritionally rich diet after weaning (days 0–23) to a phase when fed a lower-specification diet (days 23–44)[13]. In the present study, the PHY diet was found to improve the CATTD of Ca and P, the CAID of GE and P, growth performance and bone mineralisation and to increase the CAID of N when compared with the HP diet. The findings of the present study are in agreement with the findings that supplementation of a LP diet with PHY can improve nutrient digestibility, growth performance and bone mineralisation[4,29,30]. These improvements have been attributed to the capacity of PHY to release nutrients chelated by the phytate molecule. The PHY diet increased the CAID of GE compared with the LP diet and increased the CAID of N compared with the HP diet. However, there was no difference in the CATTD of N and GE among the dietary treatment groups, indicating that activity in the large intestine affects the CATTD of N and GE. Similarly, Woyengo *et al.*[31] suggested that hindgut fermentation masks the effects of PHY. This is important, as nutrients absorbed in the small intestine are used with greater efficiency than those that undergo hindgut fermentation. Nutrients such as amino acids that are not absorbed in the small intestine are of no nutritional value to pigs. Although the effects of PHY on growth performance, nutrient digestibility and bone mineralisation[32–34] are well established, the underlying biological mechanisms involved in the uptake of nutrients following the degradation of the phytate molecule are not well understood. Intestinal enterocytes respond to fluctuations in intestinal nutrients by modifying the gene expression of intestinal nutrient transporters[10,11]. This indicates that intestinal enterocytes can up-regulate the gene expression of nutrient transporters in response to increased nutrient availability. In previous studies, improvements in nutrient digestibility have been found to be accompanied by improvements in intestinal nutrient transporter

Table 5. Effect of phosphorus and phytase (PHY) levels on bone parameters in the fourth metacarpal of the right front leg of pigs during slaughter (day 43)

(Least-squares means with their standard errors)

Dietary treatments*	HP	LP	PHY	SEM	*P*
DM	753·4	723·5	742·8	12·13	0·242
Ash	444·8[a]	395·1[b]	450·4[a]	9·45	0·001
P	53·7[a]	46·4[b]	54·3[a]	0·75	0·001
Ca	116·7	114·8	117·5	3·82	0·574
Density†	1·54[a]	1·41[b]	1·55[a]	0·02	0·001

HP, high P; LP, low P.

[a,b] Least-squares mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* A total of eight replicates were used per treatment.

† Density was calculated according to the method of Giancoli *et al.*[26].

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Table 6. Effect of phosphorus and phytase (PHY) levels on the normalised relative abundance of nutrient transporter mRNA in the jejunal tissue of weaned pigs

(Least-squares means with their standard errors)

Dietary treatments*	HP	LP	PHY	SEM	*P*
Glucose					
GLUT2	1·28	0·93	1·17	0·23	0·596
GLUT5	1·04	0·94	1·16	0·13	0·473
GLUT7	1·36	1·26	0·83	0·25	0·317
GLUT8	1·29	0·88	1·19	0·24	0·477
SGLT1	0·85	0·98	1·30	0·19	0·256
Protein					
PEPT1	1·32	1·09	1·07	0·23	0·694
SLC1A4	0·98	1·12	1·11	0·19	0·839
SLC6A19	1·13	1·06	1·03	0·15	0·891
SLC7A1	1·15[a]	0·88[b]	1·01[a,b]	0·10	0·078
SLC7A11	1·24[a]	1·05[a,b]	0·78[b]	0·14	0·034
Fatty acids and vitamins					
CD36	1·45	1·27	0·82	0·45	0·605
FABP2	1·49	1·12	1·04	0·26	0·426
VDR	1·20	1·13	1·19	0·30	0·985
Minerals					
TRPV6	0·82[a]	1·13[a,b]	1·18[b]	0·14	0·086
PMCA1	1·18	1·00	1·09	0·18	0·801
Calbindin	1·00	1·03	1·08	0·12	0·909
SLC40A1	1·18	1·17	1·28	0·32	0·968
SLC17A4	0·97	1·31	1·05	0·33	0·750
SLC34A2	0·80[a]	0·98[a,b]	1·39[b]	0·21	0·061

HP, high P; LP, low P; *SGLT1*, sodium–glucose-linked transporter 1; *PEPT1*, peptide transporter 1; *CD36*, cluster of differentiation 36; *FABP2*, fatty acid-binding protein 2; *VDR*, vitamin D receptor; *TRPV6*, membrane Ca channel; *PMCA1*, plasma membrane Ca^{2+} ATPase.

[a,b] Least-squares mean values within a row with unlike superscripts were significantly different ($P<0·05$).

* A total of eight replicates were used per treatment.

gene expression[16]. Heim *et al.*[16] showed that changes in the expression of intestinal GLUT are directly related to GE digestibility. Similarly, changes in crude protein digestibility have been shown to be accompanied by changes in the gene expression of amino acid and peptide transporters[35]. Therefore, in the present study, we aimed to investigate whether dietary PHY inclusion increases the expression of glucose, galactose, fructose, amino acid, peptide, fatty acid, vitamin and mineral transporters. Although not significant, the PHY diet was found to numerically increase ileal N digestibility compared with the LP diet in the present study. The ileal mRNA expression of *PEPT1* was increased in pigs fed the PHY diet compared with that in pigs fed the HP diet. PEPT1 is a major transporter involved in the absorption of the products of protein digestion across the intestinal apical membrane. This indicates that the gene expression of this transporter was up-regulated in response to the increase in N availability as the phytate molecule was hydrolysed *in situ*[36]. Although PHY supplementation increased the gene expression of *PEPT1*, no effect was observed on the gene expression of the apical membrane amino acid transporters (*SLC1A4*, *SLC6A19* and *SLC7A1*) or the basolateral transporters (*SLC7A11* and *SLC1A2*). This indicates that the increased N availability observed in the present study could be partially influenced by the increased gene expression of *PEPT1*[37]. This is in agreement with the findings of previous studies where increases in crude protein digestibility were found to be accompanied by changes in *PEPT1* expression[35]. The HP diet increased the jejunal gene expression of the apical membrane transporter *SLC7A1* compared with the LP diet and increased the jejunal gene expression of *SLC7A11* compared with the PHY diet.

The HP diet up-regulated the gene expression of *SGLT1* compared with the LP diet. The intestinal GLUT SGLT1 is the major transporter involved in the absorption of glucose and other sugars across the luminal membrane of porcine enterocytes[38]. The high levels of Ca and P present in the inorganic P supplement may inhibit the ability of the phytate molecule to bind to free glucose, as observed when feeding the LP diet, causing an increased gene expression of *SGLT1*[39]. In a previous experiment, pigs supplemented with phytic acid have been found to exhibit a reduced expression of *SGLT1*[40]. This indicates that phytate chelates free glucose and makes it unavailable for digestion, which in turn causes the low expression observed when feeding the LP diet. Following the increased gene expression of the apical membrane transporter *SGLT1*, an increase in the gene expression of *GLUT2*, which is expressed on the basolateral membrane of intestinal enterocytes, is to be expected[38]. The gene expression of *GLUT2* was up-regulated in pigs fed the HP diet when compared with that in pigs fed the LP diet. SGLT1 and GLUT2 together are effectively responsible for glucose absorption[41]. The increased gene expression of *GLUT2* indicates increased glucose availability from the HP diet, possibly due to the inhibition of the ability of the phytate molecule to bind to free glucose by the inorganic P supplement.

Table 7. Effect of phosphorus and phytase (PHY) levels on the normalised relative abundance of nutrient transporter mRNA in the ileal tissue of weaned pigs

(Least-squares means with their standard errors)

Dietary treatments*	HP	LP	PHY	SEM	*P*
Glucose					
GLUT2	1·26[a]	0·26[b]	0·94[a,b]	0·29	0·032
GLUT5	1·31	0·62	1·09	0·30	0·315
GLUT7	1·46	0·71	0·94	0·37	0·362
GLUT8	1·02	1·01	0·99	0·10	0·973
SGLT1	1·32[a]	0·51[b]	0·79[a,b]	0·31	0·022
Protein					
PEPT1	1·09[a,b]	0·47[b]	1·40[a]	0·29	0·045
SLC1A4	1·10	1·41	1·04	0·14	0·146
SLC6A19	1·20	0·61	0·99	0·30	0·383
SLC7A1	1·07	1·26	1·01	0·13	0·362
SLC7A11	1·12	1·10	1·07	0·18	0·976
Fatty acids and vitamins					
CD36	1·11	1·31	1·93	0·51	0·487
FABP2	1·06	0·73	1·67	0·37	0·091
VDR	1·22	0·96	1·18	0·28	0·794
Minerals					
TRPV6	1·05[b]	2·71[a]	0·84[b]	0·33	0·002
PMCA1	1·01[a]	1·21[b]	1·09[a,b]	0·08	0·076
Calbindin	1·63[b]	25·03[a]	2·78[b]	0·37	0·004
SLC40A1	1·63	0·71	0·73	0·16	0·233
SLC17A4	1·24	1·47	1·33	0·32	0·823
SLC34A2	1·17	1·09	1·44	0·32	0·723

HP, high P; LP, low P; *SGLT1*, sodium–glucose-linked transporter 1; *PEPT1*, peptide transporter 1; *CD36*, cluster of differentiation 36; *FABP2*, fatty acid-binding protein 2; *VDR*, vitamin D receptor; *TRPV6*, membrane Ca channel; *PMCA1*, plasma membrane Ca^{2+} ATPase.

[a,b] Least-squares mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* A total of eight replicates were used per treatment.

Similar to that observed for N digestibility, PHY supplementation was found to increase the CAID of GE in the present study. However, PHY supplementation had no effect on the gene expression of the studied GLUT. The increase in the gene expression of *FABP2* indicates that PHY was effective at reducing the ability of the phytate molecule to bind to free fat and make it unavailable and could in part explain the increase in the CAID of GE. The fact that PHY has the ability to increase the availability of fat[8] and up-regulate the expression of the fatty acid transporter *FABP2*, as observed in the present study, indicates a role for it in increasing the availability of fat. FABP found in the brush border membranes of enterocytes may play a role in fatty acid uptake[42]. In a recent study in broilers, PHY supplementation has been found to improve the ileal digestibility of fat along with that of the fat constituents, SCFA and unsaturated fatty acids[42]. The formation of mineral–phytate complexes has been reported to prevent the utilisation of lipids. PHY supplementation may increase fat digestibility by reducing the formation of soaps in the gut[43].

Ca is a mineral that is known to be chelated by the phytate molecule[44]. During period 1, the PHY diet was found to improve the CATTD of Ca by 27 and 29% compared with the LP and HP diets, respectively. Similarly during period 2, the PHY diet was found to improve the CATTD of Ca by 31 and 28% compared with the LP and HP diets, respectively. Due to low levels of Ca and P in the LP diet used in the present study, pigs fed the LP diet exhibited reduced growth and bone mineralisation. PHY supplementation ameliorated the negative effects of the LP diet. The mineral transporters TRPV6, calbindin and PMCA1 were studied as these three Ca transporters are involved in transcellular Ca transport (uptake, intracellular movement, and extrusion)[45]. A numerical increase was observed in the gene expression of the basolateral Ca transporter *PMCA1* in the jejunum of pigs fed the PHY diet when compared with that in pigs fed the HP diet. These results indicate that the trend of an increased gene expression of *TRPV6* could partially explain the increased digestibility of Ca observed in the present study. The gene expression of the Ca channel *TRPV6* was increased in the ileum of pigs fed the LP diet when compared with that in pigs fed the other two diets. Similarly, an increased expression of calbindin was observed in pigs fed the LP diet, and a significant trend towards an increased gene expression of the basolateral Ca transporter *PMCA1* was also observed when comparing pigs fed the LP diet with those fed the HP and PHY diets. These results are in agreement with the results of the study carried out by Li[46], where low levels of Ca were found to up-regulate the expression of calbindin in broilers. It has previously been established that low levels of dietary Ca can lead to an increased expression of *PMCA1* and calbindin in the kidney of mice[45]. These results indicate that animals deficient in Ca respond by up-regulating the expression of Ca transporters to maximise the utilisation of available Ca in their ileum. In previous experiments[39], it has been shown that the degradation of the phytate molecule predominantly occurs in the stomach and proximal small intestine. This mechanism could explain why the studied Ca transporters were differentially expressed in the ileum and pigs fed the HP and PHY diets exhibited a lower expression. The majority of Ca absorption in pigs fed the HP and PHY diets will take place in the proximal region of the small intestine due to the easy absorption of Ca from the inorganic source and the increased availability of Ca following the degradation of the phytate molecule. This is supported by the trend towards an increased expression of *TRPV6* in the jejunum.

Similar to that observed for Ca digestibility, the PHY diet was found to improve the CATTD of P by 33 and 22% compared with the LP and HP diets during period 1, respectively. During period 2, the PHY diet was found to improve the CATTD of P by 33 and 17% compared with the LP and HP diets, respectively. The PHY diet improved ileal P digestibility by 25% compared with the LP diet. A trend towards an increased gene expression of the transporter *SLC34A2* was observed in the jejunum of pigs fed the PHY diet compared with the expression in those fed the HP diet. This increased gene expression could partially explain the increased P digestibility observed in the present study. In the present study, these transporters were not found to be differentially expressed in the ileum. The majority of P absorption in pigs occurs through the transcellular route and some P is transported by intestinal transporters[47]. The majority of P present in both HP and PHY diets will be released and absorbed in the proximal region of the intestine.

In summary, the PHY diet improved growth performance and bone mineralisation when compared with the HP diet and improved the ileal digestibility of GE and total tract nutrient digestibility of Ca and P when compared with the LP diet. The PHY diet improved the gene expression of the peptide transporter *PEPT1*, the Ca transporter *TRPV6*, the P transporter *SLC34A2* and the fatty acid transporter *FABP2* compared with the LP diet. The increase in the gene expression of these nutrient transporters indicates that intestinal nutrient transporter gene expression is a mechanism involved in the uptake of nutrients following the degradation of the phytate molecule.

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None of the authors has any conflicts of interest to declare.

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