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PUBLIC
REPORT 1308

WAGENINGEN
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This research was conducted by Wageningen Livestock Research as part of the Public Private Partnership "Feed4Foodure" (TKI-AF-16123), funded by Vereniging Diervoederonderzoek Nederland (VDN) and the Ministry of Agriculture, Nature and Food Quality (LNV)

Wageningen Livestock Research
Wageningen, February 2021

Public
Report 1308

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Hu, Y., J. van Baal, C. de Bruijn, J.W. Resink, and P. Bikker, 2019. *Kinetics of calcium and phosphorus digestion and absorption in pigs.* Wageningen Livestock Research, Report 1308.

Summary UK

This report describes a study in growing pigs to determine the influence of dietary calcium content and phytase inclusion on digestion, absorption, retention and excretion of calcium and phosphorus, degradation of dietary phytase and released isomers, the absorption from different gut segments, mRNA expression of transporters and other proteins involved in the digestive tract and the kidney, and potential regulatory mechanisms.

This report can be downloaded for free at https://doi.org/10.18174/545274 or at www.wur.nl/livestock-research (under Wageningen Livestock Research publications).

Public Wageningen Livestock Research Report 1308

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Foreword

This study addressed the kinetics of digestion and absorption of calcium and phosphorus from the digestive tract of growing pigs within the context of the Feed4Foodure partnership. The in vivo experiment in this study was conducted in the Swine Research Facilities (SRC) of Trouw Nutrition in Halfweg near Boxmeer, the Netherlands. The devoted coordination of Carlijn de Bruijn and skilful conduct of the study by the staff of SRC is highly appreciated. The authors additionally appreciate the discussions in the swine cluster of the Feed4Foodure project that contributed to the design and interpretation of this study. The authors are grateful for the contribution of the lab of dr. Annette Liesegang, University of Zurich, for serum hormones and metabolites and dr. Markus Rodehutscord, University of Hohenheim, for phytate analysis.

Paul Bikker
Project leader

Summary

Phosphorus (P) and calcium (Ca) are two essential nutrients in animal diets, and their absorption and metabolism are closely connected. Mineral P is the 3rd most expensive nutrient in pig diets and optimal utilisation is required to assure animal production and health and minimise environmental losses. Earlier studies have shown that inclusion of microbial phytase improves P absorption, and high dietary Ca inclusion may decrease P absorption in the gut. However, results are conflicting whether phytase inclusion interacts with dietary Ca level on P absorption and retention. We hypothesized that the negative effect of Ca on P absorption is greater in a phytase-supplemented diet. The present experiment was arranged in a 2×3 factorial design including 2 levels of phytase and 3 levels of Ca in the diet. The two phytase levels included a phytase-free diet without microbial phytase and a phytase-supplemented diet with microbial phytase at 500 FTU/kg (Axtra Phy, Danisco Animal Nutrition). The three Ca levels were a low Ca content (no limestone, 2 g/kg), a medium Ca content (5.8 g/kg) and a high Ca content (9.6 g/kg). Two batches of 30 growing pigs (BW 30.4±1.3 kg, Hypor Libra × Maxter) were allotted to the 6 treatments, with 5 replicate pigs per treatment in each batch. The experiment lasted for 21 days. During the experimental period, a 4-d balance trial was conducted to collect the faeces and urine from d 14 to 17. From d 18 onwards, feeding frequency was increased to gradually realize a constant passage rate of digesta through the gastrointestinal tract (GIT). The pigs were dissected on d 20, and digesta from stomach, proximal and distal small intestine (SI), proximal and distal large intestine (LI) were collected to determine the Ca and P flux along the gut. In the mid-jejunum and mid-colon mucosal tissue was harvested and mRNA expression levels of genes related to intestinal Ca and P absorption were determined by RT-qPCR.

From the results it was concluded that:

- Incremental dietary Ca reduced the growth performance of the pigs. This effect was negated by inclusion of phytase, indicating that it was likely mediated by a reduction in ATTD-P and a deficiency of digestible P for body gain.
- Incremental dietary Ca reduced the apparent total tract digestibility (ATTD) of P to a larger extent in phytase-supplemented diets than in phytase-free diets.
- The contribution of phytase (500 FTU/kg) to the ATTD-P content decreased with increasing Ca content from 1.5 to 1.1 g/kg.
- The two incremental levels of Ca, added as limestone, had a marginal digestibility of 44.7 and 38.5% without phytase and 54.3 and 35.2% with phytase.
- Calcium absorption was largely realised in the proximal small intestine and the large intestine and very low in the distal small intestine.
- Phosphorus absorption was low in the proximal small intestine and high in the distal small intestine in the phytase-free diets, whereas inclusion of phytase enhanced P absorption in the proximal small intestine.
- Incremental dietary Ca caused a reduction in P absorption in the proximal small intestine but not in the distal small intestine.
- Solubility of Ca and P was high in the stomach and proximal SI and decreased in distal GIT segments, especially for Ca. Phytase substantially enhanced the solubility of P in all GIT segments, but the effect on Ca solubility was not significant.
- Phytate degradation determined in the distal ileum was significantly enhanced by phytase inclusion and reduced by incremental dietary Ca.
- Incremental dietary Ca reduced the expression of the apical calcium channels in the jejunum (TRPV6) and colon (TRPV5 and TRPV6) and enhanced the expression of tight junction protein claudin 2 (CLDN-2) in the jejunum suggesting that Ca absorption switched from primarily active transcellular transport at a low dietary Ca content to primarily passive paracellular transport at a high Ca content.
- Phytase reduced the mRNA expression level of the calcium channels TRPV5 and TRPV6 and enhanced the expression of CLDN-12 in the jejunum and colon.

- The mechanism of the interaction between Ca and microbial phytase may include a direct effect on phytase efficacy, an effect on mucosal phosphatases and a complexing effect on released ortho-phosphate.
- The incremental dietary Ca level increased Ca retention (g/d), decreased the ratio between retained and absorbed Ca and increased P-retention in the phytase-supplemented diet. Phytase inclusion enhanced both Ca and P retention.
- Ash content of the 3rd metacarpal bone was enhanced by both incremental dietary Ca and phytase inclusion, with a minor effect on the Ca/P ratio in bone.
- Incremental dietary Ca reduced the expression of TRPV5, TRPV6, CaBP-28k, NCX1, CLDN12 and CLDN16 in the kidney, with a greater impact on TRPV5, CaBP-28k and NCX1 in phytase-supplemented diets, indicating an important role of these genes in the reduced reabsorption of Ca from urine in the kidney to realise Ca homeostasis.

List of abbreviations

Abbreviation	Full name
ACTB	Beta actin
ADFI	Average daily feed intake
ADG	Average daily gain
AID	Apparent ileal digestible
AP	Alkaline phosphatase
ATTD	Apparent total tract digestible
AU	Arbitrary unit
Ca	Calcium
CaSR	Ca-sensing protein receptor
cfat	Crude fat
CLDN	Claudin
Co-EDTA	Cobalt-ethylenediamine tetraacetic acid
CP	Crude protein
CX43	Connexin 43
CYP24A1	Vitamin D 24-hydroxylase
D28k	Calbindin-D28k
D9k	Calbindin-D9k
dCa	Digestible calcium
DM	Dry matter
dP	Digestible phosphorus
EEF2	Elongation factor 2
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase family member 1
FCR	Feed conversion ratio
GIT	Gastrointestinal tract
IAP	Intestinal alkaline phosphatase
IPO8	Importin 8
LI	Large intestine
MinPP1	Multiple inositol polyphosphate phosphatase 1
MRT	Mean retention time
NaPi	Sodium-dependent phosphorus transporter
NaPi-IIa	Sodium-dependent phosphorus transporter type-IIa
NaPi-IIb	Sodium-dependent phosphorus transporter type-IIb
NaPi-IIc	Sodium-dependent phosphorus transporter type-IIc
NCX	sodium-calcium exchanger
OM	Organic matter
P	Phosphorus
P2X7	Purinoceptor 7
PiT-1	Inorganic phosphate transporter 1
PiT2	Inorganic phosphate transporter 2
rCa	Retained calcium
rP	Retained phosphorus
SI	Small intestine
SLC5A1	Sodium/glucose cotransporter 1
TiO_2	Titanium dioxide
TRP	The transient receptor potential
TRPM7	Transient receptor potential cation channel, subfamily M, member 7
TRPV5	Transient receptor potential cation channel subfamily V member 5
TRPV6	Transient receptor potential cation channel subfamily V member 6
VDR	Vitamin D receptor
XPR1	Xenotropic and polytropic retrovirus receptor 1
ZO-1	Zonula occludens-1

1 Introduction

1.1 Background

Phosphorus (P) is the third most expensive nutrient in the diet of monogastric animals. Phytate-P is the major form of P storage in most cereal grains and oil seeds. Non-ruminant animals, such as pigs and poultry, cannot secrete sufficient mucosal phytase to fully release phytate-P. Inclusion of exogenous microbial phytase is one of the economic-efficient and widely-used ways to improve phytate-P absorption. Reducing dietary calcium (Ca) supply can also effectively improve P absorption. However, it remains a matter of debate whether the dietary Ca level interacts with the microbial phytase inclusion.

It is widely accepted that Ca negatively affects P absorption probably via complexation of Ca-phytate and Ca-ortho-P in the gastrointestinal tract (GIT). The phytate is negatively charged, hence it binds cations to form insoluble complexes. Due to the high inclusion level of limestone, Ca is the major cation that binds to phytate in the pig diets. On the other hand, the existence of Ca-phytate or Ca-ortho-P complexes in the digesta has not been demonstrated. The efficacy of phytase to degrade phytate is pH-dependent. The microbial phytase is mainly active in the stomach and upper part of the small intestine (SI) due to the low luminal pH. The limestone may increase the digesta pH because of its high buffer capacity. The efficacy of microbial phytase to hydrolyse phytate, therefore, was suggested to be depressed by a high-Ca level (Lei et al., 1994). It was hypothesized that the harmful effect of Ca on P digestibility was greater in phytase-supplemented diets.

It has been demonstrated that Ca and P are primarily absorbed in the SI (Marks et al., 2010). However, recent studies have shown that the stomach and large intestine (LI) may also play a role in Ca and P absorption. Gonzalez-Vega et al. (2014) reported that the Ca sources affected the absorption site probably depending on their Ca solubility; Ca from calcium carbonate was mostly absorbed in the stomach, while Ca from *L. calcareum* was mostly absorbed in the jejunum and ileum. In addition, Gonzalez-Vega et al. (2014) also observed a significant difference between the apparent ileal P digestibility and apparent total tract P digestibility, which suggested that a substantial amount of P could be absorbed posterior to the ileum. Calcium binding protein was expressed in the stomach and might be involved in the Ca absorption before the duodenum (Raeymaekers et al., 1993). However, the post-ileal Ca and P absorption needs more evidence and insight in the gene expression of the Ca and P transporters and tight junction proteins that could potentially be involved.

In mammals, Ca and P is absorbed in the gut via passive paracellular and active transcellular transport processes. For Ca absorption, two members of the transient receptor potential family (TRP), designated TRPV5 and TRPV6, are involved in the transcellular Ca uptake in the gut (González-Vega and Stein, 2016). For P absorption, type II sodium-dependent P transporters (NaPi) are shown to be involved in transcellular P uptake in the gut (Marks et al., 2010). Paracellular transport of Ca and P occurs via tight junction membrane proteins. There is increasing evidence that certain structural and functional components of tight junctions, namely the claudin (CLDN) superfamily, are involved in paracellular mineral transport. For example, CLDN-2, CLDN-7, CLDN-10 and CLDN-15 are abundantly expressed in rodent intestine. These claudins have been qualified to form paracellular pores for certain ion species, increasing ion permeability of the epithelial sheet of cells, whereas other isoforms, such as CLDN-4, tighten the paracellular pathway and increases transepithelial resistance.

This study was conducted to clarify Ca to phytase interactions on Ca and P digestion and absorption, relevant locations in the digestive tract, regulation and absorption mechanisms involved and consequences for Ca and P utilisation and retention.

1.2 Study objectives

This study was conducted:

- to obtain insight in solubilisation, passage, digestion and absorption of Ca and P throughout different segments of the digestive tract of growing pigs;
- to quantify the effect of microbial phytase, Ca and their interactions on solubilisation, passage, digestion and absorption of Ca and P throughout different segments of the digestive tract of growing pigs;
- to obtain insight in the consequences for the contribution of active (i.e. transporter mediated) and passive transport to the absorption from the GIT;
- to obtain insight in the consequences of absorption of Ca and P on their metabolic utilisation, i.e. retention and excretion.

2 Materials and methods

This experiment was conducted in the facilities of the Swine Research Centre (SRC) of Trouw Nutrition. The study was part of the project "Assessment and improvement of nutrient digestion and absorption in pigs and poultry", approved by the CCD with number 2016.D-0065. The study protocol was approved by the IVD of Wageningen University & Research and the IVD of SRC. Standard procedures for animal handling and care in the unit for growing pigs, as described in SRC SOPs were applied.

2.1 Experimental design and diets

This study was conducted in individually housed young growing pigs (Hypor Libra*Maxter, approximately 12 weeks of age, 30.4±1.3 kg BW) as experimental unit, during a 21-day period. The experiment was replicated over time in 2 batches of pigs. The study comprised 6 dietary treatments in a 2×3 factorial arrangement. The 6 dietary treatments included 3 levels of Ca and 2 levels of phytase. The 3 intended Ca levels were 2.0 (low, limestone-free), 5.8 (medium) and 9.6 g/kg (high), respectively. The 2 intended phytase levels were microbial-phytase-free (low-phytase) and microbial phytase-supplemented at 500 FTU/kg (high-phytase, Axtra Phy, Danisco Animal Nutrition). Each treatment was replicated 10 times, with 5 replicate pigs per batch. The pigs were blocked by the initial body weight (BW), and the 6 pigs of a block were adjacently housed. Within a block each pig received 1 of the 6 experimental diets.

Diets were prepared in order of increasing Ca-content and phytase-free diets were produced before phytase-supplemented diets. Titanium oxide (TiO2) was included at 5 g/kg as marker of the solid phase. A phytase-free vitamin and mineral premix was prepared by RDS, Research Diet Services in Wijk bij Duurstede.

Experimental diets were produced by a feed production plant for research diets (ABZ Diervoeding in Leusden) using a double mixing procedure to assure equal composition of the experimental diets. Prior to the feed production, a mixture of wheat, barley and soybean oil was steam pelleted in order to deactivate the intrinsic phytase activity and subsequently ground. A basal diet was made and then split into 6 equal portions. To each portion the required amount of limestone and phytase were added at the expense of diamol. The basal diet met or exceeded the minimal requirement of all nutrients except for Ca and P (CVB, 2016). The diets were pelleted in 4 mm pellets at a maximum temperature of 80 °C. Samples of the diets were continuously taken by an automatic sampling device and analysed for Ca, P and phytase prior to the start of the experiment. Experimental diets were delivered to the research facility in labelled 20 kg bags. At the research facility, cobalt-ethylenediamine tetraacetic acid (Co-EDTA) was included as marker of the liquid phase in the ration of the pigs at 2 g/kg during the last 3 days of the experiment. The dietary composition, and designed and analysed nutrient contents were shown in Table 1. The expected Ca and P content in the basal diet were 2.0 and 4.7 g/kg, respectively. No limestone was added to the basal diet, hence all Ca was plant-derived in the low-Ca diet. The calculated digestible P content in the phytase-free diets was marginal in order to maximize the phytase efficacy (1.7 g/kg). Because of the relatively high Ca content (34 mg/l) and negligible P content (0.013 mg/l) in the drinking water, the Ca content was included in the dietary Ca intake while the P content was not included.

Table 1 *Ingredient composition and nutrient contents of the experimental diets (g/kg, as-fed basis).*

Items	Dietary treatments					
Phytase addition, FTU/kg	0			500		
Ca content	2.0	5.8	9.6	2.0	5.8	9.6
Ingredients						
Wheat	249.0	249.0	249.0	249.0	249.0	249.0
Barley	249.0	249.0	249.0	249.0	249.0	249.0
Maize	173.0	173.0	173.0	173.0	173.0	173.0
Soybean meal	117.5	117.5	117.5	117.5	117.5	117.5
Rapeseed meal	79.6	79.6	79.6	79.6	79.6	79.6
Sunflower seed meal	39.8	39.8	39.8	39.8	39.8	39.8
Soybean oil	32.4	32.4	32.4	32.4	32.4	32.4
Molasses	20.0	20.0	20.0	20.0	20.0	20.0
Premix[1]	5.0	5.0	5.0	5.0	5.0	5.0
Salt	2.3	2.3	2.3	2.3	2.3	2.3
Amino acids	5.9	5.9	5.9	5.9	5.9	5.9
Monosodium phosphate	2.5	2.5	2.5	2.5	2.5	2.5
Phytase[2]	0.0	0.0	0.0	0.1	0.1	0.1
Fine limestone[3]	0.0	10.0	20.0	0.0	10.0	20.0
Diamol[4]	20.0	10.0	0.0	20	10	0.0
Titanium dioxide	4.0	4.0	4.0	4.0	4.0	4.0
Nutrients, calculated						
NE, MJ/kg	9.7	9.7	9.7	9.7	9.7	9.7
Crude protein	171	171	171	171	171	171
Crude fat	59	59	59	59	59	59
AID lysine[5]	9.1	9.1	9.1	9.1	9.1	9.1
Calcium	2.0	5.8	9.6	2.0	5.8	9.6
Phosphorus	4.7	4.7	4.7	4.7	4.7	4.7
IP-P[6]	2.9	2.9	2.9	2.9	2.9	2.9
ATTD Phosphorus[5]	1.7	1.7	1.7	2.5	2.5	2.5
Nutrients, analysed						
Dry matter	889	886	889	888	889	889
Crude ash	57	58	58	57	57	58
Crude protein	181	184	184	184	187	184
Crude fat	58	51	56	58	58	59
Starch	363	364	367	360	374	372
Sugar	46	46	45	46	48	45
Calcium	1.8	5.3	8.9	1.8	5.3	8.6
Phosphorus	4.9	4.9	4.9	4.8	4.9	4.8
IP6-P	2.3	2.3	2.3	2.3	2.3	2.3
IP5-P	0.2	0.2	0.2	0.2	0.2	0.2
IP-P	2.5	2.5	2.5	2.5	2.5	2.5
Phytase activity, FTU/kg	<100	<100	<100	568	610	547

[1] Premix contributed per kg of diet: vitamin A 10,000 IU; vitamin D_3 2,000 IU; vitamin E 40 mg, vitamin K_3 1.5 mg, vitamin B_1 1.0 mg, vitamin B_2 4.0 mg, vitamin B_6 1.5 mg, vitamin B_{12} 20 µg; niacin 30 mg, D-panthotenic acid 15 mg; choline chloride 150 mg; folic acid 0.4 mg; biotin 0.05 mg; Fe 100 mg, Cu 20 mg, Mn 30 mg; Zn 70 mg; I 0.70 mg, Se 0.25 mg.

[2] Axtra Phy, Danisco Animal Nutrition.

[3] Sibelco, Maastricht, the Netherlands.

[4] Damolin, Kønsborgvej, Denmark.

[5] AID = apparent ileal digestible, ATTD = apparent total tract digestible.

[6] IP-P, inositol phytate bound P.

2.2 Animal husbandry and feeding strategy

The experiment lasted 21 days (Table 2). From d 0-9, the pigs were individually housed in floor pens with a partly slatted concrete floor. During this period, sick pigs or pigs with feed refusal were replaced with healthy backup pigs. On d 10, all pigs were placed on fully-slatted metabolism pens with dimensions 1.6 x 1.6 m^2, partly closed fence with metal bars on top to allow animal contact, tenderfoot floor, and a feeding trough for liquid diets. After 4 d of adaptation to the metabolism pens (d 10-13), a 4-d balance trail was conducted. During the balance trial (d 14-17), the faeces were collected in plastic bags using a Velcro system according to van Kleef et al. (1994) and urine was collected using a funnel and bucket underneath the pen. Each day faeces and urine samples were stored at 4 °C. At the end of the collection period, faeces and urine were pooled per pig, subsampled and stored in the freezer at -20 °C. The environmental temperature and ventilation were automatically controlled by a climate computer. A day-night schedule (0600 to 2200 h light on) was used except for the night before dissection (d 20). On d 20, the light was on from 0200 h in order to feed the pigs according to a designed feeding schedule.

Table 2 *Timeline of the study.*

Day	0-9	10-13	14-17 (balance trial)	18-19	20
Housing	Floor pens	Metabolism pens			
Meals/d	2	2	2	6	6-10
TiO_2	+	+	+	+	+
Co-EDTA	-	-	-	+	+

The designed feeding schedule was adopted from Schop et al. (2019) and Martens et al. (2019). Daily feed allowance was three times net energy requirement for maintenance (293 KJ NE/$BW^{0.75}$, CVB protocol 2016) based on individual BW measured on d 0, 10 and 20, with daily increments based on the expected body gain. From d 0-10, the feeding level was based on the initial BW on d 0 plus an estimated average daily gain (ADG) of 500 g/d. The mean daily gain from d 0-10 was used to estimate the ADG from d 10-20 and to calculate the feed allowance accordingly. Liquid feeding was used in a water/feed ratio of 2:1 (w/w base). From d 0-17, the pigs received 2 equal meals per day at 0700 and 1500 h. On d 18 and 19, the pigs received their daily allowance through 6 equal small meals. The 6 small meals were given from 0700 to 2200 h with an interval of 3 h. The 3-hourly-feeding was applied to pigs in order to achieve a constant passage rate of digesta through the GIT. On d 20 (dissection day), the pigs were fed from 0200 h, and they were given 6 small meals at an interval of 1 h (meal size 1/12 of the daily allowance). These 6 1-hourly meals were given to the pigs until dissection. All pigs received half of their daily allowance via the 1-hourly meals before dissection. Because of the duration of dissections, extra meals (meal size 1/8 of their daily allowance) were given to the late-dissected pigs prior to the 6 hourly-feeding meals. These extra meals were given with an interval of 2 hour, in order to maintain the constant passage rate of digesta through the GIT and create steady state conditions. This meal size was chosen so that the last dissected block of pigs received half of their daily allowance via the extra meals of 2-hourly-feeding, and received the other half of their allowance via the 6 meals of 1-hourly-feeding. By applying this feeding regime, the feeding frequency was gradually increased from 3-hourly-feeding to 1-hourly-feeding. During the whole experiment, pigs were fed and dissected per block. Therefore, the 6 pigs within a block received the same number of meals and were dissected almost at the same time. The feed refusals were collected every day, dried and recorded.

2.3 Sample collection and chemical analysis

Diet samples were collected at the barn every day before feeding the pigs. They were mixed per treatment, and an aliquot was taken using a sample splitter. The dry matter (DM) content was determined by drying at 103 °C (ISO, 6492), and ash was determined by incinerated at 550 °C (ISO, 5984).

The N was analyzed by using the Kjeldahl method, and crude protein (CP) content was calculated as N×6.25 (FOSS, Hillerod, Denmark; ISO, 5983). The crude fat (cfat) was determined after acid hydrolysis (ISO 6492). The starch was determined using a photometer (Evolution 201, Thermoscientific, Waltham, MA) after enzymatic conversion into glucose (ISO 15914). The total reducing sugar content was determined photometrically (Evolution 201, Thermoscientific, Waltham, MA) after extraction with 40% ethanol. After incineration, the Ca content in the diet was determined using an AAS spectrometer (Varian, CA; ISO 6869), and the P content was determined using a photometer (Evolution 201, Thermoscientific, Waltham, MA). The Ti concentration in the diet was determined in the faeces using a photometer (Evolution 201) after destructed by H_2SO_4 (Myers et al., 2004).

On d 20, pigs were euthanized by sedating with Zoletil 100 (0.06 ml/kg BW), followed by Euthasol (24 mg/kg BW) via ear vein injection. Blood was collected from the carotid artery in serum and heparine-plasma tubes (Greiner bio-one) before exsanguination. Serum was harvested by centrifuging the blood at 3,000 g for 10 min at 4 °C. Thereafter the abdominal cavity was opened and the GIT was carefully taken out. The GIT was divided into 6 segments by tie wraps to prevent digesta flowing across compartments. The 6 gut segments included stomach, the first half of small intestine (proximal-SI), the second half of small intestine (distal-SI), caeca, the first half of large intestine (proximal-LI) and the second half of large intestine (distal-LI). The gut segments were quantitatively emptied by gentle squeezing. After thoroughly mixing, the digesta pH was determined using a pH meter (Seven2Go, Mettler Toledo). After the pH determination, the caeca and proximal-LI digesta were pooled to reduce the number of samples and stored at -20 °C until further analysis. To determine gene expression levels, mucosal scrapings from the middle of jejunum and colon mucosa were collected, snap frozen in liquid nitrogen, and stored at -80 °C. The lower left foreleg was removed for collection of the 3rd metacarpal bone and stored at -20° C before analysis.

After thawing in a cooling chamber (4 °C), the digesta were thoroughly mixed, and two aliquots were taken for analysis. One aliquot was used for the total Ca, P and Ti determination. This aliquot was freeze-dried and ground to pass a 1-mm sieve (Retsch GmbH, Germany). Subsequently, the Ca, P, Co and Ti content was determined using an ICP-OES (ThermoFisher, iCAP 7000) after destruction by a mixture of HCl (Chem-Lab), HNO_3 (Chem-lab) and HF (VWR) using a Microwave (CEM). The other aliquot was used to determine the solubility of Ca and P. This aliquot was centrifuged at 3,000 g for 5 min at 4 °C . The supernatant was quantitatively collected, and the soluble Ca and P content was determined using an ICP-OES. No destruction was applied to the supernatant, therefore, the soluble inorganic Ca and P were determined. To prevent the suspended particles blocking the ICP-OES, the supernatant was centrifuged again at 10,000 g for 10 minutes at 4 °C before determination. Because no liquid phase could be separated from the fresh digesta of the distal-LI, the distal-LI digesta was diluted with deionized water at a ratio of 1:1 (w:w). For the urine samples, an aliquot was taken during mixing on a magnet stirrer. The Ca and P content were determined using an ICP-OES after destruction. The manure samples were freeze-dried and ground to pass a 1-mm sieve (Retsch GmbH, Germany). The AAS was used to determine the Ca content, photometry was used to determine the P and Ti content in the faeces, as documented before in the determination of Ca, P and Ti content in the diets. Two methods were used for the determination of Ca (ICP and AAS), P (ICP and photometer) and Ti (ICP and photometer), because the number of samples was too big to handle in one lab within a desired time period. A pilot test was done to compare the Ca, P and Ti results from these two methods using all 6 feed samples and 1 block of faeces samples. The results showed minor differences between these two methods (data not shown).

Phytate isomers in diets and distal small intestine digesta were analysed according to Zeller et al. (2015). Briefly, diets and digesta samples were ground using a ball mill, subsequently phytate isomers were extracted for 30 min with 10 ml of a solution containing 0.2 M-EDTA and 0.1 M-sodium fluoride (pH=10) as phytase inhibitor using a rotary shaker. Afterwards samples were centrifuged at 12,000 g for 15 min and the supernatant fraction was removed and preserved on ice. The residue was re-suspended in 5 ml of aforementioned EDTA and sodium fluoride solution to further extract the phytate isomer for 30 min. The supernatant of these two extractions was combined, 1 ml of the pooled supernatant was taken, centrifuged at 14,000 g for 15 min; 0.5 ml of the resulting supernatant

fraction was filtered through a 0.2 cellulose acetate filter (VWR) into a Microcon filter device (Millipore) and centrifuged again at 14,000 g for 30 min. Filtrates were analysed by high-performance ion chromatography and UV detection at 290 nm after post-column derivatisation using an ICS-3000 system (Dionex). Phytate isomers were separated on a Carbo Pac PA 200 column and corresponding guard column. The limit of detection was defined for a signal:noise ratio of 3:1, which was 0.1 µmol/g of DM for IP3 and IP4 and 0.05 µmol/g of DM for IP5 and IP6. Data were reported on a DM basis in chyme and fresh (as is) basis in feed.

Deep-frozen jejunal and colonic mucosa was ground in liquid nitrogen and total RNA was isolated with TRIzol (ThermoFisher Scientific) according to the manufacture's instruction. Isolated RNA was subjected to on-column DNAse treatment to remove possible genomic DNA contamination with the Nucleospin II kit (Macherey Nagel). Quantity and quality of RNA was determined with the NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific) and 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies), respectively. RNA was reverse transcribed with Superscript III kit (ThermoFisher Scientific) and mRNA levels were assessed by quantitative PCR (RT-qPCR) amplification on a QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific) using the SensiFAST™ SYBR® low-ROX Kit (Bioline) under the following conditions: 95 °C for 15 seconds and 60 °C for 30 seconds for 40 cycles. Absolute expression levels of genes of interest were normalized by the corresponding mRNA level of importin 8 (IPO8) since Normfinder (Andersen et al., 2004) demonstrated that this gene was the most stable one, compared to eukaryotic translation elongation factor 2 (EEF2) or beta actin (ACTB). The quality/stability of IPO8 being reference gene was further checked by comparing to the results normalized by the geometric mean of IPO8, EEF2 and ACTB, and very similar results were obtained hence IPO8 was used to normalize the gene expression levels.

Serum Ca and P were analyzed using a Cobas 8000 modular analyzer with C701 Photometric measuring unit (Roche Diagnostics Limited, Rotkreuz, Switzerland). Commercial available test kits were used to analyze serum parathyroid hormone (PTH, Immunotopics, San Clemente, USA), 25-hydroxycholecalciferol (25(OH)D3) and 1,25-dihydroxycholecalciferol (1,25(OH)2D3, Immunodiagnostic Systems GmbH, Germany), and alkaline phosphatase (ALP, Diatools AG, Villmergen, Switzerland). The forelegs were thawed, the 3rd metacarpal bone carefully separated using a scalpel, dried in the oven at 70° C, defatted with petroleum, incinerated at 800° C for ash determination and the ash subsequently destructed in a microwave (CEM, NC) and analysed for Ca and P content (ICP-OES) (23, 24).

2.4 Calculations and statistical analysis

The Ca and P solubility were calculated using equation (1):

$$\text{Solubility, \%} = (S_{supernatant} \div m_{aliquot}) \div X_{digetsa} \times 100 \quad (1)$$

Where $S_{supernatant}$ was soluble inorganic Ca or P content in the supernatant (g), which was calculated using the soluble Ca or P concentration in the supernatant (g/l) multiplied by the quantitative volume of the supernatant (l); $m_{aliquot}$ was the mass of the fresh digesta used for centrifugation, expressed on freeze dried base (kg, FDM base), which was calculated using the mass of the aliquot (fresh base) multiplied by the freeze-dried DM content (g/kg); $X_{digesta}$ was the total Ca or P content in the digesta (g/kg, FDM base).

The apparent total tract digestibility (ATTD) was calculated using equation (2)

$$\text{ATTD, \%} = (1-(X_{digesta} \div X_{diet}) \times (Ti_{diet} \div Ti_{digesta})) \times 100 \quad (2)$$

Where $X_{digesta}$ and X_{diet} were the nutrient (e.g. Ca, P, CP, DM) content in the faeces (g/kg, FDM base) and diet (g/kg), respectively; the Ti_{diet} and $Ti_{digesta}$ were the Ti content in the faeces (g/kg, FDM base) and diet (g/kg), respectively. In this calculation, the Ca content in the diet included the Ca from drinking water based on the water:feed ratio of 2:1.

The daily Ca and P retention was calculated using the equation (3)

$$\text{Retention, g/d} = (X_{absorption} - X_{excretion}) \div 4 \quad (3)$$

Where $X_{absorption}$ was the absorbed Ca or P during the entire balance period (g), which was calculated using the Ca or P intake (g) multiplied by the ATTD (%) of Ca or P and then divided by 100; $X_{excretion}$ was the urinary Ca or P excretion during the balance period (g), which was calculated using the urinary Ca or P content (g/kg) multiplied by the quantitative weight of the urine (kg). Results were divided by 4, being the days of the balance trial (d) to express Ca and P retention on a daily basis.

The Ca and P flux along the GIT was calculated using equation (4):

$$\text{Flux, g/kg} = X_{digesta} \times Ti_{diet} \div Ti_{digesta} \quad (4)$$

Where $X_{digesta}$ was the Ca or P content in the digesta (g/kg, FDM base); $Ti_{digesta}$ and Ti_{diet} were the Ti content in the digesta (g/kg, FDM base) and diet (g/kg), respectively

The mean retention time (MRT) of the solid and liquid phase of the digesta in different GIT segments was calculated using the equation (5) according to Schop et al. (2019):

$$\text{MRT, h} = M_{digesta} \div M_{rate} \quad (5)$$

Where $M_{digesta}$ was the quantitative marker mass in the digesta (g), which was calculated using the marker content in the digesta (g/kg, FDM base) multiplied by the quantitative weight of digesta (kg, FDM base); the M_{rate} was the marker intake rate (g/h), which was calculated using the marker intake (g) divided by the corresponding feeding time (h). The 6 1-hourly meals were used to calculate the M_{rate} for the stomach and SI. However, because the digesta MRT from stomach to the caeca was longer than 6 hours, the marker intake from d 19 to dissection was used to calculate the M_{rate} in the proximal and distal LI. The Ti was the marker for the solid phase, while Co was the marker for the liquid phase.

The percentage of P bound to IPs (IPs-P/P) in the distal small intestine was calculated using equation (6):

$$\text{IPs-P/P, \%} = IPs * S * 31/1000/P_{digesta} * 100 \quad (6)$$

Where IPs was content of IPs in the distal small intestine (μmol/g, FDM base); S was the number of P bound to IPs (e.g. 6 for IP6, 5 for IP5); 31 was the molecular weight of P (g/mol), $P_{digesta}$ was the P content in the freeze-dried digesta (g/kg, FDM base).

The percentage of individual IP isomers in the total IP content (∑IP) in the distal ileal digesta were calculated as:

$$IP_s/\sum IP, \% = IP_s/\sum IP_s * 100$$

where IP_s is the content of individual IP isomers (s=3, 4, 5 and 6, μmol/g) and $\sum IP_s$ the sum of IP_s (n=3, 4, 5 and 6, μmol/g).

Pig was the experimental unit for all analysis and calculations. All data were submitted to a 2-way ANOVA using the MIXED procedure of SAS (version 9.4, SAS Institute, Cary, NC). Phytase, Ca content and their interaction were included as fixed effects, batch and block were included as random effects. The residual diagnosis was plotted using the ODS GRAPHICS procedure. The distribution and variance homogeneity of the studentized residuals were visually checked via these graphics. The LSMEANS procedure with a PDIFF option was used to separate the means. Probability was considered significant at $P \leq 0.05$ and a trend at $0.05 < P < 0.1$.

3 Results

3.1 General

The pigs in the first batch grew well and readily ate their daily ration. In the second batch, one pig suddenly died due to a twisted intestine and one pig received a 1-d medical treatment because of diarrhoea. The other pigs remained healthy throughout the experiment.

3.2 Growth performance

The growth performance was summarized in Table 3. The incremental Ca level impaired the ADG (*P*=0.016) and FCR (*P*=0.002) in the entire experimental period (d 0-20), while phytase inclusion improved both ADG (*P*=0.025) and FCR (*P*=0.001) in this period. In the period d 10-20, the ADG (*P*=0.034) and FCR (*P*=0.063) were affected by the interaction between the Ca and phytase. The incremental Ca content impaired the ADG and the FCR in the phytase-free diet but not in the phytase-supplemented diet. In the 20 d period, incremental dietary Ca impaired the FCR in phytase-free diets (interaction *P*=0.067).

Table 3 *Effect of dietary calcium (Ca) content and phytase inclusion on growth performance in growing pigs*.*

Ca, g/kg	Phytase, FTU/kg	D 0-10			D 10-20			D 0-20		
		ADG, g/d	ADFI, kg/d	FCR g/g	ADG, g/d	ADFI, kg/d	FCR g/g	ADG, g/d	ADFI, kg/d	FCR g/g
1.8	0	589	1.20	2.12	882[a]	1.52	1.73	736	1.29	1.77
5.3	0	576	1.20	2.15	824[ab]	1.52	1.85	700	1.30	1.86
8.8	0	519	1.19	2.45	765[b]	1.49	2.00	642	1.27	2.01
1.8	500	593	1.15	1.97	865[a]	1.50	1.75	729	1.27	1.74
5.3	500	632	1.22	1.97	884[a]	1.53	1.74	752	1.31	1.75
8.8	500	548	1.17	2.16	875[a]	1.51	1.74	712	1.28	1.80
SEM		33	0.03	0.12	34	0.02	0.08	28	0.02	0.05
Ca level										
1.8		591[a]	1.18	2.05[b]	874	1.51	1.74	732[a]	1.28	1.76[b]
5.3		604[a]	1.21	2.06[b]	853	1.52	1.79	725[a]	1.30	1.81[b]
8.8		534[b]	1.18	2.30[a]	820	1.50	1.87	677[b]	1.27	1.90[a]
SEM		24	0.02	0.09	24	0.01	0.06	20	0.02	0.04
Phytase										
0		561	1.20	2.24	824	1.51	1.86	693	1.29	1.88
500		591	1.18	2.04	874	1.52	1.74	730	1.28	1.77
SEM		19	0.02	0.07	20	0.01	0.05	17	0.01	0.03
P-value										
Ca		0.011	0.236	0.008	0.085	0.147	0.081	0.016	0.208	0.002
Phytase		0.160	0.332	0.009	0.013	0.487	0.021	0.025	0.742	0.001
Ca*phytase		0.459	0.367	0.608	0.034	0.326	0.063	0.132	0.463	0.067

[a-c] Mean values lacking a common letter within a column differ (*P*<0.05).
*ADG=average daily gain; ADFI=average daily feed intake; FCR=feed conversion ratio.
Dietary P content was 4.87 g/kg in all diets.

3.3 Digestibility and retention of Ca and P

The Ca intake, absorption and retention were summarized in Table 4. All observations in Table 4 were affected by the interactive effect between the Ca content and phytase inclusion (P<0.001) except for the Ca intake (P=0.477). The Ca intake increased with incremental dietary Ca content but was equal between the low and high phytase diet as intended. Compared to the low-Ca content, medium Ca content increased urinary Ca output in phytase-free (0.08 vs 1.17) but not in phytase-supplemented diets (0.05 vs 0.10). Overall phytase inclusion improved the Ca digestion and retention (P<0.001). The incremental Ca content improved the ATTD of Ca in the phytase-free diets, while it reduced the ATTD of Ca in the phytase-supplemented diets. Compared to the medium Ca content, the high Ca content increased Ca retention in the phytase-free diet (2.44 vs 2.01) and to a lesser extent in the high phytase diet (4.68 vs 4.49). The ratio of retained Ca to ingested Ca (rCa/Ca intake) and retained Ca to digested Ca (rCa/dCa) was reduced by an incremental Ca content, but the reduction was much larger in the phytase-free diet. The Ca digestibility of limestone at the first supplementation (medium Ca) was 44.7 and 54.3% for the phytase-free and phytase-supplemented diets, respectively. The higher supply of limestone (high Ca) reduced Ca digestibility, as the Ca digestibility of limestone at the second supplementation was 38.5 and 35.2% for the phytase-free and phytase-supplemented diets. Phytase improved Ca digestibility of limestone at the first supplementation but not at the second supplementation.

The P intake, absorption and retention were summarized in Table 5. The P intake was similar between treatment groups (P>0.10). Compared to medium Ca, high Ca decreased urinary P output in phytase-supplemented diets (0.22 vs 0.02) but not in phytase-free diets (0.04 vs 0.02). The ATTD of P was reduced by the incremental Ca content in both low and high phytase diets, but it was reduced to a higher extent in the phytase-supplemented diets compared to the phytase-free diets. Compared to the low Ca content, medium Ca did not influence the ATTD of P in the phytase-free diets (27.0 vs 29.7), while it reduced the ATTD of P in the phytase-supplemented diets (52.0 vs 60.1). The P retention (rP), both expressed in g/d and as rP/P intake, was not affected by dietary Ca content in the phytase-free diets whereas it was increased from the low to medium Ca content in the phytase-supplemented diets. The ratio retained P to dP intake (rP/dP) increased with the incremental Ca content, with a larger effect in the phytase-supplemented diets.

Table 4 *Effect of dietary calcium (Ca) content and phytase inclusion on Ca intake, absorption and retention in growing pigs**

Ca g/kg	Phytase, FTU/kg	Ca intake g/d	Ca ATTD, %	Urinary Ca output, g/d	rCa, g/d	rCa/dCa, %	rCa/Ca, intake %	ATTD Ca in limestone, %
1.8	0	2.59	36.9^{e}	0.08^{d}	0.87^{d}	91.0^{b}	33.7^{d}	
5.3	0	7.57	42.0^{d}	1.17^{c}	2.01^{c}	63.2^{d}	26.6^{e}	44.7
8.8	0	12.38	40.6^{de}	2.59^{a}	2.44^{b}	48.3^{e}	19.6^{f}	38.5
1.8	500	2.57	71.3^{a}	0.05^{d}	1.78^{c}	97.4^{a}	69.5^{a}	
5.3	500	7.64	60.1^{b}	0.10^{d}	4.49^{a}	97.8^{a}	58.8^{b}	54.3
8.8	500	12.50	50.2^{c}	1.60^{b}	4.68^{a}	74.2^{c}	37.4^{c}	35.2
SEM		0.08	1.88	0.13	0.18	3.11	2.01	
Ca								
1.8		2.58^{c}	54.1	0.06	1.33	94.2	51.6	
5.3		7.60^{b}	50.6	0.66	3.18	79.6	41.9	
8.8		12.44^{a}	45.4	2.10	3.56	61.3	28.5	
SEM		0.06	1.35	0.09	0.13	2.23	1.40	
Phytase								
0		7.51	39.9	1.28	1.77	67.5	26.7	
500		7.57	60.6	0.60	3.62	89.5	55.1	
SEM		0.05	1.10	0.07	0.10	1.81	1.14	
P-value								
Ca		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
Phytase		0.288	<0.001	<0.001	<0.001	<0.001	<0.001	
Ca*phytase		0.477	<0.001	<0.001	<0.001	<0.001	<0.001	

[a-f] Mean values lacking a common superscript letter within a column differ (*P*<0.05).
* ATTD=apparent total tract digestible; rCa=retained Ca; dCa=apparently digestible Ca intake, g/d.
Dietary P content was 4.87 g/kg in all diets.

Table 5 *Effect of dietary calcium (Ca) content and phytase inclusion on phosphorus (P) intake, absorption and retention in growing pigs*.*

Ca g/kg	Phytase, FTU/kg	P intake, g/d	P ATTD, %	Urinary P, output, g/d	rP, g/d	rP/dP, %	P retention, %
1.8	0	6.91	29.7^{d}	0.32^{b}	1.73^{d}	84.2^{c}	25.0^{cd}
5.3	0	6.90	27.0^{d}	0.04^{c}	1.82^{d}	98.0^{a}	26.4^{c}
8.8	0	6.80	23.4^{e}	0.02^{c}	1.69^{d}	99.1^{a}	23.2^{d}
1.8	500	6.85	60.1^{a}	1.41^{a}	2.71^{c}	65.5^{d}	39.5^{b}
5.3	500	6.96	52.0^{b}	0.22^{b}	3.40^{a}	93.9^{b}	48.8^{a}
8.8	500	6.87	45.8^{c}	0.02^{c}	3.13^{b}	99.4^{a}	45.5^{a}
SEM		0.06	1.22	0.07	0.11	1.75	1.6
Ca							
1.8		6.88	44.9	0.82	2.22	74.9	32.2
5.3		6.93	38.8	0.12	2.57	96.1	37.1
8.8		6.83	35.8	0.02	2.49	99.2	36.2
SEM		0.05	0.87	0.05	0.08	1.28	1.2
Phytase							
0		6.87	27.2	0.13	1.75	93.4	25.4
500		6.89	52.7	0.55	3.07	86.0	44.5
SEM		0.04	0.72	0.04	0.07	1.04	1.0
P-value							
Ca		0.116	<0.001	<0.001	<0.001	<0.001	<0.001
Phytase		0.570	<0.001	<0.001	<0.001	<0.001	<0.001
Ca*phytase		0.258	0.023	<0.001	0.002	<0.001	0.001

[a-e] Mean values lacking a common superscript letter within a column differ (*P*<0.05).
* ATTD=apparent total tract digestible; rP=retained P, g/d; dP= apparently digestible P intake, g/d.
Dietary P content was 4.87 g/kg in all diets.

3.4 Calcium and phosphorus flux

3.4.1 Phosphorus

The flux of P through the digestive tract, expressed per kg of feed intake, was summarized in Table 6. The P flux gradually decreased from the stomach to the distal-SI, increased in the proximal-LI and decreased again in the distal-LI. The average P flux was 4.6, 3.8, 3.0, 3.6, 3.0 and 2.9 g/kg in the stomach, proximal-SI, distal-SI, proximal-LI, distal-LI and faeces, respectively. Overall, the flux was lower in each segment with phytase-supplemented diets, indicating a higher absorption of P. The P flux was higher in each segment posterior to the stomach, with incremental dietary Ca, indicating a lower absorption of P. The P flux was drastically reduced in the proximal and distal SI using phytase-supplemented diets, whereas in phytase-free diets, a relatively large reduction was only observed in the distal SI. The P flux was affected by the interaction between phytase and Ca level in the distal-SI (P=0.007) and faeces (P<0.001). In the distal-SI, increasing Ca level had no impact on P flux in the phytase-free diets, while it increased P flux for the phytase-supplemented diets. A similar trend was observed in the faeces, in which the P flux was increased by the incremental Ca level to a greater extent in the phytase-supplemented diets compared to the phytase-free diets. These effects were further quantified by calculation of the net disappearance (absorption) or appearance (secretion) of P in each segment of the GIT (Table 7), and P digestibility or absorption (as percentage of dietary P) in different GIT segments (Appendix 1). The majority of P absorption was realised in the distal SI for phytase-free diets and the proximal and distal SI in phytase-supplemented diets. Interestingly, the P absorption in the distal SI increased with increasing Ca content, in contrast to the effect of Ca in the proximal SI. The net P absorption in the entire LI was small, as can be derived from the similarity in flux in the distal SI and the faeces.

Table 6 *Effect of dietary calcium (Ca) content and phytase inclusion on phosphorus (P) flux through the gastrointestinal tract in growing pigs, expressed in g/kg ingested feed *.*

Ca g/kg	Phytase FTU/kg	Stomach	Prox-SI	Distal-SI	Prox-LI	Distal-LI	Faeces
1.8	0	4.82	4.21	3.61[a]	4.07	3.48	3.42[b]
5.3	0	4.62	4.30	3.65[a]	4.84	3.97	3.55[b]
8.8	0	4.69	4.91	3.85[a]	4.71	3.99	3.73[a]
1.8	500	4.35	2.76	1.81[c]	2.14	1.65	1.94[e]
5.3	500	4.22	3.11	2.39[b]	2.60	2.17	2.33[d]
8.8	500	4.62	3.71	2.55[b]	3.19	2.58	2.64[c]
SEM		0.18	0.27	0.13	0.22	0.16	0.07
Ca level							
1.8		4.58	3.48[b]	2.71	3.10[b]	2.56[b]	2.68
5.3		4.43	3.74[b]	3.05	3.78[a]	3.12[a]	2.98
8.8		4.65	4.31[a]	3.20	3.95[a]	3.29[a]	3.18
SEM		0.13	0.19	0.09	0.16	0.12	0.02
Phytase							
0		4.71	4.47	3.70	4.54	3.81	3.57
500		4.40	3.19	2.24	2.64	2.13	2.30
SEM		0.11	0.15	0.08	0.13	0.09	0.04
P-value							
Ca		0.231	<0.001	<0.001	<0.001	<0.001	<0.001
Phytase		0.007	<0.001	<0.001	<0.001	<0.001	<0.001
Ca*phytase		0.274	0.718	0.007	0.135	0.156	0.001

[a-c] Mean values lacking a common letter within a column differ (P<0.05).
* SI=small intestine; LI=large intestine.
Dietary P content was 4.87 g/kg in all diets.

Table 7 *Effect of dietary calcium (Ca) content and phytase inclusion on phosphorus (P) absorption and secretion through the gastrointestinal tract in growing pigs, expressed in g/kg ingested feed.*

Ca g/kg	phytase FTU/kg	Mouth~ stomach	Stomach~ Prox-SI	Prox-SI~ distal-SI	Distal-SI~ prox-LI	Prox-LI~ distal-LI	Distal-LI~ anus
1.8	0	0.05	0.62	0.59	-0.46	0.60	0.06
5.3	0	0.25	0.32	0.65	-1.19	0.87	0.42
8.8	0	0.18	-0.22	1.05	-0.86	0.72	0.26
1.8	500	0.52	1.59	0.95	-0.33	0.49	-0.29
5.3	500	0.65	1.11	0.72	-0.20	0.43	-0.16
8.8	500	0.25	0.91	1.16	-0.64	0.61	-0.06
SEM		0.19	0.30	0.28	0.25	0.24	0.16
Ca							
1.8		0.29	1.10[a]	0.77	-0.39	0.54	-0.12
5.3		0.44	0.69[ab]	0.68	-0.72	0.66	0.14
8.8		0.22	0.35[b]	1.11	-0.75	0.67	0.10
SEM		0.13	0.21	0.20	0.18	0.17	0.12
Phytase							
0		0.16	0.24	0.76	-0.84	0.73	0.25
500		0.47	1.21	0.95	-0.40	0.51	-0.17
SEM		0.108	0.174	0.16	0.15	0.139	0.09
P-value							
Ca		0.231	0.003	0.090	0.092	0.722	0.063
Phytase		0.007	<0.001	0.271	0.006	0.128	<0.001
Ca*phytase		0.274	0.734	0.737	0.068	0.569	0.574

[a-b] Mean values lacking a common letter within a column differ (*P*<0.05).
* SI=small intestine; LI=large intestine; prox=proximal.
Dietary P content was 4.87 g/kg in all diets.

3.4.2 Calcium

The flux of Ca through the digestive tract, expressed per kg of feed intake, was summarized in Table 8. The Ca flux drastically decreased from the stomach to the proximal-SI, with a further reduction in the LI. The mean Ca flux was 5.4, 3.2, 3.3, 3.1, 2.5 and 2.8 g/kg in the stomach, proximal-SI, distal-SI, proximal-Li, distal-LI and faeces, respectively. Increasing dietary Ca content increased the Ca flux in all gut segments (*P*<0.001), while phytase reduced the Ca flux in all gut segments (*P*<0.05) except in the stomach (*P*=0.475). The Ca flux was affected by the interaction between the Ca level and phytase in the proximal-LI (*P*=0.018) and distal-LI (*P*=0.082) but not in faeces (*P*=0.174). The Ca flux in these segments was lower at low dietary Ca contents in phytase-supplemented diets compared to phytase–free diets, but it increased more with the highest increment in dietary Ca content. These effects were further quantified by calculation of the net disappearance (absorption) or appearance (secretion) of Ca in each segment of the GIT (Table 9), and Ca digestibility (as percentage of dietary Ca) in different GIT segments (Appendix 2). The majority of Ca absorption was realised in the proximal SI for both phytase-free and phytase-supplemented diets. Phytase enhanced the Ca absorption in both the proximal and distal SI. The increase in dietary Ca increased the absorption mainly in the proximal SI. A remarkably high Ca absorption was observed in the proximal LI in the treatment with high Ca in the phytase-free diet. In contrast to P, the LI contributed substantially to the net Ca absorption.

Table 8 *Effect of dietary calcium (Ca) content and phytase inclusion on Ca flux through the gastrointestinal tract in growing pigs, expressed in g/kg ingested feed**

Ca g/kg	Phytase FTU/kg	Stomach	Prox-SI	Distal-SI	Prox-LI	Distal-LI	Faeces
1.8	0	2.12	1.39	1.61	1.38^{d}	1.29	1.15
5.3	0	5.49	3.48	3.79	4.10^{b}	3.34	3.09
8.8	0	8.79	5.66	6.31	4.91^{ab}	4.11	5.26
1.8	500	2.09	0.73	0.65	0.69^{d}	0.49	0.52
5.3	500	5.13	2.59	2.35	2.45^{c}	2.00	2.13
8.8	500	8.81	5.31	4.99	5.24^{a}	3.91	4.41
SEM		0.31	0.29	0.28	0.46	0.34	0.12
Ca							
1.8		2.10^{c}	1.06^{c}	1.13^{c}	1.03	0.89^{c}	0.84^{c}
5.3		5.32^{b}	3.06^{b}	3.11^{b}	3.32	2.70^{b}	2.64^{b}
8.8		8.80^{a}	5.48^{a}	5.65^{a}	5.07	4.01^{a}	4.84^{a}
SEM		0.22	0.21	0.20	0.33	0.25	0.09
Phytase							
0		5.47	3.51	3.90	3.46	2.91	3.17
500		5.35	2.89	2.68	2.80	2.14	2.36
SEM		0.17	0.17	0.16	0.66	0.20	0.07
P-value							
Ca		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Phytase		0.475	0.001	<0.001	0.017	<0.001	<0.001
Ca*phytase		0.638	0.442	0.591	0.018	0.082	0.174

$^{a-d}$ Mean values lacking a common letter within a column differ (P<0.05).
*SI=small intestine; LI=large intestine, prox=proximal.
Dietary P content was 4.87 g/kg in all diets.

Table 9 *Effect of dietary calcium (Ca) content and phytase inclusion on Ca absorption through the gastrointestinal tract in growing pigs, expressed in g/kg ingested feed**

Ca g/kg	Phytase FTU/kg	Mouth~ stomach	Stomach~ Prox-SI	Prox-SI~ distal-SI	Distal-SI~ prox-LI	Prox-LI~ distal-LI	Distal-LI~ anus
1.8	0	-0.36	0.73	-0.22	0.23^{b}	0.09	0.14
5.3	0	-0.22	2.01	-0.32	-0.31^{b}	0.77	0.24
8.8	0	0.00	3.14	-0.65	1.40^{a}	0.80	-1.15
1.8	500	-0.33	1.36	0.08	-0.04^{b}	0.19	-0.03
5.3	500	0.14	2.53	0.24	-0.09^{b}	0.44	-0.13
8.8	500	-0.02	3.50	0.32	-0.25^{b}	1.33	-0.50
SEM		0.31	0.38	0.44	0.50	0.59	0.32
Ca							
1.8	-0.34	1.04^{c}	-0.07	0.10	0.14	0.05^{a}	
5.3	-0.05	2.26^{b}	-0.05	-0.21	0.61	0.07^{a}	
8.8	-0.01	3.32^{a}	-0.17	0.58	1.06	-0.83^{b}	
SEM	0.22	0.27	0.32	0.36	0.42	0.23	
Phytase							
0	-0.19	1.96	-0.40	0.44	0.55	-0.26	
500	-0.08	2.46	0.21	-0.13	0.66	-0.22	
SEM	0.17	0.22	0.26	0.29	0.34	0.18	
P-value							
Ca	0.234	<0.001	0.954	0.118	0.098	<0.001	
Phytase	0.477	0.023	0.036	0.068	0.762	0.848	
Ca*phytase	0.638	0.877	0.515	0.027	0.605	0.067	

$^{a-b}$ Mean values lacking a common letter within a column differ (P<0.05).
*SI=small intestine; LI=large intestine, prox=proximal.
Dietary P content was 4.87 g/kg in all diets.

3.5 Phytate degradation

Phytate degradation in the distal small intestine was summarized in Table 10 (reported as phytate distribution) and Table 11 (reported as phytate-P distribution).

Microbial phytase supplementation significantly increased IP6 degradation from an average of 5.8% to 78.6% of the dietary content and reduced ∑IP content in the freeze-dried digesta at the distal-SI. Dietary Ca addition significantly reduced IP6 degradation but not ∑IP in the digesta (P=0.014 and 0.137, respectively). The IP6 was largely degraded in the phytase-supplemented diets, with an average of 25.8, 7.2, 37.3 and 29.7% for the IP6/∑IP, IP5/∑IP, IP4/∑IP and IP3/∑IP, respectively, remaining in the ileal chyme. In contrast, in the phytase-free diets, IP6 was largely intact with an average of 76.5, 16.7, 5.3 and 1.5% for the IP6/∑IP to IP3/∑IP, respectively, in ileal chyme. Increasing dietary Ca content from 1.8 to 8.8 g/kg increased undegraded IP6/∑IP more in the phytase-supplemented compared to phytase-free diets (16 vs. 4% unit, P=0.025). Incremental dietary Ca content reduced both IP4/∑IP and IP3/∑IP but only for the phytase-supplemented diets (P=0.018 and 0.009, respectively). The IP5/∑IP was inconsistently affected by dietary Ca content (P=0.001).

Table 10 *Effect of dietary calcium (Ca) content and phytase inclusion on phytate or inositol phosphate (IP) content and distribution in the distal small intestine in growing pigs.*

Ca g/kg	Phytase FTU/kg	IP content µmol/g	IP3/IP %	IP4/IP %	IP5/IP %	IP6/IP %
1.8	0	46.3	2.6^{c}	6.2^{c}	18.2^{a}	72.9^{b}
5.3	0	45.0	1.0^{c}	4.2^{c}	15.5^{b}	79.3^{a}
8.8	0	41.2	1.0^{c}	5.5^{c}	16.4^{ab}	77.2^{ab}
1.8	500	26.7	37.5^{a}	39.4^{a}	5.8^{d}	17.4^{e}
5.3	500	36.1	24.0^{b}	39.4^{a}	9.6^{c}	26.9^{d}
8.8	500	28.6	26.9^{b}	33.5^{b}	6.4^{d}	33.3^{c}
SEM		3.9	2.7	1.8	1.2	3.0
Ca						
1.8		36.5	20.1	22.8	12.0	45.2
5.3		40.6	12.5	21.8	12.6	53.1
8.8		34.9	13.9	19.5	11.4	55.2
SEM		2.8	2.0	1.3	0.86	2.1
Phytase						
0		44.2	1.5	5.3	16.7	76.5
500		30.3	29.7	37.3	7.2	25.8
SEM		2.3	1.4	1.0	0.70	1.7
P-value						
Ca		0.137	0.001	0.033	0.387	<0.001
Phytase		<0.001	<0.001	<0.001	<0.001	<0.001
Ca*phytase		0.170	0.009	0.018	0.001	0.025

[a-c] Mean values lacking a common letter within a column differ (*P*<0.05).
Dietary P content was 4.87 g/kg in all diets.

Table 11 *Effect of dietary calcium (Ca) content and phytase inclusion on phytate or inositol phosphate (IP6) degradation and IP-P distribution and flux in the distal small intestine in growing pigs *.*

Ca g/kg	Phytase FTU/kg	IP6 degradation %	IP3-P/P %	IP4-P/P %	IP5-P/P %	IP6-P/P %	IP-P/P %	IP-P flux g/kg	P flux g/kg
1.8	0	8.4	1.1^{d}	3.7^{c}	13.4^{a}	63.7^{a}	81.9	2.99^{a}	3.61^{a}
5.3	0	3.0	0.4^{d}	2.4^{c}	11.0^{b}	67.3^{a}	81.1	2.96^{a}	3.65^{a}
8.8	0	6.1	0.4^{d}	2.9^{c}	10.9^{b}	61.7^{a}	75.8	2.92^{a}	3.85^{a}
1.8	500	88.9	15.4^{a}	23.1^{a}	4.6^{d}	15.0^{c}	58.1	1.07^{c}	1.81^{c}
5.3	500	73.9	11.4^{b}	25.9^{a}	8.1^{c}	26.9^{b}	72.4	1.74^{b}	2.39^{b}
8.8	500	73.1	9.1^{c}	16.9^{b}	4.4^{d}	26.7^{b}	57.2	1.45^{b}	2.55^{b}
SEM		5.1	1.1	2.3	1.2	3.6	6.1	0.18	0.13
Ca									
1.8		48.6^{a}	8.3	13.4	9.0	39.3	70.0^{ab}	2.03	2.71
5.3		38.4^{b}	5.9	14.1	9.6	47.1	76.7^{a}	2.35	3.05
8.8		39.6^{b}	4.8	9.9	7.6	44.2	66.5^{b}	2.18	3.20
SEM		3.6	0.8	1.6	0.8	2.6	4.4	0.13	0.09
Phytase									
0		5.8	0.66	3.0	11.8	64.2	79.6	2.95	3.70
500		78.6	12.0	21.8	5.6	22.7	62.2	1.41	2.24
SEM		3.0	0.61	1.3	0.7	2.1	3.6	0.11	0.08
P-value									
Ca		0.014	<0.001	0.022	0.065	0.015	0.067	0.055	<0.001
Phytase		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Ca*phytase		0.168	0.002	0.015	0.003	0.036	0.216	0.030	0.01

[a-b] Mean values lacking a common letter within a column differ ($P<0.05$).
Dietary P content was 4.87 g/kg in all diets.

3.6 Ca and P solubility

The P solubility was summarized in Table 12. Overall, the P solubility was high in stomach and proximal-SI and decreased along the distal GIT segments, with an average P solubility of 49.8, 49.1, 24.7, 19.7 and 21.7% in the stomach, proximal-SI, distal-SI, proximal-LI and distal-LI, respectively. No interactive effects between dietary Ca and phytase on P solubility were observed in any of the gut segment (P>0.10). High Ca decreased P solubility (P<0.10) and phytase increased P solubility (P<0.001) in all gut segments.

The Ca solubility was summarized in Table 13. Overall, the Ca solubility was high in the stomach, somewhat lower in the proximal SI and decreased sharply in the distal-SI. The mean Ca solubility was 49.3, 36.6, 7.6, 6.8 and 4.7% in the stomach, proximal-SI, distal-SI, proximal-LI and distal-LI, respectively. The incremental dietary Ca content increased Ca solubility in the stomach (P<0.001) and distal-SI (P=0.045), with less consistent effects in the other segments. Phytase tended to increase the Ca solubility in all gut segments (P<0.10) except in the proximal-SI (P=0.633). The Ca solubility was inconsistently affected by the interaction between dietary Ca content and phytase in the proximal-LI (P<0.001).

Table 12 *Effect of dietary calcium (Ca) content and phytase inclusion on phosphorus (P) solubility (%) through the gastrointestinal tract in growing pigs*.*

Ca g/kg	Phytase FTU/kg	Stomach	Prox-SI	Distal-SI	Prox-LI	Distal LI
1.8	0	43.7	56.8	15.0	20.2	21.7
5.3	0	47.3	28.9	12.2	15.6	15.9
8.8	0	39.5	32.4	14.6	14.0	16.1
1.8	500	55.4	70.8	44.0	26.3	29.0
5.3	500	57.8	51.3	31.8	24.0	28.7
8.8	500	55.1	54.5	30.6	18.1	19.1
SEM		2.94	7.15	5.33	1.72	3.39
Ca						
1.8		49.6	63.8[a]	29.5	23.3[a]	25.4[a]
5.3		52.3	39.5[b]	21.5	19.6[b]	21.9[a]
8.8		47.3	43.4[b]	22.6	16.0[c]	17.6[ab]
SEM		2.11	5.13	3.72	1.24	2.43
Phytase						
0		43.5	39.4	13.9	16.6	17.9
500		56.1	59.1	35.6	22.8	25.5
SEM		1.71	4.17	3.02	1.00	1.98
P-value						
Ca		0.052	<0.001	0.096	<0.001	0.009
Phytase		<0.001	<0.001	<0.001	<0.001	<0.001
Ca*phytase		0.454	0.631	0.206	0.244	0.149

[a-b] Mean values lacking a common letter within a column differ (P<0.05).
* SI=small intestine; LI=large intestine; prox=proximal.
Dietary P content was 4.87 g/kg in all diets.

Table 13 *Effect of dietary calcium (Ca) content and phytase inclusion on Ca solubility (%) through the gastrointestinal tract in growing pigs.*

Ca g/kg	Phytase FTU/kg	Stomach	Prox-SI	Distal-SI	Prox-LI	Distal-LI
1.8	0	43.6	43.8	3.8	9.3[a]	5.4
5.3	0	49.9	21.6	5.8	4.4[b]	2.7
8.8	0	48.7	41.2	9.1	4.8[b]	4.2
1.8	500	43.1	30.2	6.8	6.9[b]	5.3
5.3	500	56.1	32.7	9.2	9.4[a]	5.2
8.8	500	54.4	50.0	11.1	5.7[b]	5.2
SEM		3.42	10.7	2.62	1.12	1.09
Ca						
1.8		43.4[b]	37.0	5.3[b]	8.1	5.3
5.3		52.8[a]	26.8	7.4[ab]	6.8	3.9
8.8		51.5[a]	45.6	10.1[a]	5.2	4.7
SEM		2.46	7.67	1.88	0.80	0.78
Phytase						
0		47.4	35.5	6.2	6.2	4.1
500		51.0	37.8	9.0	7.3	5.2
SEM		2.00	6.23	1.53	0.65	0.64
P-value						
Ca		<0.001	0.095	0.045	0.003	0.219
Phytase		0.057	0.633	0.072	0.089	0.082
Ca*phytase		0.304	0.170	0.936	<0.001	0.289

[a-b] Mean values lacking a common letter within a column differ (*P*<0.05).
* SI=small intestine; LI=large intestine; prox=proximal.
Dietary P content was fixed at 4.87 g/kg.

3.7 Dry matter digestibility

The DM digestibility through the GIT was summarized in Table 14. The DM digestibility was negative in the stomach, thereafter it increased along the GIT, with an average of -60, 31.6, 60, 77 and 79% in the stomach, proximal-SI, distal-SI, proximal-LI and distal-LI, respectively. It was inconsistently affected by dietary Ca content. The incremental Ca content decreased DM digestibility in the distal-SI (*P*=0.011) and increased it in the total tract (*P*=0.026). Phytase inclusion increased the DM digestibility in the distal-SI, distal-LI and the total tract (*P*=0.035).

Table 14 *Effect of dietary calcium (Ca) content and phytase inclusion on dry matter (DM) digestibility (%) through the gastrointestinal tract in growing pigs*.*

Ca g/kg	Phytase FTU/kg	Stomach	Prox-SI	Distal-SI	Prox-LI	Distal LI	ATTD
1.8	0	-75.2	27.1	59.7	76.4	78.4	81.1
5.3	0	-41.0	35.8	59.4	75.2	77.6	82.2
8.8	0	-47.7	21.4	55.9	77.5	78.0	81.9
1.8	500	-81.3	40.4	65.6	75.8	79.7	82.0
5.3	500	-64.2	38.5	61.3	77.8	78.8	82.8
8.8	500	-52.0	26.5	58.3	76.2	79.1	82.3
SEM		16.5	7.43	2.47	1.35	0.94	0.47
Ca							
1.8		-78.2^{b}	33.8^{ab}	62.6^{a}	76.1	79.1	81.5^{b}
5.3		-52.6^{a}	37.1^{a}	60.4^{ab}	76.5	78.2	82.5^{a}
8.8		-49.9^{a}	24.0^{b}	57.1^{b}	76.9	78.6	82.1^{a}
SEM		11.8	5.32	1.77	0.97	0.67	0.34
Phytase							
0		-54.6	28.1	58.3	76.3	78.0	81.7
500		-65.9	35.0	61.8	76.6	79.2	82.3
SEM		9.60	4.33	1.44	0.79	0.55	0.27
P-value							
Ca		0.041	0.050	0.011	0.716	0.406	0.026
Phytase		0.213	0.123	0.034	0.784	0.035	0.034
Ca*phytase		0.603	0.549	0.379	0.146	0.988	0.687

* Calculated using 88.9% and 96% as the DM content for the diet and freeze-dried digesta, respectively.
SI=small intestine; LI=large intestine; prox=proximal.
Dietary P content was 4.87 g/kg in all diets.

3.8 Digestibility of proximate components

The ATTD of proximate nutrients was summarized in Table 15. Dietary Ca content and phytase inclusion did not affect the ATTD of organic matter and crude protein but the ATTD of ash was enhanced both by the increment in dietary Ca content and phytase inclusion (P<0.001). The incremental Ca level reduced the digestibility of crude fat (P<0.001).

Table 15 *Effect of dietary calcium (Ca) content and phytase inclusion on apperent total tract digestibility (ATTD, %) of proximate components in growing pigs*.*

Ca level	Phytase	DM	CP	Ash	OM	cfat
1.8	0	81.1	79.3	22.6^{e}	85.1	81.6
5.3	0	82.2	80.8	32.5^{d}	85.6	81.2
8.8	0	81.9	80.9	38.2^{c}	85.0	78.3
1.8	500	82.0	78.9	33.5^{d}	85.3	81.4
5.3	500	82.8	81.1	39.9^{b}	85.7	80.8
8.8	500	82.3	80.6	45.9^{a}	84.8	78.7
SEM		0.47	1.32	0.74	0.50	0.97
Ca level						
1.8		81.5^{b}	79.1	28.0	85.2	81.5^{a}
5.3		82.5^{a}	80.9	36.0	85.6	81.0^{a}
8.8		82.1^{a}	80.7	42.0	84.9	78.5^{b}
SEM		0.34	0.95	0.53	0.35	0.70
Phytase						
0		81.7	80.3	31.1	85.2	80.4
500		82.3	80.2	39.7	85.3	80.3
SEM		0.27	0.77	0.43	0.28	0.57
P-value						
Ca		0.026	0.123	<0.001	0.104	<0.001
Phytase		0.034	0.834	<0.001	0.891	0.852
Ca*phytase		0.687	0.932	0.002	0.841	0.800

$^{a-e}$ Mean values lacking a common letter within a column differ (P<0.05).
*CP=crude protein; OM=organic matter; cfat=crude fat.
Dietary P content was 4.87 g/kg in all diets.

3.9 Serum characteristics

The serum concentration of Ca, P, *alkaline phosphatase (AP), parathyroid hormone (PTH), 25 hydroxycholecalciferol (25(OH)D) and 1,25 dihydroxycholecalciferol (1,25(OH)$_2$D)* were summarized in Table 16. Overall, incremental dietary Ca enhanced the serum Ca and AP concentration and reduced the serum P concentration (P<0.001). Dietary phytase reduced the serum Ca (P<0.001) and AP (P=0.03) concentration and enhanced the serum P concentration (P<0.001). The effect of dietary Ca content on serum Ca and P concentration was larger in the phytase-free diets than in the phytase-supplemented diets, as indicated by the interaction between phytase and Ca content (P<0.001). Serum PTH was reduced by incremental dietary Ca content (P<0.001), while it was enhanced by phytase but only at medium Ca level (P=0.011). Both dietary Ca level and phytase inclusion enhanced serum 25(OH)D (P<0.001). In contrast, serum 1,25(OH)$_2$D was reduced by dietary limestone inclusion but only in the phytase-supplemented diets (P=0.010).

Table 16 *Effect of dietary calcium (Ca) content and phytase inclusion on serum Ca, P, alkaline phosphatase (AP), parathyroid hormone (PTH), 25 hydroxycholecalciferol (25(OH)D) and 1,25 dihydroxycholecalciferol (1,25(OH)$_2$D) in growing pigs.*

Ca level	Phytase	AP, u/l	Ca, mmol/l	P, mmol/l	PTH, pg/ml	25(OH)D, nmol/l	1,25(OH)$_2$D, pmol/l
1.8	0	100	2.38^{cd}	2.90^{b}	118.2^{a}	70.2	1048^{a}
5.3	0	113	2.78^{b}	2.19^{d}	4.8^{c}	85.9	1007^{a}
8.8	0	141	3.38^{a}	1.85^{e}	1.2^{c}	89.4	1003^{a}
1.8	500	98	2.35^{d}	3.23^{a}	127.3^{a}	84.4	1013^{a}
5.3	500	99	2.48^{c}	3.09^{ab}	73.0^{b}	101.7	873^{b}
8.8	500	116	2.82^{b}	2.63^{c}	3.7^{c}	110.5	860^{b}
SEM		10	0.07	0.10	16.3	6.35	26
Ca							
1.8		99^{a}	2.37	3.07	122.8	77.3	1030
5.3		106^{a}	2.64	2.62	37.1	93.4	943
8.8		129^{b}	3.10	2.24	2.48	99.9	932
SEM		7	0.05	0.07	11.40	4.55	19
Phytase							
0		118	2.85	2.31	41.4	81.8	1019
500		105	2.55	2.98	67.8	98.7	916
SEM		6	0.04	0.06	9.27	3.70	15
P-value							
Ca		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Phytase		0.030	<0.001	<0.001	0.006	<0.001	<0.001
Ca*phytase		0.280	<0.001	<0.001	0.011	0.710	0.010

$^{a-d}$ Mean values lacking a common letter within a column differ (P<0.05).

3.10 Composition of metacarpal bone

The defatted bone mass, ash content and composition of the 3rd metacarpal bone was presented in Table 17. Both incremental dietary Ca content and phytase inclusion enhanced defatted bone mass of the 3rd metacarpal bone (P=0.001). The first limestone inclusion enhanced ash content of the defatted metacarpus irrespective of phytase level; however, the second increment in limestone inclusion reduced ash content in the phytase-free but not in the phytase-supplemented diets (Interaction P=0.034). Total bone ash mass increased with incremental dietary Ca content and phytase inclusion. The Ca content in bone ash was not affected by dietary treatments, whereas the P content was somewhat enhanced by inclusion of phytase. Accordingly, the Ca/P ratio in the bone only tended to be enhanced by dietary Ca/P ratio (P=0.056).

Table 17 *Effect of dietary calcium (Ca) content and phytase inclusion on defatted bone mass, ash content and composition of the defatted 3rd metacarpal bone in growing pigs*

Ca level	Phytase	Defatted bone, g	Ash in defat bone, %	Ash in bone, g	Ca in bone ash, g/kg	P in bone ash, g/kg	Ca/P
1.8	0	3.37	56.9^{cd}	1.92	196	95.2	2.06
5.3	0	3.59	57.5^{bc}	2.07	197	92.3	2.13
8.8	0	3.74	56.7^{d}	2.12	205	91.9	2.27
1.8	500	3.59	57.0^{cd}	2.05	199	94.9	2.10
5.3	500	4.15	58.5^{a}	2.42	203	98.9	2.06
8.8	500	4.04	58.1^{ab}	2.35	206	97.1	2.12
SEM		0.15	0.03	0.08	5.74	2.89	0.07
Ca							
1.8		3.48	56.9	1.98	198	95.1	2.08
5.3		3.86	58.0	2.24	200	95.5	2.09
8.8		3.89	57.5	2.23	205	94.5	2.19
SEM		0.11	0.02	0.06	4.12	2.02	0.05
Phytase							
0		3.57	57.0	2.03	199	93.2	2.15
500		3.93	57.9	2.27	203	97.0	2.09
SEM		0.09	0.02	0.05	3.35	1.64	0.04
P-value							
Ca		0.001	<0.001	<0.001	0.162	0.910	0.056
Phytase		<0.001	<0.001	<0.001	0.293	0.030	0.173
Ca*phytase		0.329	0.034	0.216	0.856	0.232	0.189

$^{a-d}$ Mean values lacking a common letter within a column differ (P<0.05).

3.11 Digesta pH and passage rate

The pH of the fresh digesta determined immediately after collection was summarized in Table 18. Overall the pH of the digesta was low in the stomach, increased in the proximal and distal SI, decreased in the caeca and increased somewhat in the distal LI. The average digesta pH in the stomach, proximal-SI, distal-SI, caeca, proximal-LI and distal-LI were 3.64, 5.88, 6.42, 5.46, 5.53 and 5.94, respectively. The pH was differentially affected by the diet treatments through the GIT.

Incremental Ca level increased the pH of the digesta in stomach (P<0.001), caeca (P=0.003), proximal-LI (P<0.001) and distal-LI (P=0.051), while it did not consistently affect the pH in the proximal SI. An interactive effect between the Ca level and phytase was observed in the distal-SI (P=0.022), with a decrease in pH with incremental Ca content in the phytase-free diet but not in the phytase-supplemented diet. Phytase inclusion decreased digesta pH in the stomach (P=0.005) and caeca (P=0.059), while it increased digesta pH in the proximal-SI (P=0.081). In the distal-SI, phytase enhanced the pH at the high Ca diets, but not at the low Ca diets (interaction, P=0.022).

Table 18 *Effect of dietary calcium (Ca) content and phytase inclusion on digesta pH through the gastrointestinal tract in growing pigs*.*

Ca g/kg	Phytase FTU/kg	Stomach	Prox-SI	Distal-SI	Caeca	Prox-LI	Distal-LI
1.8	0	3.23	5.76	6.55[a]	5.50	5.39	5.83
5.3	0	4.08	5.98	6.25[bc]	5.52	5.52	5.92
8.8	0	4.13	5.72	6.20[c]	5.75	5.68	5.96
1.8	500	2.96	6.03	6.48[a]	5.41	5.43	5.84
5.3	500	3.46	5.99	6.47[ab]	5.44	5.48	6.02
8.8	500	3.97	5.79	6.55[a]	5.60	5.70	6.07
SEM		0.21	0.11	0.11	0.09	0.07	0.10
Ca level							
1.8		3.09[c]	5.89[ab]	6.51	5.45[b]	5.41[b]	5.84
5.3		3.78[b]	5.99[a]	6.36	5.48[b]	5.51[b]	5.97
8.8		4.05[a]	5.75[b]	6.38	5.67[a]	5.69[a]	6.02
SEM		0.15	0.78	0.08	0.07	0.05	0.08
Phytase							
0		3.81	5.82	6.33	5.59	5.53	5.90
500		3.46	5.93	6.50	5.48	5.54	5.98
SEM		0.12	0.06	0.06	0.05	0.04	0.06
P-value							
Ca		<0.001	0.020	0.099	0.003	<0.001	0.051
Phytase		0.005	0.081	0.010	0.059	0.839	0.256
Ca*phytase		0.262	0.193	0.022	0.824	0.698	0.806

[a-c] Mean values lacking a common letter within a column differ (*P*<0.05).
* SI=small intestine; LI=large intestine; prox=proximal.
Dietary P content was 4.87 g/kg in all diets.

The passage rate of the solid and liquid digesta were summarized in Table 19 and 20, respectively. Overall, the mean MRT of the solid fraction among all treatments was 2.29, 0.53, 1.65, 10.1 and 7.51 h in the stomach, proximal-SI, distal-SI, proximal-LI, and distal-LI, respectively. The mean MRT of the liquid fraction among all treatments was 1.40, 0.45, 1.35, 8.24 and 4.99 h in the stomach, proximal-SI, distal-SI, proximal-LI, and distal-LI, respectively. Both the MRT of the liquid and solid digesta were not influenced by the diet treatments in all gut segments (*P*>0.10).

Table 19 *Effect of dietary calcium (Ca) content and phytase inclusion on mean retention time (MRT, h) of the solid phrase through the gastrointestinal tract in growing pigs**

Ca g/kg	Phytase FTU/kg	Stomach†	Prox-SI†	Distal-SI†	Prox-LI‡	Distal-LI‡
1.8	0	2.62	0.59	1.57	10.6	7.63
5.3	0	2.11	0.56	1.56	9.5	7.61
8.8	0	2.36	0.42	1.75	10.3	8.88
1.8	500	2.28	0.55	1.87	10.0	7.03
5.3	500	1.98	0.55	1.63	10.9	6.71
8.8	500	2.41	0.49	1.52	9.4	7.21
SEM		0.41	0.10	0.18	1.22	1.13
Ca						
1.8		2.45	0.57	1.72	10.3	7.33
5.3		2.05	0.56	1.59	10.1	7.18
8.8		2.39	0.45	1.64	9.8	8.04
SEM		0.28	0.07	0.13	0.88	0.81
Phytase						
0		2.36	0.52	1.63	10.1	8.04
500		2.23	0.53	1.68	10.1	6.99
SEM		0.23	0.06	0.10	0.71	0.63
P-value						
Ca		0.318	0.168	0.627	0.869	0.523
Phytase		0.538	0.834	0.624	0.950	0.121
Ca*phytase		0.794	0.720	0.121	0.440	0.788

*SI=small intestine; LI=large intestine; prox=proximal.
† Calculation using the 6 hourly feeding meals on d 20.
‡ Calculation using the feed intake on d 19 and 20.
Dietary P content was 4.87 g/kg in all diets.

Table 20 *Effect of dietary calcium (Ca) content and phytase inclusion on mean retention time (MRT, h) of the liquid phrase through the gastrointestinal tract in growing pigs**

Ca level	Phytase	Stomach†	Prox-SI†	Distal-SI†	Prox-LI‡	Distal-LI‡
1.8	0	1.61	0.44	1.33	8.21	4.85
5.3	0	1.28	0.48	1.25	8.08	5.08
8.8	0	1.46	0.42	1.41	7.70	5.77
1.8	500	1.32	0.46	1.46	8.09	4.53
5.3	500	1.11	0.51	1.38	8.91	4.77
8.8	500	1.57	0.42	1.29	8.44	4.95
SEM		0.30	0.08	0.16	0.91	0.74
Ca						
1.8		1.47	0.45	1.39	8.15	4.69
5.3		1.20	0.49	1.31	8.47	4.93
8.8		1.52	0.42	1.35	8.07	5.36
SEM		0.22	0.06	0.12	0.65	0.53
Phytase						
0		1.45	0.44	1.33	8.00	5.23
500		1.34	0.46	1.37	8.47	4.75
SEM		0.17	0.05	0.09	0.53	0.43
P-value						
Ca		0.286	0.428	0.806	0.781	0.439
Phytase		0.505	0.729	0.645	0.362	0.274
Ca*phytase		0.622	0.980	0.456	0.717	0.857

*SI=small intestine; LI=large intestine.
† Calculation using the 6-hourly-feeding meals on d 20.
‡ Calculation using the feed intake on d 19 and 20.
Dietary P content was 4.87 g/kg in all diets.

3.12 Jejunal mRNA expression levels

Jejunal tissues from pigs of the lowest and highest dietary Ca treatment groups were subjected to RT-qPCR analysis to determine the expression of genes related to intestinal Ca and P absorption (Table 21).

3.12.1 Ca and P transporters

No interaction effect was observed between dietary calcium and phytase level (P>0.10). The mRNA level of the apical Ca channel TRPV6 was decreased by both high Ca and phytase level (P<0.001 and 0.003, respectively). The other apical Ca channel TRPV5 was decreased by phytase inclusion (P=0.003) but not affected by dietary Ca level. Dietary treatments did not affect (P>0.10) expression of Calbindin-D28k (CaBP-28k) and Calbindin-D9k (CaBP-9k), or basolateral Na^+/Ca^{2+} exchanger (NCX1). The mRNA level of Ca-sensing receptor (CaSR) was below the limit of detection. TRPM7 mRNA, encoding a non-selective divalent cation transporter, was increased by high Ca (P=0.040), but not by phytase (P=0.285). Expression of XPR1, a putative basolateral P channel, was decreased by high Ca (P=0.024), but not by phytase (P=0.734). Dietary treatments did not alter (P>0.10) expression of the apical sodium-dependent P transporters NaPi-IIc, PiT-1 and PiT-2, or endogenous mucosa phosphatase (MinPP1) and osmolyte regulator, Na-inositol cotransporter (SLC5A3/SMIT).

3.12.2 Claudins and other proteins

As a measure of modulation of tight junction permeability, we measured the mRNA expression of several claudins known to be expressed as integral membrane proteins in tight junctions of all epithelia. As shown in Table 21, CLDN2 mRNA was increased by high Ca (P<0.001), while CLDN12 mRNA was elevated by phytase (P=0.013). On the contrary, high Ca decreased (P<0.001) CLDN10 mRNA. Dietary treatment had no (P>0.10) effect on expression levels of CLDN4, CLDN7 and CLDN15. In addition, ZO-1 mRNA, encoding another tight junction-associated protein, was numerically decreased (P=0.086) by phytase.

Furthermore, high Ca increased transcript levels of vitamin D receptor (VDR) (P<0.001), ATP-gated ion channel P2X7 (P=0.048) and gap junction protein CX43 (P=0.049). Dietary treatments had no influence on ectonucleotide pyrophosphatase (ENPP1) mRNA (P>0.10) (Table 21).

Table 21 *Effect of phytase (PHY) and Ca level on gene expressions of Ca and P transporters and tight junction proteins in the jejunum, in arbitrary units*.*

Items	Dietary treatments					*P*-value		
Ca, g/kg	1.8		8.8		SEM	Ca	PHY	Ca*PHY
PHY, FTU/kg	0	500	0	500				
Genes								
TRPV5	0.025	0.020	0.029	0.017	0.004	0.746	0.003	0.207
TRPV6	1.15	0.93	0.61	0.31	0.160	<0.001	0.003	0.630
CaBP-28k	0.099	0.071	0.064	0.060	0.03	0.333	0.486	0.605
CaBP-9k	261	229	308	254	37	0.179	0.112	0.671
NCX1	1.57	1.56	1.68	1.69	0.10	0.105	0.992	0.879
XPR1	7.90	8.10	7.17	7.20	0.48	0.024	0.734	0.797
NaPi-IIc	0.339	0.339	0.384	0.339	0.04	0.407	0.397	0.411
PiT-1	4.49	4.33	4.89	4.35	0.32	0.367	0.135	0.401
PiT-2	28.6	30.2	28.8	22.5	4.7	0.276	0.492	0.250
MinPP1	7.79	7.37	7.38	7.71	0.42	0.915	0.869	0.221
SLC5A3	5.69	5.21	5.60	5.51	0.41	0.719	0.339	0.511
CLDN2	18.7	17.3	24.8	28.4	2.9	<0.001	0.598	0.233
CLDN4	66.9	69.3	69.4	61.9	7.0	0.622	0.606	0.323
CLDN7	23.5	24.2	23.9	24.3	1.3	0.811	0.558	0.910
CLDN10	0.87	0.71	0.40	0.40	0.14	<0.001	0.408	0.417
CLDN12	9.7	11.2	10.7	11.6	0.66	0.150	0.013	0.544
CLDN15	17.1	17.8	17.4	18.5	1.6	0.665	0.433	0.851
ZO-1	3.61	3.51	3.64	3.49	0.11	0.949	0.086	0.695
TRPM7	15.5	14.5	16.0	16.0	0.65	0.040	0.285	0.302
IAP	34.3	37.5	35.1	37.9	3.6	0.828	0.248	0.934
VDR	9.1	10.1	19.5	21.1	2.2	<0.001	0.390	0.862
P2X7	0.56	0.45	0.69	0.88	0.19	0.048	0.741	0.271
ENPP1	0.364	0.356	0.362	0.343	0.03	0.746	0.578	0.823
CX43	1.47	1.36	1.55	1.62	0.12	0.049	0.753	0.282

*TRPV5, Transient receptor potential cation channel subfamily V member 5; TRPV6, Transient receptor potential cation channel subfamily V member 6; CABP28k, Calbindin-D28k; CABP9k, Calbindin-D9k; NCX1, sodium-calcium exchanger; TRPM7, Transient receptor potential cation channel, subfamily M, member 7; IAP, Intestinal alkaline phosphatase; MinPP1, Multiple inositol polyphosphate phosphatase 1; SLC5A3/SMIT, Sodium/myo-inositol cotransporter; NaP-IIc, Type II sodium-dependent phosphate transport protein c; PiT1, Inorganic phosphate transporter 1; PiT2, Inorganic phosphate transporter 2; XPR1, Xenotropic and Polytropic Retrovirus Receptor 1; CLDN, claudin; ZO-1, Zonula occludens-1; VDR, Vitamin D receptor; P2X7, ATP-gated ion channel 7; ENPP1, Ectonucleotide pyrophosphatase/phosphodiesterase family member 1; CX43/connexin 43, gap junction protein 43.

3.13 Colonic mRNA expression levels

The effects of the treatments with the lowest and highest dietary Ca content treatments on the colonic expression of genes related to Ca and P absorption is presented in Table 22.

3.13.1 Ca and P transporter

In colon, high Ca and phytase decreased the mRNA of apical Ca channels TRPV5 and TRPV6, as well as that of CaBP-9k (P<0.001). However, expression of basolateral Na^+/Ca^{2+} exchanger (NCX1) and non-selective divalent cation transporter (TRPM7) was not affected by dietary treatment (P>0.10). Furthermore, high Ca decreased (P=0.026) expression level of the osmolyte regulator Na-inositol cotransporter (SLC5A3/SMIT) and numerically increased (P=0.055) apical P transporter (PiT-1). Dietary treatment did not significantly change expression XPR1 or apical P transporter PiT-2 and endogenous mucosal phytase (MinPP1). Colon did not express CaBP-28k, Ca sensing receptor (CaSR) and Type II sodium-dependent phosphate transporters (NaPi-II) at the level of detection (data not shown).

3.13.2 Claudins and other proteins

Expression of colonic CLDN7 displayed an interaction effect (P=0.046) between Ca and phytase level with an increased CLDN7 mRNA by high Ca in the phytase-free, but not phytase-supplemented diet (Table 22). Expression of CLDN4 (P=0.027) and CLDN10 (P<0.001) was lower by high Ca, while expression of CLDN12 tended to be elevated (P=0.072) by phytase. Contrary to jejunum, colon did not expression of CLD15 and intestinal alkaline phosphatase (IAP) (data not shown).

Expression of vitamin D receptor (VDR) tended to be increased by phytase (P=0.052). Dietary treatments did not significantly alter colonic expression of ZO-1, P2X7, ENPP1 and CX43 (Table 22).

Table 22 *Effect of phytase and Ca level on gene expressions of Ca and P transporters and tight junction proteins in the colon, in arbitrary units*.*

Items	Dietary treatments					*P*-value		
Ca, g/kg	1.8		8.8		SEM	Ca	Phytase	Ca*phytase
PHY, FTU/kg	0	500	0	500				
Genes								
TRPV5	0.034	0.022	0.025	0.011	0.008	0.030	0.004	0.778
TRPV6	1.85	1.43	1.03	0.49	0.216	<0.001	0.002	0.572
CaBP-9k	215	159	108	44	18	<0.001	<0.001	0.738
NCX1	1.77	1.72	1.56	1.74	0.14	0.350	0.521	0.258
TRPM7	20.9	20.5	20.1	20.4	0.74	0.421	0.896	0.556
MinPP1	9.27	9.47	9.40	9.27	0.47	0.913	0.914	0.622
SLC5A3	11.9	11.8	10.8	10.7	0.65	0.026	0.893	0.957
PiT-1	17.0	16.1	17.5	18.2	0.92	0.055	0.827	0.211
PiT-2	122	134	115	119	21	0.452	0.610	0.781
XPR1	9.9	10.7	10.4	10.5	0.71	0.714	0.385	0.534
CLDN2	12.5	12.0	12.1	12.6	1.08	0.891	0.962	0.511
CLDN4	62.5	57.6	43.8	53.8	6.8	0.027	0.603	0.136
CLDN7	32.5[a]	29.7[b]	29.4[b]	30.3[ab]	1.2	0.153	0.279	0.046
CLDN10	1.90	2.17	1.19	1.24	0.26	<0.001	0.406	0.555
CLDN12	22.8	25.2	24.4	26.0	1.5	0.246	0.072	0.711
VDR	7.06	7.85	6.74	7.60	0.57	0.498	0.052	0.935
ZO-1	5.19	5.33	5.00	5.30	0.19	0.390	0.101	0.551
P2X7	0.526	0.490	0.517	0.528	0.03	0.551	0.621	0.355
ENPP1	0.481	0.448	0.446	0.479	0.03	0.927	0.985	0.128
CX43	3.47	3.80	4.20	3.74	0.32	0.153	0.786	0.090

*TRPV5, Transient receptor potential cation channel subfamily V member 5; TRPV6, Transient receptor potential cation channel subfamily V member 6; CABP9k, Calbindin-D9k; NCX1, sodium-calcium exchanger; TRPM7, Transient receptor potential cation channel, subfamily M, member 7; MinPP1, Multiple inositol polyphosphate phosphatase 1; SLC5A3/SMIT, Sodium/myo-inositol cotransporter; PiT1, Inorganic phosphate transporter 1; PiT2, Inorganic phosphate transporter 2; XPR1, Xenotropic and polytropic retrovirus receptor 1; CLDN, claudin; ZO-1, Zonula occludens-1; VDR, Vitamin D receptor; P2X7, ATP-gated ion channel 7; ENPP1, Ectonucleotide pyrophosphatase/phosphodiesterase family member 1; CX43/connexin 43, gap junction protein 43.

3.14 mRNA expression levels in the kidney

The effects of the treatments with the lowest and highest dietary Ca content treatments on the expression of genes related to Ca and P re-absorption in the kidney is presented in Table 23.

3.14.1 Ca and P transporter

Renal expression of CaSR was enhanced by dietary Ca content but only in the phytase-free diets (P=0.031). In contrast, renal expression of Ca transporters, including TRPV5, TRPV6, CaBP-28k and NCX1, were reduced by a high compared to low dietary Ca content (P<0.020). Moreover, this reducing impact of dietary Ca level on TRPV5, CaBP-28k and NCX was greater in the phytase-supplemented compared to phytase-free diet (P=0.022, 0.024 and 0.021, respectively). Renal expression of CaBP-9k was enhanced by dietary Ca level but only in phytase-free diets (P=0.001).

Expression of all type II transporters, including NaPi-IIa, IIb and IIc, as well as PiT-2 were not affected by dietary treatments. However, renal expression of PiT-1 and XPR1 was enhanced by dietary Ca level and reduced by phytase inclusion.

3.14.2 Claudins and other proteins

Renal expression of CLDN12 and 16 was reduced by a high compared to low dietary Ca level (P=0.053 and <0.001, respectively). Expression of other tight junction proteins, including ZO-1, CLDN2, 4, 7 and 10, was not affected by dietary Ca content. Phytase inclusion reduced CLDN7 and 10 (P=0.004 and 0.002, respectively) but had no impact on other tight junctions.

Both CYP24 and VDR were enhanced by dietary Ca level and microbial phytase. Expression of MinPP1 was reduced by phytase while it tended to be enhanced by dietary Ca level (P=0.025 and 0.055, respectively). Expression of SLC5A3 was not affected by dietary treatments. Dietary Ca level enhanced renal expression of CX43 (P=0.012) but had no impact on P2X7 or ENPP1. Phytase reduced ENPP1 and tended to reduce CX43 (P=0.010 and 0.058, respectively) but had no impact on P2X7.

Table 23 *Effect of phytase and Ca level on gene expressions of Ca and P transporters and tight junction proteins in the kidney,* in arbitrary units*.

Items	Dietary treatments				SEM	*P*-value		
Ca, g/kg	1.8		8.8			Ca	Phytase	Ca*Phytase
PHY, FTU/kg	0	500	0	500				
Genes								
CaSR	0.40^{c}	0.44^{bc}	0.67^{a}	0.54^{b}	0.052	<0.001	0.265	0.031
TRPV5	0.90^{a}	1.11^{a}	0.91^{a}	0.35^{b}	0.222	0.020	0.272	0.022
TRPV6	0.172	0.156	0.111	0.100	0.020	0.004	0.343	0.867
TRPM7	4.26	4.07	4.28	4.09	0.19	0.893	0.159	0.999
CaBP-9k	0.20^{b}	0.14^{b}	1.08^{a}	0.12^{b}	0.18	0.002	<0.001	0.001
CaBP-28k	63.10^{a}	68.99^{a}	40.39^{b}	15.74^{c}	8.99	<0.001	0.151	0.024
NCX1	1.36^{ab}	1.53^{a}	1.06^{b}	0.66^{c}	0.21	0.001	0.326	0.021
NaPi-2a	12.48	12.21	13.10	12.93	0.57	0.107	0.592	0.898
NaPi-2b	0.006	0.006	0.006	0.004	0.002	0.416	0.469	0.591
NaPi-2c	0.42	0.44	0.47	0.39	0.05	0.985	0.420	0.192
PiT-1	6.35	5.71	9.27	7.54	0.57	<0.001	0.006	0.234
PiT-2	33.50	34.82	32.83	30.12	6.37	0.491	0.878	0.656
XPR1	1.45	1.36	1.58	1.41	0.07	0.086	0.016	0.411
ZO1	0.87	0.89	0.89	0.85	0.037	0.749	0.693	0.263
CLDN2	0.54	0.55	0.52	0.58	0.025	0.730	0.114	0.253
CLDN4	0.40	0.41	0.44	0.34	0.045	0.629	0.162	0.102
CLDN7	0.07	0.06	0.08	0.06	0.006	0.347	0.004	0.209
CLDN10	10.49	9.65	10.91	9.36	0.48	0.871	0.002	0.313
CLDN12	1.11	1.08	1.07	0.96	0.06	0.053	0.114	0.359
CLDN16	0.56	0.58	0.34	0.38	0.03	<0.001	0.216	0.550
SLC5A3	1.55	1.48	1.63	1.46	0.14	0.842	0.255	0.640
CYP24	1.73	2.69	7.26	9.40	0.95	<0.001	0.030	0.395
VDR	1.59	2.16	2.33	2.78	0.30	0.004	0.026	0.765
MinPP1	1.32	1.17	1.32	1.30	0.05	0.055	0.025	0.078
CX43	0.30	0.27	0.36	0.31	0.03	0.012	0.058	0.591
P2X7	0.041	0.040	0.047	0.044	0.003	0.108	0.491	0.754
ENPP1	0.70	0.66	0.71	0.55	0.05	0.129	0.010	0.128

*TRPV5, Transient receptor potential cation channel subfamily V member 5; TRPV6, Transient receptor potential cation channel subfamily V member 6; CaBP-28k, Calbindin-D28k; CaBP-9k, Calbindin-D9k; NCX1, sodium-calcium exchanger; TRPM7, Transient receptor potential cation channel, subfamily M, member 7; IAP, Intestinal alkaline phosphatase; MinPP1, Multiple inositol polyphosphate phosphatase 1; SLC5A3/SMIT, Sodium/myo-inositol cotransporter; NaP-IIc, Type II sodium-dependent phosphate transport protein c; PiT1, Inorganic phosphate transporter 1; PiT2, Inorganic phosphate transporter 2; XPR1, Xenotropic and Polytropic Retrovirus Receptor 1; CLDN, claudin; ZO-1, Zonula occludens-1; CYP24, vitamin D 24 hydroxylase; VDR, Vitamin D receptor; P2X7, ATP-gated ion channel 7; ENPP1, Ectonucleotide pyrophosphatase/phosphodiesterase family member 1; CX43/connexin 43, gap junction protein 43.

4 Discussion

The aim of this study was to obtain insight in kinetics of solubilisation, passage, digestion, excretion and retention, as well as the transcellular and paracellular absorption of Ca and P throughout different segments of the digestive tract of growing pigs. The experiment was conducted without major problems. The realized dietary Ca and phytase activity in the diets closely reflected the nutrient contents as planned on forehand. Pigs of the first batch ate and grew very well, while in the second batch 1 pig died and 1 pig received a 1-d medical treatment. The sick and dead pig of the second batch should not affect the power of this study, because each treatment group was designed to replicate 10 times.

4.1 Total tract digestibility of calcium and phosphorus

As shown in Table 5, the ATTD of P was improved by phytase supplementation of the diet, and reduced by the incremental Ca content. These results are in line with many other studies (González-Vega et al., 2016; She et al., 2017; Bournazel et al., 2018; Broomhead et al., 2018). We hypothesised that the negative effect of high Ca would be higher in the phytase-supplemented diet because of the Ca-phytate and Ca-P complexation in the GIT and a direct effect on phytase efficacy. Although this was hypothesised or stated by several other authors (Lei et al., 1994; Selle et al., 2009; Poulsen et al., 2010; Walk, 2016; Wu et al., 2018), results are conflicting and do not allow a firm conclusion. In this study, a significant interaction between phytase and Ca content was observed (Table 5). The ATTD of P was reduced from 29.7 to 23.4% by the incremental Ca content in the phytase-free diet and from 60.1 to 45.8% in the phytase-supplemented diet. The ATTD P content of the diet was reduced by 0.3 and 0.7 g/kg in the phytase-free and phytase-supplemented diets, respectively. Hence, the negative effect of Ca was 2 to 2.5 times bigger in the presence of phytase. The contribution of the phytase supplement to the ATTD P content was 1.48, 1.22 and 1.09 g/kg in the diets with low, medium and high Ca content, respectively. Hence, the efficacy of phytase was Ca dependent and decreased with increasing dietary Ca.

The ATTD of Ca slightly increased with incremental dietary Ca in phytase-free diets and slightly decreased with incremental dietary Ca in phytase-supplemented diets. The latter results indicate a down-regulation of Ca absorption when the Ca supply was increased. The Ca from limestone had a higher digestibility than Ca from feed materials in the phytase-free diet, and presumably a large proportion of Ca was bound with phytate in the feed materials. This explains the increase in ATTD of Ca with incremental Ca from limestone in phytase free diets. Phytase inclusion enhanced the ATTD of Ca. This effect of phytase on ATTD as percentage of dietary Ca decreased with increasing Ca content, but slightly increased when expressed as the contribution of the phytase supplement to the ATTD Ca content, being 0.63, 0.96, and 0.85 g/kg in the diets with low, medium and high Ca content, respectively. This indicates that supplemented Ca from limestone was partly bound by phytate and partly released by inclusion of phytase. The marginal digestibility of Ca from limestone in the first (low to medium) and second (medium to high) supplementation step was 44.7 and 38.5% in phytase-free diets and 54.3 and 35.2% in phytase-supplemented diets.

The incremental Ca content reduced the growth performance (ADG, FCR) of the pigs whereas phytase inclusion had a beneficial effect (Table 3). The interaction between these effects was significant from d10-20 and a tendency in the total period. The results indicate that the inclusion of phytase only improved the growth performance in medium and high calcium diets. The negative impact of incremental Ca was only observed in the phytase-free diets. These results suggest that the negative effect of incremental Ca was mediated by the effect on digestible P. The increase in Ca presumably caused a deficiency in P due to a reduction in P digestibility (see Table 5) and consequently reduced the growth performance. This effect was fully compensated by inclusion of phytase. In phytase-supplemented diets, with higher digestible P content, incremental Ca reduced P-digestibility without

effect on growth performance. This interpretation is supported by results of Gonzalez-Vega et al. (2016) who observed a negative effect of high Ca on growth performance of pigs which could be compensated by an increase in dietary P-content.

4.2 Ca-phytate and Ca-P complexation in the GIT

Microbial phytase activity is pH dependent and may be most active in the proximal compared to distal GIT. The complexation between Ca and phytate has been widely accepted as a cause for Ca hampering phytase activity. The formation of a Ca-phytate complex is pH dependent, as is the efficacy of phytase. A survey covering 17 different types of phytase indicated that the optimal pH for most types of phytase is below 6 (Lei et al., 2013), and the optimal pH for almost all investigated commercial phytases is below 5 (Dersjant-Li et al., 2015). The luminal pH is critical for Ca-phytate complexation, pH 5 is pivotal to Ca-phytate complexation and below 5 the precipitation is minor (Selle et al., 2009). The pH was 3.6, 5.9 and 6.4 in the stomach, proximal-SI and distal-SI, respectively, in the present study (Table 18). Hence the phytase was mostly active in the stomach and presumably inactivated in the distal-SI due to the high luminal pH. In addition, digesta pH was increased with incremental dietary Ca content in the stomach, caeca and proximal-LI, while IP is better soluble at a low compared to high pH. The phytase used in the present study was reported by the manufacturer to have the highest efficacy at a pH of 3.5-4.5 and a gradual decrease in efficacy below and above this range. Thus, the limestone used to increase dietary Ca content, with its high pH buffering capacity, might also inhibit phytase efficacy by increasing digesta pH and reducing IP solubility. The trypsin and pancreas juice secretion may be another reason for the inactivity of microbial phytase in the distal-SI (Dersjant-Li et al., 2015). This assumption was supported by Jongbloed et al. (1992) and Pagano et al. (2007), who reported that no phytase activity could be detected in the ileum digesta, regardless of microbial phytase supplementation. In the present study, the P was absorbed in both the proximal and distal SI, and for the phytase-free diet more P was absorbed in the distal-SI compared to the proximal SI (0.24 vs 0.76, Table 7); while for the phytase-supplemented diet, the P absorption was about equally high in the proximal and distal SI (1.21 vs 0.95, Table 7). The microbial phytase was mostly active in the stomach, and degraded the phytate to release the phytate-P, thus allowing a substantial P absorption in the proximal-SI in the phytase-supplemented diets compared to the minor absorption for the phytase-free diets (1.21 vs 0.24). Subsequently the microbial phytase likely was inactivated in the SI, especially in the distal-SI, therefore the P absorption in the distal-SI was similar between the low and high phytase diet (0.76 vs 0.95).

Previous studies in broilers indicated that a high compared to low dietary Ca content reduced ileal IP degradation accompanied with a reduced mucosal phosphatase activity (Applegate et al., 2003). Impact of dietary Ca content on mucosal phosphatases expression in pigs has not been reported before. To clarify this aspect, we measured the mRNA expression of two endogenous mucosal phosphatases using qPCR technology. The candidate genes were multiple inositol polyphosphate phosphatase 1 (Windhorst et al., 2013) and intestinal alkaline phosphatase (IAP) (Schlemmer et al., 2001). The expression of both phosphatases was not affected by dietary Ca content or phytase supplementation in the jejunal and colonic mucosa. A literature survey indicated that precaecal IP6 degradation upon feeding diets devoid of phytase is remarkably lower in pigs than in broilers, suggesting that endogenous phosphatases can be more active in broilers than pigs (Rodehutscord and Rosenfelder, 2016). This might explain why effects of Ca supplementation were exerted in the aforementioned broiler study (Applegate et al., 2003) but not in the present study.

In contrast to our study, Poulsen et al. (2010) and Létourneau-Montminy et al. (2010) reported no detrimental impact of a high dietary Ca content on phytase efficacy to improve P apparent digestibility. Dietary Ca content was 4 vs. 8 g/kg and 7 vs. 10 g/kg in Poulsen et al. (2010) and Létourneau-Montminy et al. (2010), respectively. Hence the contrast of dietary Ca in these two studies was not as great as that in the present study (2.0 vs. 9.6 g/kg). Besides, Poulsen et al. (2010) used wheat and barley to formulate their basal diet, which resulted in a high intrinsic phytase activity (650 FTU/kg) that might have masked the microbial phytase activity (750 FTU/kg). Wheat and barley were also used in the present study, but these ingredients were preheated at 80° C before diet inclusion to minimise the intrinsic phytase activity, as confirmed by the analysed phytase activity below the

detection limit of the assay in the diets not supplemented with phytase (Table 1). Létourneau-Montminy et al. (2010) used a basal diet containing a higher digestible P content (2.6 g/kg) than our study, which may have reduced intestinal P absorption and underestimated phytase efficacy.

4.3 Ca and P absorption kinetics

Calcium was primarily absorbed in the proximal-SI and might also be absorbed in the LI. The stomach was reported to play a role in Ca and P absorption. It was estimated that approximately 30% and 10-20% of the Ca and P was absorbed in the stomach, respectively (Mesina et al., 2018). However, no Ca absorption was observed in the stomach in the present study. Rather, some endogenous Ca secretion was observed in the stomach probably due to the secretion of gastric juice. On average, the Ca absorption was -0.13, 2.21, -0.09, 0.16, 0.60 and -0.24 g/kg for the stomach, proximal-SI, distal-SI, proximal-LI, distal-LI and until the anus, respectively (Table 9). The proximal-SI was the major site for Ca absorption in the present study, and the negligible Ca absorption in the distal-SI was probably caused by the low solubility of limestone at high pH condition. Interestingly, we also observed substantial Ca absorption in the proximal-LI. Our results were in line with Rutherfurd et al. (2014) but conflicted with Gonzalez-Vega et al. (2014). Rutherfurd et al. (2014) did not observe any Ca absorption from limestone in the stomach, whereas Ca was mostly absorbed in the jejunum with negligible absorption in the ileum; Gonzalez-Vega et al. (2014) reported that Ca from limestone was mostly absorbed in the upper GIT, even before the duodenum, while Ca from *L. calcareum* was mostly absorbed in the jejunum and ileum. It is possible that Ca source might affect the absorption site, which was probably related to the Ca solubility.

The presence of post-ileal Ca and P absorption is controversial and varies substantially among studies. In some studies the apparent ileal digestible (AID) Ca and P was similar to the apparent total tract digestible (ATTD) Ca and P, suggesting that the post-ileal Ca and P absorption was minor (Rutherfurd et al., 2014; Mesina et al., 2018). However, in the present study, a substantial amount of P was secreted to the proximal LI, presumably in the caeca, and then reabsorbed in the distal LI (Table 7). Moreover, the P absorption in distal LI was greater for phytase-free compared to phytase-supplemented diets (0.25 vs. -0.17 g/kg). Nonetheless, the net absorption in the LI based on ileal and total tract digestibility was very small, 0.13 and -0.06 g/kg, in phytase-free and phytase-supplemented diets, respectively. Our results were partly in line with Gonzalez-Vega et al. (2014), who demonstrated that in a phytic acid free diet, the AID P was similar to ATTD of P; while in a phytic acid supplemented diet, about 10-20% of P was absorbed in the LI, which caused the ATTD of P to be significantly higher than the AID of P. In addition, it was noteworthy that 20-30% of the digestible Ca was absorbed in the colon especially for the phytase-free diets (Table 9). Mechanism might be that microbiota degraded phytate and released phytate bound Ca and P, which caused a substantial Ca and minor P absorption in the LI especially for the phytase-free diets. Therefore, the post-ileal Ca absorption seems to be important and might contribute to the body Ca homeostasis. Nevertheless, insight in the expression of the Ca transporters in the LI is needed to confirm the pathways and regulation of post-ileal Ca absorption.

4.4 Calcium and phosphorous transporters in the GIT

4.4.1 Phosphorous transporters and claudins in the GIT

The NaPi-IIb is generally regarded as the major P transporter in the GIT of different species, e.g. rodents, while it was beyond detection limit in both jejunum and colon in the present study. In contrast, we observed that NaPi-IIc mRNA was abundantly expressed in the GIT of pigs in our study, but not affected by dietary treatments. Thus NaPi-IIc might be the major P transporter in the GIT of pigs and might be regulated at posttranscription level. Our results were in line with Wubuli et al. (2019), who reported that NaPi-IIa mRNA was beyond detection limit in the entire GIT, NaPi-IIb was only expressed at a low level in the distal jejunum, ileum, caecum and colon, while NaPi-IIc was abundantly expressed in the entire SI. Hence NaPi-IIc seemed to be the major P transporter in the

GIT of pigs. Expression of NaPi-IIb in the GIT of pigs is still under debate. While expression of NaPi-IIb was detected by qPCR or immunoblotting (Saddoris et al. 2010; Vigors et al., 2014; Saddoris et al. 2019), others could not detect significant levels of NaPi-IIb RNA in duodenum, jejunum and colon of pigs (Just et al., 2018; Oster et al., 2018). None of the determined P transporters (PiT-1, PiT-2 and NaPi-IIc) was influenced by dietary treatment in both jejunum and colon in the present study. However, the xenotropic and polytropic retrovirus receptor 1 (XPR1), which is postulated to be a functional cellular phosphate exporter (Giovannini et al., 2013; Ansermet et al., 2017), was downregulated by high Ca level in the jejunum, suggesting that high Ca had an inhibitory effect on transcellular P transport across the enterocytes. This observation is in line with the reducing effect of Ca on P absorption in this segment as indicated by the lower digestibility of P in this segment.

Paracellular P permeation is less documented but may be modulated to maintain systematic P homeostasis. Previous studies (e.g. Stein et al., 2008; Xue et al., 2017) suggested that P might be primarily absorbed via a paracellular pathway, since absorbed P gradually increases with incremental dietary P intake. Herein we found that soluble inorganic P content was much higher in the GIT lumen than that in the serum (appendix Table 3), which generated a P gradient across epithelium cell and facilitated paracellular P absorption. Paracellular P absorption primarily occurs via pores formed by claudins. However, the specific claudins for P permeation are not identified. A high Ca level reduced the mRNA expression of CLDN10 in jejunum, as well as that of CLDN4 and CLDN10 in colon. The charge specificity of CLDN4 and CLDN10 is inconsistent and under debate. It has been shown that CLDN4 decreases paracellular conductance through a selective decrease in sodium permeability in cultured MDCK cells (Van Itallie et al., 2001). As for CLDN10, several splicing variants of this gene are reported, each likely with different ion selectivity. For example, CLDN10b, abundantly expressed in rat kidney, is involved in paracellular sodium and chloride permeability and in cell cultures, both CLDN10a and CLDN10b can form either anion or cation selective pores (Van Itallie et al., 2006). Either way, the observed down regulation of intestinal CLDN4 and CLDN10 suggests that gut permeability for certain ions was reduced in response to high Ca level. The barrier function of the GIT may have been altered and paracellular P permeation might be modulated by dietary Ca level.

4.4.2 Ca transporters and claudins in the GIT

The modulation of Ca transporters in the GIT is in line with Ca digestibility in our study and previous reports. The molecular mechanism of transcellular Ca absorption is well documented (Diaz de Barboza et al., 2015; Ichai, 2018). Transcellular Ca starts with the passive entry of Ca via apical Ca channels TRPV5 and TRPV6. Once inside the enterocyte, Ca binds to either CaBP-28k or CaBP-9k, freely located in the cytosol, which transports the ions to the opposite basolateral membrane where it is exported into the blood by the action of either the NCX1 or PMCA1. Here, we found that both TRPV5 and TRPV6 expression were downregulated by high Ca and high phytase in jejunum and colon. These results were in line with the reduced Ca digestibility (Table 4) and also reported by González-Vega et al. (2016), showing a linear or quadratic decreased of expression of TRPV6 and CaBP-28k responding to incremental dietary Ca levels.

A high dietary Ca content might enhance Ca absorption via the paracellular pathway. In the present study, the CLDN2 mRNA level was upregulated by high Ca in the jejunum, and CLDN12 mRNA level was upregulated by high phytase in both jejunum and colon. The CLDN2, CLDN12 and CLDN15 may be involved in the Ca permeation in the gut (Alexander et al., 2014). Enhanced expression of CLDN2 and CLDN12 created paracellular cation-selective pores permeable to Ca^{2+} in MDCK I cell line (Yu et al., 2010; Alexander et al., 2014). In addition, the expression level of CLDN2 and CLDN12 was upregulated by vitamin D_3 [1,25$(OH)_2$D] treatment in a Caco-2 cell line (Fujita et al., 2008). The high dietary Ca level and phytase inclusion increased the luminal Ca^{2+} concentration and generated a positive electrochemical gradient across the epithelium in our study (appendix Table 3). Thus paracellular Ca absorption might be small at a low Ca diet while substantial at a high Ca diet. This assumption was in line with the expression of CLDN2 and 12, which were enhanced by an incremental dietary Ca level or phytase in the jejunum and colon (Table 21 and 22). Interestingly, in the phytase-free diets increasing dietary Ca content improved Ca digestibility (Table 4), while it reduced mRNA expression of Ca transporters but enhanced CLDN2 and 12 in the GIT. Thus it seemed that paracellular Ca permeation might partly compensate a reduction in active transcellular Ca absorption with

incremental dietary Ca level. In conclusion, the GIT is actively involved in Ca absorption via downregulation of the Ca transporters (TRPV5, TPRV6 and calbindin-D9k) pathway and upregulating the Ca related claudins (CLDN2 and CLDN12) with incremental dietary Ca.

4.5 Ca and P reabsorption in the kidney

4.5.1 Regulation pattern of Ca transporters in the kidney

In the kidney, primary urine with minerals including Ca and P is produced by filtration. Subsequently, required minerals are re-absorbed by active and passive transport mechanisms. Similar to the GIT, Ca and P reabsorption in the kidney is realised via transporters (transcellular) and CLDN (paracellular). Incremental dietary Ca level reduced renal expression of Ca transporters more in the phytase-supplemented diets. We found that a high compared to low dietary Ca level reduced renal expression of TRPV6, D28k and NCX1 (Table 23), which was in line with the enhanced urinary Ca excretion at a high Ca level (Table 4). A similar result was obtained by Gonzalez-Vega et al. (2016), who reported that incremental dietary Ca content linearly reduced expression of TRPV5, TRPV6, D9k and D28k in the kidney of pigs. The mechanism might be that an incremental dietary Ca content enhanced intestinal Ca absorption (in gram not in percentage, Table 4) and enhanced serum Ca content, which signalled to reduce serum PTH (Table 16). The reduced PTH circulation downregulated renal expression of TRPV5, TPRV6, D28k and NCX1 thus increased urinary Ca excretion. This reducing impact of PTH on Ca transporters might be dependent on $1,25(OH)_2D_3$, because the reduced serum PTH was accompanied with a reduced $1,25(OH)_2D_3$ in the serum and an upregulated renal expression of CYP24, the vitamin D 24 hydroxylase which inactivates vitamin D. In addition, the high serum Ca content could be sensed by CaSR on the basolateral membrane of the kidney, which directly signalled to reduce Ca reabsorption in mice (Lee et al., 2019; Riccardi and Valenti, 2016). Our results confirmed this model in pigs, as expression of CaSR in the kidney was upregulated by a high serum Ca content, accompanied with a reduced renal expression of TRPV6 and CaBP-28k and an enhanced urinary Ca excretion. Phytase inclusion degraded phytate (Table 10 and 11) and improved intestinal Ca absorption (Table 4), hence it strengthened the negative impact of a high dietary Ca on renal expression of Ca transporters. Taken together, our results indicated that a high dietary Ca reduced renal expression of Ca transporters, with a greater effect in the phytase-supplemented diets, which was mediated via PTH and $1,25(OH)_2D_3$ or CaSR in the kidney.

4.5.2 Regulation of claudins in the kidney

The Ca reabsorption via the paracellular pathway in the kidney of pigs might also be modulated in response to dietary Ca level or phytase inclusion. Renal expression of TRPV6, CaBP-28k and NCX1 was accompanied with a similar modulation pattern on CLDN2, 12 and 16 (Table 23). As documented before, CLDN2 and 12 might form pores selectively permeable to Ca. Studies in mice indicated that CLDN16 was abundantly expressed in the distal convoluted tubule and interacted with Ca transporter (e.g. TRPV5) to mediate Ca reabsorption in the kidney (Hou et al., 2013, 2019). Thus the enhanced renal expression of CLDN2, 12 and 16 might suggest an enhanced Ca reabsorption via the paracellular pathway in the present study. We also found that dietary treatments impacted renal expression of CLDN7 and 10. As documented before, physiological function of CLDN7 and 10 is under debate, although alteration of their expression might suggest an enhanced or reduced permeability to certain ions.

4.5.3 Posttranscription regulation of P transporters in the kidney

Incremental dietary Ca content reduced urinary P excretion (Table 5), which was in line with Stein et al. (2011), who also found that a high dietary Ca level reduced urinary P output in pigs. However, renal expression of P transporters was hardly affected by dietary treatments except for PiT-1 (enhanced by dietary Ca level) and XPR1 (reduced by phytase, Table 23). Thus expression of P transporters in the kidney of pigs was not the explanatory mechanism for the observed reduction in urinary P excretion, which might suggest the existence of a posttranscription regulation of these transporters. Several reviews indicated that that NaPi-IIa was responsible for 70-90% of renal P

reabsorption and was primarily regulated at post-transcriptional level, i.e. turnover rate of the proteins and trafficking to the apical membrane via its PDZ binding motif (Biber et al., 2013; Hernando et al., 2020; Levi et al., 2019). It was possible that a high dietary Ca level enhanced the amount of NaPi-IIa protein on the apical membrane of renal epithelium thereby reduced urinary P excretion, although NaPi-IIa mRNA expression was not affected. We did not further test the posttranscription regulation of NaPi-IIa or other P transporters as it was beyond the scope of this study. In conclusion, the variation in urinary excretion between dietary treatments was not mediated by expression of P transporter, hence a posttranscription regulation might exist to modulate expression of P transporters in the kidney of pigs.

4.6 Ca and P retention

The dietary Ca:P ratio plays an important role in Ca and P homeostasis. The utilization of absorbed Ca and P depends on the absolute Ca and P intake, and the ratio between both. In a factorial model to estimate Ca and P-requirements, it was assumed that approximately 98% of the absorbed Ca and P can be retained in a Ca and P balanced diet (Bikker and Blok, 2017). In the present study, the absolute Ca absorption was increased while the absolute P absorption was reduced by the incremental Ca content. Therefore the absorbed P was in excess in the low Ca level, while the absorbed Ca was in excess in the high Ca level. The excess Ca and P was excreted via urine causing waste of P and risk of environmental pollution. In the phytase-free diets, the retention of Ca and P was relatively low and limited by the digestible P supply in the medium and high Ca diets. In the phytase-supplemented diets, digestible Ca supply limited the Ca and P retention at the low Ca diet. The greatest utilization of absorbed Ca and P was observed in phytase-supplemented diet at the medium Ca level (Table 4 and 5), with 97.7 and 93.9% of the absorbed Ca and P retained, respectively. This suggests an optimal ratio between ATTD Ca and P somewhat above 1.27.

5 Conclusions

From the results of this study it was concluded that:

- Incremental dietary Ca reduced the growth performance of the pigs. This effect was negated by inclusion of phytase, indicating that it was likely mediated by a reduction in ATTD-P and a deficiency of digestible P for body gain.
- Incremental dietary Ca reduced the apparent total tract digestibility (ATTD) of P to a larger extent in phytase-supplemented diets than in phytase-free diets.
- The contribution of phytase (500 FTU/kg) to the ATTD-P content decreased with increasing Ca content from 1.5 to 1.1 g/kg.
- The two incremental levels of Ca, added as limestone, had a marginal digestibility of 44.7 and 38.5% without phytase and 54.3 and 35.2% with phytase.
- Calcium absorption was largely realised in the proximal small intestine and the large intestine and very low in the distal small intestine.
- Phosphorus absorption was low in the proximal small intestine and high in the distal small intestine in the phytase-free diets, whereas inclusion of phytase enhanced P absorption in the proximal small intestine.
- Incremental dietary Ca caused a reduction in P absorption in the proximal small intestine but not in the distal small intestine.
- Solubility of Ca and P was high in the stomach and proximal SI and decreased in distal GIT segments, especially for Ca. Phytase substantially enhanced the solubility of P in all GIT segments, but the effect on Ca solubility was not significant.
- Phytate degradation determined in the distal ileum was significantly enhanced by phytase inclusion and reduced by incremental dietary Ca.
- Incremental dietary Ca reduced the expression of the apical calcium channels in the jejunum (TRPV6) and colon (TRPV5 and TRPV6) and enhanced the expression of tight junction protein claudin 2 (CLDN-2) in the jejunum suggesting that Ca absorption switched from primarily active transcellular transport at a low dietary Ca content to primarily passive paracellular transport at a high Ca content.
- Phytase reduced the mRNA expression level of the calcium channels TRPV5 and TRPV6 and enhanced the expression of CLDN-12 in the jejunum and colon.
- The mechanism of the interaction between Ca and microbial phytase may include a direct effect on phytase efficacy, an effect on mucosal phosphatases and a complexing effect on released ortho-phosphate.
- The incremental dietary Ca level increased Ca retention (g/d), decreased the ratio between retained and absorbed Ca and increased P-retention in the phytase-supplemented diet. Phytase inclusion enhanced both Ca and P retention.
- Ash content of the 3rd metacarpal bone was enhanced by both incremental dietary Ca and phytase inclusion, with a minor effect on the Ca/P ratio in bone.
- Incremental dietary Ca reduced the expression of TRPV5, TRPV6, CaBP-28k, NCX1, CLDN12 and CLDN16 in the kidney, with a greater impact onTRPV5, CaBP-28k and NCX1 in phytase-supplemented diets, indicating an important role of these genes in the reduced reabsorption of Ca from urine in the kidney to realise Ca homeostasis.

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Appendix 1 P digestibility in gut segments

Table 1 *Effect of dietary calcium (Ca) content and phytase inclusion on phosphorus (P) digestibility through the gastrointestinal tract in growing pigs, in % of dietary P**

Ca g/kg	Phytase FTU/kg	Stomach	Prox-SI	Distal-SI	Prox-LI	Distal-LI	Total tract
1.8	0	1.0	13.6	25.8[c]	16.4	28.6	29.7[d]
5.3	0	5.2	11.8	25.0[c]	0.68	18.5	27.0[e]
8.8	0	3.8	-0.74	20.9[c]	3.22	18.0	24.7[e]
1.8	500	10.7	43.4	62.9[a]	56.2	66.2	60.1[a]
5.3	500	13.4	36.1	50.9[b]	46.7	55.5	52.0[b]
8.8	500	5.1	23.9	47.7[b]	34.5	47.0	45.8[c]
SEM		3.78	5.37	2.65	4.74	3.31	1.22
Ca							
1.8		5.9	28.5[a]	44.4	36.3[a]	47.4[a]	44.9
5.3		9.1	23.3[a]	37.3	22.5[b]	36.0[b]	38.8
8.8		4.4	11.6[b]	34.3	18.8[b]	32.5[b]	35.8
SEM		2.72	3.85	1.90	3.30	2.37	0.87
Phytase							
0		3.3	8.2	23.9	6.8	21.7	27.2
500		9.6	34.4	53.9	45.7	56.2	52.7
SEM		2.21	3.13	1.54	2.68	1.93	0.72
p-value							
Ca		0.232	<0.001	<0.001	<0.001	<0.001	<0.001
phytase		0.007	<0.001	<0.001	<0.001	<0.001	<0.001
Ca*phytase		0.273	0.717	0.007	0.137	0.157	<0.001

[a-e] Mean values lacking a common letter within a column differ ($P<0.05$).
* SI=small intestine; LI=large intestine; prox=proximal.
Dietary P content was fixed at 4.87 g/kg.

Appendix 2 Ca digestibility in gut segments

Table 2 ` *Effect of dietary calcium (Ca) content and phytase inclusion on Ca digestibility through gastrointestinal tract in growing pigs, in % of dietary Ca.**

Ca g/kg	Phytase FTU/kg	Stomach	Prox-SI	Distal-SI	Prox-LI	Distal-LI	Total tract
1.8	0	-15.9	23.9^{c}	11.9^{d}	24.6^{c}	29.5^{c}	36.9^{e}
5.3	0	-2.9	34.8^{b}	28.9^{c}	23.1^{c}	37.5^{c}	42.0^{d}
8.8	0	0.7	36.1^{b}	28.8^{c}	44.6^{b}	53.6^{b}	40.6^{d}
1.8	500	-14.2	60.1^{a}	64.3^{a}	62.3^{a}	72.9^{a}	71.3^{a}
5.3	500	3.9	51.4^{a}	55.9^{b}	54.2^{ab}	62.4^{b}	60.1^{b}
8.8	500	0.6	40.1^{b}	43.7^{b}	40.9^{b}	55.9^{b}	50.2^{c}
SEM		5.51	5.27	5.06	6.41	4.59	1.88
Ca							
1.8		-15.1^{b}	42.0	38.1	43.4	51.2	54.1
5.3		0.33^{a}	42.7	41.7	37.8	49.3	50.6
8.8		0.66^{a}	38.1	36.2	42.7	54.7	45.4
SEM		3.85	3.67	3.52	4.46	3.29	4.0
Phytase							
0		-6.0	31.6	23.2	30.8	40.2	39.9
500		-3.5	50.5	54.6	52.4	63.8	60.6
SEM		3.13	2.99	2.86	3.62	2.67	3.2
p-value							
Ca		<0.001	0.367	0.428	0.463	0.210	<0.001
Phytase		0.384	<0.001	<0.001	<0.001	<0.001	<0.001
Ca*phytase		0.649	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a-e}$ Mean values lacking a common letter within a column differ (P<0.05).
* SI=small intestine; LI=large intestine; prox=proximal.
Dietary P content was fixed at 4.87 g/kg.

Appendix 3 Soluble inorganic Ca and P in gut segments

Table 3 *Effect of dietary calcium (Ca) content and phytase inclusion on soluble Ca and P content in the serum and digesta through gastrointestinal tract in growing pigs, mmol/l**

Ca g/kg	phytase FTU/kg	Serum	Stomach	Prox-SI	Distal-SI	Prox-LI	Distal-LI
		soluble inorganic Ca					
1.8	0	2.38	6.8	3.9	0.6	5.4	1.3
	500	2.35	6.8	1.6	0.6	2.0	0.5
5.3	0	2.78	23.9	5.3	2.6	7.0	1.7
	500	2.48	21.6	6.0	2.7	9.2	1.8
8.8	0	3.38	37.2	12.5	4.6	7.0	2.6
	500	2.82	36.0	13.6	4.9	9.6	3.0
		soluble inorganic P					
1.8	0	2.90	20.9	20.6	7.4	45.8	18.5
	500	3.23	23.9	19.4	15.1	28.8	12.6
5.3	0	2.19	24.4	12.0	7.2	38.7	16.9
	500	3.09	23.8	17.1	12.5	34.0	13.9
8.8	0	1.85	21.2	12.3	5.9	31.6	15.8
	500	2.63	25.0	15.8	9.7	27.2	11.2

* SI=small intestine; LI=large intestine; prox=proximal.
Dietary P content was 4.87 g/kg in all diets.

To explore the potential of nature to improve the quality of life

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