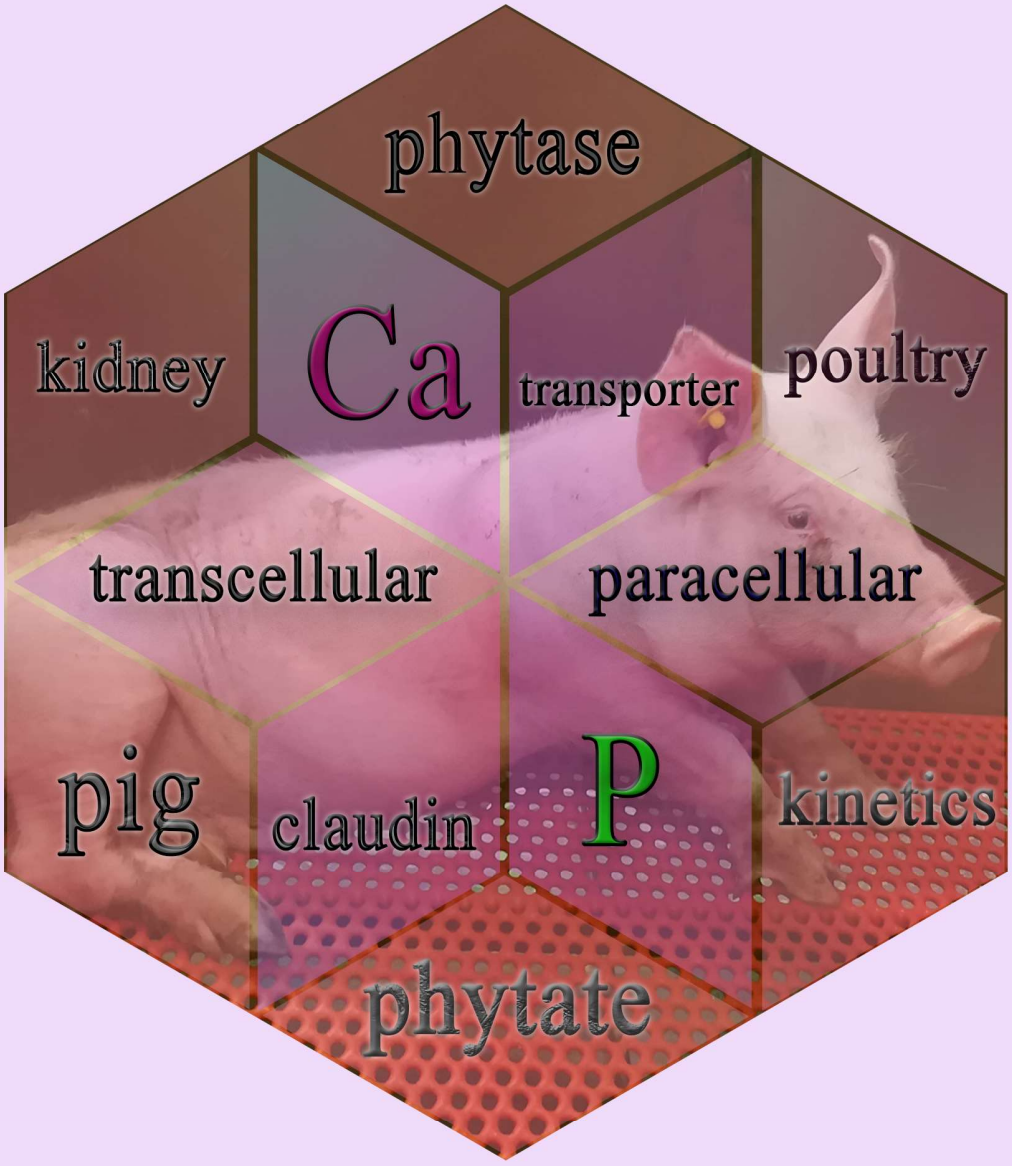
New insights into porcine/poultry calcium/phosphorus metabolism and nutrition



Hu Yixin

Propositions

1. The main difference in Ca and P homeostasis between pigs and chickens lies in the balance between the intestine and kidney.
   (this thesis)

2. Intestinal expression of Ca related transporters is determined by, rather than a determinant for, intestinal Ca absorption in pigs and chickens.
   (this thesis)

3. A corner stone of science lies in ruins, if the conclusion of Baker's study that: "more than 70% of researchers have tried and failed to reproduce another scientist's experiments, and more than half have failed to reproduce their own experiments" (Nature, 2016, 533:452-454), can be reproduced.

4. The use of statistical significance in arguments regarding someone's health is not a comfort.

5. Counterintuitively, it is toilet paper rather than personal protective equipment, food or clean water that offers a sense of safety and control at the start of a pandemic.

6. Writing propositions is like cooking a culinary delight, the appreciation depends on the reviewer's hedonic values.

Proposition belonging to the thesis, entitled

New insights into porcine/poultry calcium/phosphorus metabolism and nutrition

Hu Yixin

Wageningen, 8 September 2021

New insights into porcine/poultry calcium/phosphorus metabolism and nutrition

Hu Yixin

Thesis committee

Promoter

Prof. Dr W.H. Hendriks

Professor of Animal Nutrition

Wageningen University & Research

Co-promoter

Dr P. Bikker

Senior researcher, Wageningen Livestock Research

Wageningen University & Research

Dr J. van Baal

Researcher, Animal Nutrition Group

Wageningen University & Research

The late Dr M.M. van Krimpen

Senior researcher, Wageningen Livestock Research

Wageningen University & Research

Other members

Prof. Dr E.J.M. Feskens, Wageningen University & Research

Dr C.H. Smits, Trouw Nutrition, Amersfoort

Dr A. Narcy, INRAE, Nouzilly, France

Dr E. Delezie, ILVO, Merelbeke, Belgium

This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Science (WIAS).

New insights into porcine/poultry calcium/phosphorus metabolism and nutrition

Hu Yixin

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 8 September 2021
at 4 p.m. in the Aula

Hu Yixin
New insights into porcine/poultry calcium/phosphorus metabolism and nutrition, 221 pages

PhD thesis, Wageningen University, Wageningen, the Netherlands (2021)

With references, with summary in English.

ISBN 978-94-6395-869-1

DOI https://doi.org/10.18174/549277

Table of Contents

CHAPTER 1

General introduction

Background

The global human population has increased rapidly over the past few decades and is predicted to peak at 9.73 billion in 2064 [1]. Together with the increasing wealth and income, the global meat consumption particularly pork and chicken has also been substantially increasing [2]. In animal production, phosphorus (P) is an indispensable dietary macro element for all animal species where it plays an important role in many biological processes, including cell signalling and differentiation, muscle contraction and bone development [3; 4]. In most grains and cereals used in the formulation of compound feeds for pigs and poultry, P is predominantly bound in various forms of inositol phosphate (IP, **Table 1**) such as inositol hexakisphosphate (IP6). Other forms of IP with lower degree of phosphorylation (IP5-IP1) are also found in cereal grains but only at marginal levels [5]. All these IP require enzymatic hydrolysis before P can be absorbed from the intestinal lumen into the enterocytes [6]. In addition, the various IP forms are considered to be antinutritional factors since they can bind dietary cations and

Table 1. Content of total phosphorus (P), total inositol phosphate (IP), IP/P, standardized digestible P (dP) content in pigs and retainable P (rP) content in poultry in commonly used feed ingredients [1]

Ingredients	P	IP	IP/P	dP in pigs	rP in poultry
	g/kg	g/kg	%	%	%
Plant seeds					
Barley	3.1	2.3	74.2	35	38
Lupin	2.9	1.5	51.7	60	49
Maize	2.4	2.1	87.5	27	30
Oat	3.2	2.1	65.6	30	50
Rice	2.6	2.3	88.5	13	16
Rye	3.1	2.0	64.5	30	38
Sorghum	2.7	1.9	70.4	25	30
Soybean	5.0	3.5	70.0	55	41
Sunflower seed	7.7	6.2	80.5	17	27
Triticale	3.4	2.2	64.7	30	NA
Wheat	2.9	1.9	65.5	30	38
By products from plant seeds					
Cotton seed meal	10.7	8.0	74.8	30	30
Maize gluten meal	4.3	3.0	69.8	20	40
Rapeseed meal	10.5	7.9	75.2	28	33
Rice bran meal	16.5	14.9	90.3	13	16
Soybean meal	6.8	4.7	69.1	42	42
Sunflower seed meal	10.3	8.2	79.6	17	27
Wheat bran	9.8	8.3	84.7	18	27
Mineral P					
$NaH_2PO_4 \cdot H_2O$				89	91
$Ca(H_2PO_4)_2 \cdot H_2O$				83	85
$CaHPO_4 \cdot H_2O$				65	55
$CaHPO_4 \cdot 2H_2O$				71	78

[1] Data from CVB [7].
NA=not available.

positively charged proteins, amino acids and polysaccharides [8]. Although non-ruminant animals such as pigs and poultry, secrete endogenous phosphatases into the lumen of the gastrointestinal tract (GIT), the secreted quantities are insufficient to fully degrade IP. To meet the minimal requirement for P of modern-day production animals, mineral P such as monocalcium phosphate (MCP) and dicalcium phosphate (DCP) which are an expensive and finite resource collected from Permian sediments and predicted to be depleted in a few decades [9], is routinely added to the diets. The P not deposited in body tissues is excreted via urine and faeces which contribute to environmental pollution including surface water eutrophication [10]. Due to being an easily collected and as such finite resource as well as the environmental burden and economics of lowering feed costs, improving P absorption and retention in pigs and poultry has been intensively studied.

Phosphorus digestion in the GIT of pigs and poultry

The various forms of IP in cereal grains require cleavage of P from the inositol ring prior to absorption in the GIT. In this context, I regard the enzymatic hydrolysis of dietary IP as a digestion process, since "the enzymes convert the macromolecules into absorbable molecules in a process termed digestion. The products of digestion, as well as secretion from the upper parts of the GIT, are then transported across the epithelium to enter the blood or lymph by a process termed absorption" [11]. Other minerals such as calcium (Ca), which is mostly derived from inorganic sources such as limestone ($CaCO_3$), are solubilized in the GIT and then absorbed by the enterocytes. Thus, in the example of dietary Ca, this mineral is not directly involved in enzymatic digestion in the GIT, although terminologies such as digestible Ca and Ca digestibility are widely used in nutritional studies in pigs (e.g. González-Vega and Stein [12]) and poultry (e.g. Anwar *et al.* [13]). In this thesis, Ca and P digestibility is also used to be consistent with previous publications with the understanding that this terminology is scientifically correct for IP bound P (IP-P) but not for inorganic P or Ca.

The IP-P can be digested in the GIT of pigs and poultry by phytases and phosphatases derived from exogenous microbial phytase added to the diet, intrinsic plant phytases, endogenous mucosal phosphatases and endogenously formed microbial phytases [14]. In the stomach (pigs) and crop (poultry), P digestion is predominantly catalysed by intrinsic plant and exogenous microbial phytases.

Endogenous mucosal phosphatases, including intestinal alkaline phosphatase (IAP) and putative multiple inositol polyphosphate phosphatase (MINPP1) [15; 16], catalyse IP dephosphorylation in the small intestine. In the caeca and colon that harbour an abundant microbial flora, most of the dietary IP6 is degraded by microbial phytases and consequently faecal IP6 recovery is low [17]. Of note is that efficacy of phytase and phosphatases is dependent on luminal pH [6] which gradually increases from the proximal to distal GIT.

Phosphorus is predominantly absorbed in the small intestine via transcellular and paracellular pathways. Recent studies in pigs indicate that the stomach and colon may also contribute to intestinal P absorption. For instance, using T-cannulated pigs, Gonzalez-Vega *et al.* [18] showed that gastric and colonic P absorption could be substantial depending on dietary P sources. Liu *et al.* [19; 20] also indicated that post-ileal P absorption was dependent on dietary P source and body weight of pigs. It is conceivable that the microbiota in the caeca and colon degrade IP [17] and release IP-P, which may increase soluble inorganic P concentration in the GIT lumen and generate a high concentration gradient of soluble inorganic P across enterocytes, thereby, facilitating P absorption in the colon of pigs. Thus, other GIT segments beside the small intestine may also substantially contribute to P absorption, depending on dietary P source. Since most published studies focussed on the cumulative P absorption in the distal ileum of broilers or total tract in pigs, they do not provide full insight into P digestion and absorption along the entire GIT.

In most of the commonly used feed ingredients, P digestibility appears to be higher in broilers than in pigs (Table 1). This finding is in line with the fact that poultry seems to have a greater specific activity of IAP and higher ratio of villus height to crypt depth in the small intestine (**Table 2**), indicating a greater potential to catalyse IP dephosphorylation and larger surface area per unit volume to absorb P compared to pigs. In addition, previous studies showed that IP6 degradation was 7-37% in the duodenum and 10-60% in the ileum, with only one study [21] reaching a value above 40% in the ileum of pigs fed corn and soybean-meal based diets with low phytase activity (**Table 3**). In contrast, broilers fed corn and soybean-meal based diets with low phytase activity had greater IP6 degradation, i.e. 7-9% in the crop, 14-59% in the duodenum and 16-75% in the ileum, with only two studies [22; 23] reporting values below 40% in the ileum (Table 3). Of note is that the stepwise degradation of IP6 may generate IP with lower degree of phosphorylation (e.g. IP3 and IP4) [22], which

also require to be dephosphorylated prior to mucosal P absorption. Furthermore, unlike mammals, the GIT of poultry consists of a crop to store and wet the ingested feed, a proventriculus and gizzard to grind the digesta and function as a real stomach, caeca to ferment, reflux of digesta in various parts of the intestine and a cloaca where urine is secreted [24]. These differences suggest that insights obtained in avian studies cannot be extrapolated to pigs and *vice versa*.

Table 2. Comparison of intestinal morphology and specific activity of alkaline phosphatases in the small intestine of pigs and broilers

Item	Pigs	Broilers
Morphology of small intestine, μm		
Reference	Lee *et al.* [25]	Wu *et al.* [26]
Body weight, kg	18.7 (42 days of age)	0.77 (21 days of age)
Jejunum		
Villus height	274	872
Crypt depth	245	119
Villus height: crypt depth	1.11	7.32
Ileum		
Villus height	254	784
Crypt depth	205	101
Villus height: crypt depth	1.24	7.76
Specific activity of alkaline phosphatases, U/mg of protein		
Reference	Pointillart *et al.* [27]	Iji *et al.* [28]
Body weight, kg	57.7 (132 days of age)	NR (21 days of age)
Duodenum	0.06	1.27
Jejunum	0.19	1.36
Ileum	0.12	0.58

NR=not reported.

Table 3. *Myo*-inositol hexakisphosphate (IP6) degradation in the gastrointestinal tract of pigs and broilers fed diets mainly based on maize and soybean meal with a low phytase activity and free of exogenous microbial phytase inclusion

Species Reference	Dietary Ca g/kg	Dietary P g/kg	Sampling location	IP6 degradation %
Pigs				
Baumgärtel *et al.* [21]	9.1	2.9	Ileum	60
Jongbloed *et al.* [29]	5.2	3.3	Duodenum	22
			Ileum	10
Kemme *et al.* [30]	5.8	5.0	Duodenum	7
			Ileum	27
Kemme *et al.* [31]	5.5	3.0	Ileum	35
Lu *et al.* [32]	6.8	5.4	Duodenum/jejunum	8
			Ileum	26
			Faeces	87
Rapp *et al.* [33]	5.6	4.3	Duodenum	9
			Ileum	19
Rosenfelder-Kuon *et al.* [17]	7.0	3.7	Ileum	18
			Faeces	97
Rutherfurd *et al.* [34]	3.9	4.0	Stomach	37
			Jejunum	38
			Ileum	39
			Faeces	87
Zeng *et al.* [35]	4.1	3.8	Ileum	11
Broilers				
Ajuwon *et al.* [23]	6.8	6.4	Duodenum/jejunum	14
			Ileum	27
Amerah *et al.* [36]	5.1	5.1	Ileum	51
Applegate *et al.* [37]	3.6	3.7	Ileum	75
Li *et al.* [22]	7.0	5.0	Ileum	16
Shastak *et al.* [38]	4.3	3.0	Ileum	62
Siegert *et al.* [39]	9.2	4.1	Ileum	45
Sommerfeld *et al.* [40]	6.5	4.9	Crop	7
			Duodenum/jejunum	35
			Ileum	42
Tamim and Angel [41]	1.8	4.1	Ileum	67
Tamim *et al.* [42]	1.8	4.0	Ileum	69
Zeller *et al.* [43]	7.5	5.2	Crop	9
			Duodenum/jejunum	59
			Ileum	74
			Caeca	91
Zeller *et al.* [44]	6.0	4.4	Duodenum/jejunum	55
			Ileum	67

Approaches to improve P digestion and absorption

Many factors can affect dietary P digestion and absorption, including, but not limiting to, dietary Ca content [45-49], P [40; 50], intrinsic plant phytase activity [51], exogenous microbial phytase supplementation [32; 52-56], vitamin D_3 supply [57; 58]; diet acidification [59-61], diet soaking [62] and processing [63]. Among these, a low dietary Ca supply [45-49] and supplementation with exogenous microbial phytase [32; 52-56] have been shown to effective in improving dietary P digestion and absorption.

Dietary Ca and P interactions

Calcium is the 5th most abundant macro element in the world and plays an important role in many biological processes including bone formation by binding P to form hydroxyapatite ($Ca_5(PO_4)_3(OH)$) [64]. Probably due to its low cost and abundant sources, Ca is generally oversupplied in the diets for pigs and poultry. Moreover, the variation in Ca content of feed materials is not well known. According to a survey based on 795 broiler and pig diets from 2010-2015 [65], the analysed Ca content is on average 2.2 g/kg higher than the formulated Ca content. A high level of dietary Ca can reduce P digestion and absorption probably by generating insoluble Ca-IP and Ca-P complexes [66]. In addition to Ca-IP and Ca-P complexation, Ca may also hamper dietary P digestion by reducing IP solubilisation and inhibiting efficacy of phytase and phosphatases. In particular, IP is better soluble in water at a low pH [8; 67], while limestone ($CaCO_3$), the major source of dietary Ca, may increase luminal pH in the proximal GIT (stomach for pigs and proventriculus and gizzard for poultry) during limestone solubilisation [36]. Furthermore, a high luminal Ca concentration can aggregate IAP, thereby, reducing its efficacy to catalyse IP hydrolysis [68]. For instance, Applegate *et al.* [37] reported that mucosal phosphatase activity in the duodenum and jejunum was 9% greater in broilers fed diets containing 4 compared to 9 g/kg Ca, accompanied with 12% greater IP hydrolysis in the ileum digesta.

Reducing dietary Ca content increases dietary P absorption. However, a too low Ca supply may reduce post-absorptive P metabolism and compromise growth performance in both pigs [69; 70] and broilers [71]. The surplus of absorbed P above the optimal ratio between Ca and P is not retained but excreted via the kidneys in urine [72]. Thus, a certain dietary Ca supply is critical to optimize post-absorptive

P utilization. To lower dietary Ca supply without compromising growth performance and P utilisation, the use of Ca sources of higher digestibility seems to be a promising approach. For example, compared to fine limestone, coarse limestone is less soluble and has been shown to improve Ca digestibility in the distal ileum of broilers [73-75]. In addition, dietary phytase inclusion substantially increased apparent Ca digestibility from 44 to 68% in the presence of coarse but not fine limestone in broilers [76]. Thus, coarse compared to fine limestone seems to increase dietary Ca absorption, thereby, allowing a lower dietary Ca content without compromising growth performance and bone formation.

Use of exogenous microbial phytase

As mentioned above, endogenous mucosal phosphatases in the small intestine of pigs and poultry are insufficient to fully degrade the various forms of dietary IP. The application of microbial phytase, therefore, has been extensively studied and widely practised in the pig and poultry feed industry [77]. A meta-analysis of 88 published experiments in pigs indicated that inclusion of microbial phytase (1000 FTU/kg) enhanced dietary P absorption by 21% unit on average [78]. This estimated improvement of P absorption is largely in line with results of Wang *et al.* [79], who analysed 245 published studies in pigs and reported that dietary microbial phytase inclusion reduced total P excretion by 31% unit. Phytase dosage was not included in the latter meta-analysis model. Efficacy of microbial phytase to improve dietary P absorption, however, appears to be lower in poultry compared to pigs. In particular, a meta-analysis of 103 broiler and 26 layer experiments indicated that a mean dietary inclusion of 1039 FTU microbial phytase per kg improved P retention by 8.6% unit in broilers [80]. This difference in phytase efficacy further supports that broilers may have a greater intrinsic potential to digest various forms of IP and absorb IP-P.

Dietary phytase inclusion may also increase absorption of other minerals (e.g. Ca [81], zinc and copper [82]), and increase the digestibility of amino acids [83] and energy [84] in broilers and pigs. The main mechanism behind this positive impact of microbial phytase may be that the various forms of IP bind cations and positively charged proteins and polysaccharides [8]. Microbial phytase degrades IP, thereby, reducing the binding affinity and improving the absorption of nutrients

that potentially bind to IP. In addition, recent studies in pigs have shown that dietary phytase inclusion reduces endogenous Ca losses [85] and increases P absorption from fish meal [86]. Furthermore, the final end product of IP hydrolysis, *myo*-inositol, with its insulin-mimetic property [87], has been shown to improve growth performance in both pigs [88] and poultry [89]. Thus, microbial phytase may improve growth performance via multiple mechanisms in addition to dietary IP degradation.

Mechanisms of intestinal Ca and P absorption

It is widely acknowledged that Ca and P are absorbed from the GIT lumen via transcellular and paracellular pathways [90]. The transcellular absorption pathway is recognized as an active, ATP-consuming process and is mediated by transporters spanning the apical and basolateral plasma membrane of the enterocytes. Studies in rodents have indicated that transcellular Ca absorption in the GIT begins with apical Ca diffusion into the enterocyte via transient receptor potential cation channel subfamily V member 6 (TRPV6) and to a lesser extent via member 5 (TRPV5), followed by Ca diffusion across cytoplasm by binding calbindin-D9k and -D28k (CaBP-D9k/D28k, in birds exclusively CaBP-D28k), and ends with active Ca exportation via basolateral sodium-Ca exchanger (NCX1/SLC8A1) and plasma membrane Ca-ATPase 1 (PMCA1/ATP2B1) [91]. These transporters may also participate in transcellular Ca absorption in the GIT of pigs [92] and poultry [93] due to their relative high degree in protein sequence homology, implicating functional similarity. Of note is that TRPV6 mRNA expression in the GIT of broilers is below the detection limit [94], and transient receptor potential channel 1 (TRPC1) is speculated to function as a substitute apical Ca channel in layers [95].

The transporters involved in P absorption are less well understood. In rodents, apical P entry into the enterocyte is primarily mediated via sodium-dependent phosphate transporter type IIb (NaPi-IIb/SLC34A2) [96]. Other P transporters such as inorganic phosphate transporter 1 (PiT-1/SLC20A1) and 2 (PiT-2/SLC20A2) are also suggested to play a role in transepithelial P absorption. However, their substantial role in whole body P homeostasis is still under debate. Some researchers argued that these two PiT channels are responsible for extracellular P binding and signalling [97]. Furthermore, basolateral P exportation

is not fully clarified. Xenotropic and polytropic retrovirus receptor 1 (XPR1) is reputed to function as a candidate P exporter on the basolateral membrane [98]. However, its contribution to transepithelial P absorption remains elusive. These P transporters may also play a role in intestinal P absorption in pigs and poultry, but more evidence about their expression as modulated by dietary intervention is needed.

Paracellular Ca and P absorption has been studied to a much lesser extent than transcellular absorption. The Ca and P paracellular absorption in the GIT has been considered to be static and not regulated [99; 100]. Recently some researchers, however, claim that paracellular Ca and P absorption may be rather flexible [101]. Some claudins (CLDN) form channels which are selectively permeable to certain small ions, and their expression level can be modulated by several systemic hormones and regulators [102]. For instance, CLDN-2 and -12 can form pores permeable to Ca, and their expression level is elevated by 1,25-dihydroxycalciferol ($1,25(OH)_2D_3$, the bioactive form of vitamin D_3) treatment in the GIT of mice [103]. CLDN-4 [104] and -7 [105] can form pores permeable for chloride but a barrier to sodium in MDCK (a canine kidney cell line) and LLC-PK1 (a porcine kidney cell line) cell cultures. However, reports about expression and modulation of Ca permeable CLDN in pigs and poultry is scarce. Furthermore, a CLDN that forms a potent channel permeable to P has not been identified yet.

Renal Ca and P reabsorption and excretion

Whole body homeostasis of Ca and P is maintained by regulating their absorption in the GIT, deposition and resorption in bone and excretion via the kidney [106]. The surplus of absorbed Ca or P above the optimal ratio between Ca and P cannot be deposited in bone of broilers and pigs, since hydroxyapatite ($Ca_5(PO_4)_3(OH)$) has a constant Ca to P ratio of approximately 2.1. Thus, surplus of Ca and P is eliminated via the kidney in urine. Urinary Ca and P excretion are under tight control of systemic hormones and regulators such as $1,25(OH)_2D_3$, parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) [107]. It has been reported that a high serum Ca concentration can activate Ca sensing receptor (CaSR) in the parathyroid gland and signal a reduction in PTH expression and secretion; a lower PTH concentration in the serum decreases $1,25(OH)_2D_3$, thereby, reducing renal

Ca reabsorption in pigs [92] and broilers [108]. The kidney, therefore, is generally acknowledged to play a central role in whole body Ca and P homeostasis.

In the mammalian kidney, blood is filtrated in the renal capsule (**Figure 1**) where after the filtrate, which is also called primary urine, flows through the renal tubules. During transport of primary urine through these tubules, most of the Ca and P is reabsorbed and urinary Ca and P excretion is low under normal physiological conditions. A study in rodents has shown that P is predominantly reabsorbed in the proximal convoluted tubules via transcellular and paracellular pathways [109]. Bulk Ca is reabsorbed mainly in the proximal convoluted tubules and thick ascending limb via a paracellular pathway while the fine-tuned Ca reabsorption takes place in the distal and connecting tubule via a transcellular pathway [110]. The transporters involved in renal Ca and P reabsorption are largely similar to those in the GIT, except that apical Ca entry is predominantly mediated via TRPV5 [91] and apical P entry is mostly mediated by NaPi-IIa and NaPi-IIc instead of NaPi-IIb [111]. Of note is that TRPV5 is not identified in birds, thus apical Ca entry is mostly meditated via TRPV6 by the avian kidney. Furthermore, CLDN-16 is expressed in the distal convoluted tubules by murine kidneys and may interact with TRPV5 and mediate paracellular Ca reabsorption [112].

The structure of the avian kidneys differs from that of mammals (Figure 1). There are two types of nephrons in the avian kidney: reptilian-type and mammalian-type. Unlike mammals, 70-90% of the avian nephron is reptilian-type which does not contain a loop of Henle [113]. In addition, the boundary between renal cortex and medulla is not distinct in the avian kidney, since the avian renal cortex contains complete reptilian-type nephrons [114]. These differences indicate that renal Ca and P reabsorption may not be the same between mammals and poultry. Urinary Ca and P excretion in poultry is more difficult to measure because urine is secreted into the cloaca and mixed with undigested components in the excreta [115]. Measuring the expression level of Ca- and P-related transporters and CLDN in the kidney, therefore, seems to be a promising approach to shed more light on the difference in control of Ca and P reabsorption and their excretion via urine in the kidney between pigs and poultry.

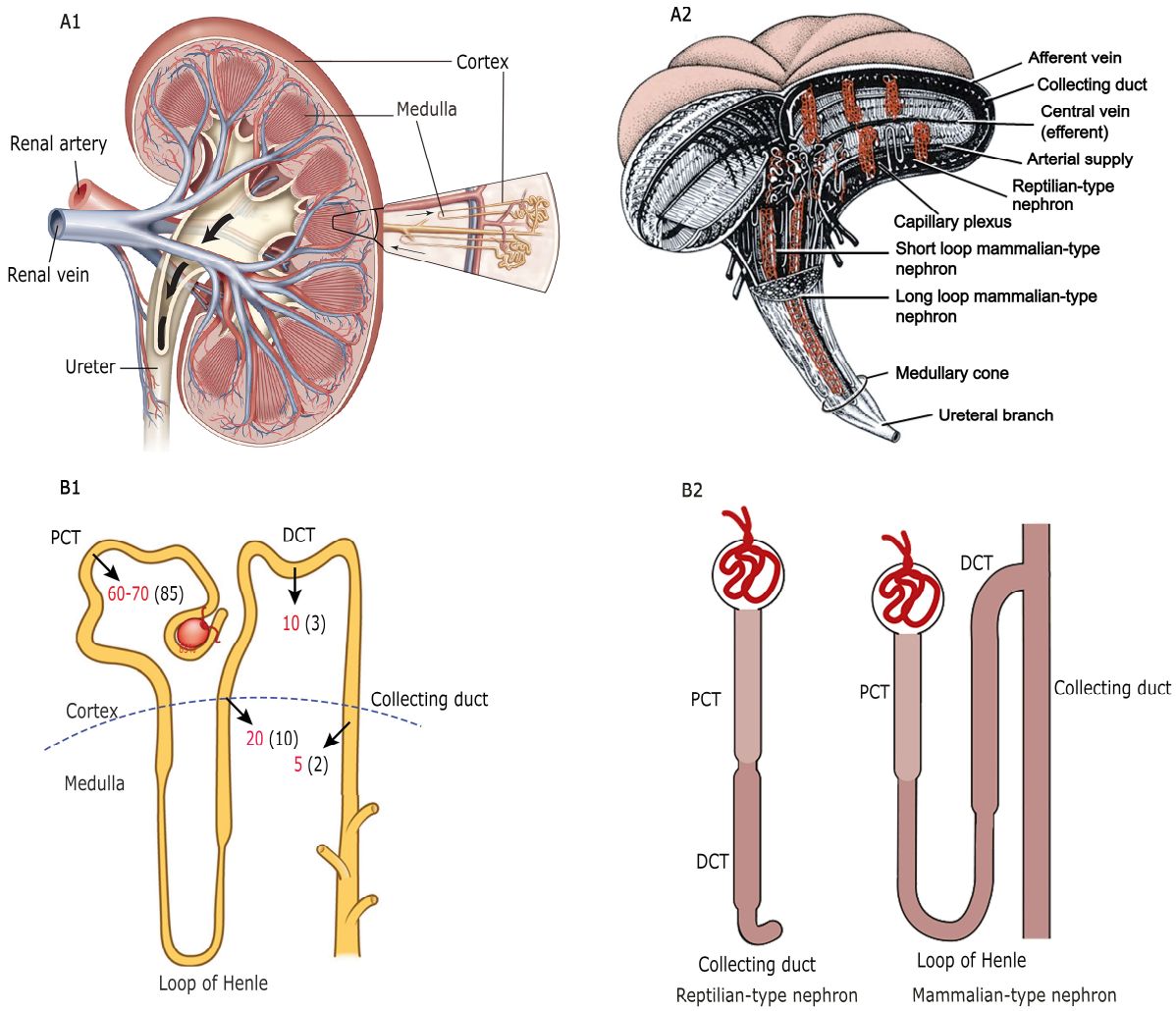


Figure 1. *Schematic illustration of the mammalian kidney (A1, adapted from Rogers [116]) and nephron (B1, adapted from Blaine et al. [117]), and avian kidney (A2, adapted from Sherwood et al. [114]) and nephron (B2, adapted from Romagnani et al. [118]). The outer portion of mammalian kidney, i.e. renal cortex, contains proximal and distal convoluted tubules (PCT and DCT); the innermost part of mammalian kidney, i.e. renal medulla, consists of loop of Henle (A1 and B1). The boundary between renal cortex and medulla is not distinct in the avian kidney, and the renal cortex of avian kidney contains complete nephrons without loop of Henle (A2). The nephrons without loop of Henle, i.e. reptilian-type nephron, are the primary type of nephron in avian kidney (70-90%). The other type of nephron in avian kidney, i.e. mammalian-type nephron, contains a loop of Henle (B2). The percentage of Ca (red number) and P (black number in parentheses) reabsorption (%) in different segments of mammalian nephrons is shown in B1: bulk Ca is reabsorbed mainly in the PCT (60-70%) and thick ascending limb (20%) via a paracellular pathway while the fine-tuned Ca reabsorption takes place in the DCT (10%) via a transcellular pathway; P is predominantly reabsorbed in the PCT (85%) via transcellular and paracellular pathways.*

Formulation of knowledge gaps

Intestinal Ca and P absorption involves several different processes: Ca and P solubilisation, IP degradation, endogenous Ca and P secretion, transcellular and paracellular Ca and P absorption, and more. Most of the previous studies provide end-point values related to the cumulative Ca and P absorption up to the terminal ileum of broilers or total tract of pigs, with or without the correction for basal endogenous Ca and P losses. They, however, do not quantify Ca and P absorption along the GIT. Since IP dephosphorylation is a dynamic process affected by many factors (e.g. dietary Ca level, phytase activity, dietary P sources and diet processing), monitoring Ca and P absorption in different GIT segments may significantly contribute to a better understanding of IP degradation and synchronisation of post-absorptive Ca and P utilisation. Furthermore, although some studies showed that a high dietary Ca content may inhibit microbial phytase efficacy, results are inconsistent, and a firm conclusion cannot be drawn yet. Insight into Ca and phytase interactions in different GIT segments helps to optimize dietary P absorption, save feed cost and reduce P excretion in animal production. In addition, Ca and P are (re)absorbed via transporters and CLDN, which are under tight control of systemic hormones and regulators such as PTH and $1,25(OH)_2D_3$. Most of the previous studies in pigs and poultry, however, did not characterise expression of Ca- and P-related transporters and CLDN. Insight into modulation of Ca and P transporters and CLDN in the GIT and kidneys allows for the development of concepts to further improve intestinal P absorption and utilisation and reduce P renal excretion in pigs and poultry.

Aim and outline of the thesis

The research described in this thesis aimed to provide further insights into the interacting effects between dietary limestone particle size and inclusion level, and microbial phytase inclusion on absorption of Ca and P along the GIT, their deposition in bone and excretion via the kidney, as well as expression of Ca- and P-related transporters and CLDN in the GIT and kidney of pigs and broilers. I first investigated the interacting effects between dietary Ca content and microbial phytase inclusion on Ca and P absorption along the GIT, their deposition in bone and excretion via urine (Chapter 2), as well as expression of Ca- and P-related

transporters and CLDN (Chapter 3) in the jejunal and colonic mucosa in pigs. This was followed by a study in broilers to clarify the impact of limestone particle size and inclusion level in the diet of broilers on Ca and P absorption in different GIT segments, and characteristics of tibia breaking strength (Chapter 4) as well as the expression of Ca- and P-related transporters and CLDN (Chapter 5) in the duodenal and jejunal mucosa. In chapter 6, I investigated the interacting impacts between dietary Ca content and microbial phytase inclusion on Ca and P absorption along the small intestine, characteristics of tibia breaking strength and duodenal expression of Ca- and P-related transporters and CLDN in broilers. I found in a literature survey that broilers may have a greater intrinsic potential to liberate and absorb dietary IP-P than pigs, and microbial phytase having a greater potential to improve P absorption in pigs than broilers. Because of the similar experimental designs used in chapters 2, 4 and 6, these studies provided a basis for comparison of the impact of dietary Ca and microbial phytase intake on Ca and P absorption and excretion between pigs and broilers. Hence, I compared in Chapter 7 the impact of dietary limestone particle size and inclusion level, as well as microbial phytase inclusion on renal expression of Ca- and P-related transporters and CLDN in the kidney of broilers and pigs. Finally, I summarized and discussed the results presented in this thesis in Chapter 8, with special emphasis on the similarities and differences between pigs and poultry as related to Ca and P homeostasis.

References

1. Vollset SE, Goren E, Yuan C-W *et al.* (2020) Fertility, mortality, migration, and population scenarios for 195 countries and territories from 2017 to 2100: a forecasting analysis for the global burden of disease study. *Lancet* **396**, 17-23.

2. Godfray HCJ, Aveyard P, Garnett T *et al.* (2018) Meat consumption, health, and the environment. *Science* **361**, 243.

3. Michigami T & Ozono K (2019) Roles of phosphate in skeleton. *Front Endocrinol* **10**, 180.

4. Vervloet MG, Sezer S, Massy ZA *et al.* (2017) The role of phosphate in kidney disease. *Nat Rev Nephrol* **13**, 27.

5. Rodehutscord M, Ruckert C, Maurer HP *et al.* (2016) Variation in chemical composition and physical characteristics of cereal grains from different genotypes. *Arch Anim Nutr* **70**, 87-107.

6. Dersjant-Li Y, Awati A, Schulze H *et al.* (2015) Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. *J Sci Food Agric* **95**, 878-896.

7. CVB (2018) *Chemische Samenstellingen en Nutritionele Waarden van Voedermiddelen (Chemical Compositions and Nutritional Values of Feed Ingredients).* Wageningen, the Netherlands: Federatie Nederlandse Diervoederketen.

8. Wang R & Guo S (2021) Phytic acid and its interactions: contributions to protein functionality, food processing, and safety. *Compr Rev Food Sci Food Saf* **20**, 2081–2105.

9. Cordell D & White S (2014) Life's bottleneck: sustaining the world's phosphorus for a food secure future. *Annu Rev Environ Resour* **39**, 161-188.

10. Abbasi F, Fakhur-un-Nisa T, Liu J *et al.* (2019) Low digestibility of phytate phosphorus, their impacts on the environment, and phytase opportunity in the poultry industry. *Environ Sci Pollut Res* **26**, 9469–9479.

11. Pandol SJ, Raybould HE & Yee Jr HF (2008) Integrative responses of the gastrointestinal tract and liver to a meal. In *Textbook of Gastroenterology (Fifth Edition),* pp. 3 [Yamada T, editor]. Oxford: Wiley Blackwell Publishing.

12. Gonzalez-Vega JC & Stein HH (2014) - Invited review - Calcium digestibility and metabolism in pigs. *Asian-Australas J Anim Sci* **27**, 1-9.

13. Anwar MN, Ravindran V, Morel PCH *et al.* (2018) Measurement of the true ileal calcium digestibility of some feed ingredients for broiler chickens. *Anim Feed Sci Technol* **237**, 118-128.

14. Rodehutscord M & Rosenfelder P (2016) Chapter 1 Update on phytate degradation pattern in the gastrointestinal tract of pigs and broiler chickens. In *Phytate Destruction-Consequences for Precision Animal Nutrition*, pp. 15-28 [Walk CL, Kühn I, Stein HH, Kidd MT and Rodehutscord M, editors]. Wageningen, the Netherlands: Wageningen Academic Publishers.

15. Cho J, Choi K, Darden T *et al.* (2006) Avian multiple inositol polyphosphate phosphatase is an active phytase that can be engineered to help ameliorate the planet's "phosphate crisis". *J Biotechnol* **126**, 248-259.

16. Briviba K, Schollenberger M, Rodehutscord M *et al*. (2018) Dephosphorylation of myo-inositol phosphates in the *in vitro* intestinal Caco-2 cell model. *Int J Food Sci Nutr* **69**, 46-51.

17. Rosenfelder-Kuon P, Klein N, Zegowitz B *et al.* (2020) Phytate degradation cascade in pigs as affected by phytase supplementation and rapeseed cake inclusion in corn-soybean meal-based diets. *J Anim Sci* **98**, 1-12.

18. Gonzalez-Vega JC, Walk CL, Liu Y *et al.* (2014) The site of net absorption of Ca from the intestinal tract of growing pigs and effect of phytic acid, Ca level and Ca source on Ca digestibility. *Arch Anim Nutr* **68**, 126-142.

19. Liu JB, Shen XY, Zhai HX *et al.* (2017) Dietary sources of phosphorus affect postileal phosphorus digestion in growing pigs. *J Anim Sci* **95**, 4490-4498.

20. Liu JB, Yan HL, Cao SC *et al.* (2018) Effect of feed intake level on the determination of apparent and standardized total tract digestibility of phosphorus for growing pigs. *Anim Feed Sci Technol* **246**, 137-143.

21. Baumgärtel T, Metzler BU, Mosenthin R *et al.* (2008) Precaecal and postileal metabolism of P, Ca and N in pigs as affected by different carbohydrate sources fed at low level of P intake. *Arch Anim Nutr* **62**, 169-181.

22. Li W, Angel R, Kim S-W *et al.* (2017) Impacts of dietary calcium, phytate, and phytase on inositol hexakisphosphate degradation and inositol phosphate release in different segments of digestive tract of broilers. *Poult Sci* **96**, 3626-3637.

23. Ajuwon KM, Sommerfeld V, Paul V *et al.* (2020) Phytase dosing affects phytate degradation and Muc2 transporter gene expression in broiler starters. *Poult Sci* **99**, 981-991.

24. Svihus B (2014) Function of the digestive system. *J Appl Poult Res* **23**, 306-314.

25. Lee DN, Liu SR, Chen YT *et al.* (2007) Effects of diets supplemented with organic acids and nucleotides on growth, immune responses and digestive tract development in weaned pigs. *J Anim Physiol Anim Nutr (Berl)* **91**, 508-518.

26. Wu YB, Ravindran V, Thomas DG *et al.* (2004) Influence of phytase and xylanase, individually or in combination, on performance, apparent metabolisable energy, digestive tract measurements and gut morphology in broilers fed wheat-based diets containing adequate level of phosphorus. *Br Poult Sci* **45**, 76-84.

27. Pointillart A, Fourdin A & Fontaine N (1987) Importance of cereal phytase activity for phytate phosphorus utilization by growing pigs fed diets containing triticale or corn. *J Nutr* **117**, 907-913.

28. Iji PA, Saki A & Tivey DR (2001) Body and intestinal growth of broiler chicks on a commercial starter diet. 2. Development and characteristics of intestinal enzymes. *Br Poult Sci* **42**, 514-522.

29. Jongbloed AW, Mroz Z & Kemme PA (1992) The effect of supplementary *Aspergillus niger* phytase in diets for pigs on concentration and apparent digestibility of dry matter, total phosphorus, and phytic acid in different sections of the alimentary tract. *J Anim Sci* **70**, 1159-1168.

30. Kemme PA, Schlemmer U, Mroz Z *et al.* (2006) Monitoring the stepwise phytate degradation in the upper gastrointestinal tract of pigs. *J Sci Food Agric* **86**, 612-622.

31. Kemme PA, Jongbloed AW, Mroz Z *et al.* (1999) Digestibility of nutrients in growing–finishing pigs is affected by *Aspergillus niger* phytase, phytate and lactic acid levels 2. Apparent total tract digestibility of phosphorus, calcium and magnesium and ileal degradation of phytic acid. *Livest Prod Sci* **58**, 119-127.

32. Lu H, Shin S, Kuehn I *et al.* (2020) Effect of phytase on nutrient digestibility and expression of intestinal tight junction and nutrient transporter genes in pigs. *J Anim Sci* **98**, 1-12.

33. Rapp C, Lantzsch HJ & Drochner W (2001) Hydrolysis of phytic acid by intrinsic plant or supplemented microbial phytase (*Aspergillus niger*) in the stomach and small intestine of minipigs fitted with re-entrant cannulas. *J Anim Physiol Anim Nutr (Berl)* **85**, 406-413.

34. Rutherfurd SM, Chung TK & Moughan PJ (2014) Effect of microbial phytase on phytate P degradation and apparent digestibility of total P and Ca throughout the gastrointestinal tract of the growing pig. *J Anim Sci* **92**, 189-197.

35. Zeng Z, Wang D, Piao X *et al.* (2014) Effects of adding super dose phytase to the phosphorus-deficient diets of young pigs on growth performance, bone quality, minerals and amino acids digestibilities. *Asian Australas J Anim Sci* **27**, 237.

36. Amerah A, Plumstead P, Barnard L *et al.* (2014) Effect of calcium level and phytase addition on ileal phytate degradation and amino acid digestibility of broilers fed corn-based diets. *Poult Sci* **93**, 906-915.

37. Applegate TJ, Angel R & Classen HL (2003) Effect of dietary calcium, 25-hydroxycholecalciferol, or bird strain on small intestinal phytase activity in broiler chickens. *Poult Sci* **82**, 1140-1148.

38. Shastak Y, Zeller E, Witzig M *et al.* (2014) Effects of the composition of the basal diet on the evaluation of mineral phosphorus sources and interactions with phytate hydrolysis in broilers. *Poult Sci* **93**, 2548-2559.

39. Siegert W, Zuber T, Sommerfeld V *et al.* (2019) Prececal amino acid digestibility and phytate degradation in broiler chickens when using different oilseed meals, phytase and protease supplements in the feed. *Poult Sci* **98**, 5700-5713.

40. Sommerfeld V, Van Kessel AG, Classen HL *et al.* (2019) Phytate degradation in gnotobiotic broiler chickens and effects of dietary supplements of phosphorus, calcium, and phytase. *Poult Sci* **98**, 5562-5570.

41. Tamim NM & Angel R (2003) Phytate phosphorus hydrolysis as influenced by dietary calcium and micro-mineral source in broiler diets. *J Agric Food Chem* **51**, 4687-4693.

42. Tamim NM, Angel R & Christman M (2004) Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. *Poult Sci* **83**, 1358-1367.

43. Zeller E, Schollenberger M, Kühn I *et al.* (2015) Hydrolysis of phytate and formation of inositol phosphate isomers without or with supplmtented phytases in different segments of the digestive tract of broilers. *J Nutr Sci* **4**, 1-12.

44. Zeller E, Schollenberger M, Witzig M *et al.* (2015) Interactions between supplemented mineral phosphorus and phytase on phytate hydrolysis and inositol phosphates in the small intestine of broilers. *Poult Sci* **94**, 1018-1029.

45. Schlegel P & Gutzwiller A (2020) Dietary calcium to digestible phosphorus ratio for optimal growth performance and bone mineralization in growing and finishing pigs. *Animals* **10**, 178.

46. Mansilla WD, Franco-Rossello R, Torres CA *et al.* (2020) The effect of reducing dietary calcium in prestarter diets (0-4 D) on growth performance of broiler chickens, tibia characteristics, and calcium and phosphorus concentration in blood. *Poult Sci* **99**, 4904-4913.

47. Misiura MM, Filipe JAN, Walk CL *et al.* (2018) Do not neglect calcium: a systematic review and meta-analysis (meta-regression) of its digestibility and utilisation in growing and finishing pigs. *Bri J Nutr* **119**, 1207-1219.

48. Lee SA, Lagos LV, Bedford MR *et al.* (2020) Increasing calcium from deficient to adequate concentration in diets for gestating sows decreases digestibility of phosphorus and reduces serum concentration of a bone resorption biomarker. *J Anim Sci* **98**, skaa076.

49. Zanu HK, Kheravii SK, Morgan NK *et al.* (2020) Interactive effect of 2 dietary calcium and phytase levels on broilers challenged with subclinical necrotic enteritis: part 1 - broiler performance, gut lesions and pH, bacterial counts, and apparent ileal digestibility. *Poult Sci* **99**, 4861-4873.

50. Omara, II, Mou CT, Persia ME *et al.* (2020) Effects of available phosphorus source and concentration on performance and expression of sodium phosphate type IIb cotransporter, vitamin D-1α-hydroxylase, and vitamin D-24-hydroxylase mRNA in broiler chicks. *Poult Sci* **99**, 1822-1831.

51. Morgan N, Walk C, Bedford M *et al.* (2015) Contribution of intestinal- and cereal-derived phytase activity on phytate degradation in young broilers. *Poult Sci* **94**, 1577-1583.

52. Zanu HK, Kheravii SK & Morgan NK *et al.* (2020) Interactive effect of dietary calcium and phytase on broilers challenged with subclinical necrotic enteritis: 3. Serum calcium and phosphorus, and bone mineralization. *Poult Sci* **99**, 3617-3627.

53. Bassi LS, Teixeira LV, Sens RF *et al.* (2021) High doses of phytase on growth performance, bone mineralization, diet utilization, and plasmatic myo-inositol of turkey poults. *Poult Sci* **100**, 101050.

54. Kriseldi R, Johnson JA, Walk CL *et al.* (2021) Influence of exogenous phytase supplementation on phytate degradation, plasma inositol, alkaline phosphatase, and glucose concentrations of broilers at 28 days of age. *Poult Sci* **100**, 224-234.

55. Wang J & Kim WK (2021) Evaluation of a novel corn-expressed phytase on growth performance and bone mineralization in broilers fed different levels of dietary nonphytate phosphorus. *J Appl Poult Res* **30**, 100120.

56. Lee J, Kim JW & Nyachoti CM (2021) Standardized total tract digestibility of phosphorus in high-protein sunflower meal fed to growing pigs with or without phytase supplementation. *Anim Feed Sci Technol* **274**, 114853.

57. Adhikari R, White D, House JD *et al.* (2019) Effects of additional dosage of vitamin D_3, vitamin D_2, and 25-hydroxyvitamin D_3 on calcium and phosphorus utilization, egg quality and bone mineralization in laying hens. *Poult Sci* **99**, 364-373.

58. Aye Cho T-Z, Sadiq MB, Srichana P *et al.* (2020) Vitamin D_3 enhanced intestinal phosphate cotransporter genes in young and growing broilers. *Poult Sci* **99**, 2041-2047.

59. Votterl J, Klinsoda J, Zebeli Q *et al.* (2020) Dietary phytase and lactic acid-treated cerealgrains differently affected calcium and phosphorus homeostasis from intestinal uptake to systemic metabolism in a pig model. *Nutrients* **12**, 1542.

60. Kristoffersen S, Itani K, Benzertiha A *et al.* (2021) Effect of crop retention time and acidification of the feed on phytase efficacy in broiler chickens. *Br Poult Sci* **62**, 443-451.

61. Vieira BS, Silva FG, Oliveira CFS *et al.* (2017) Does citric acid improve performance and bone mineralization of broilers when combined with phytase? A systematic review and meta-analysis. *Anim Feed Sci Technol* **232**, 21-30.

62. Vötterl JC, Zebeli Q, Hennig-Pauka I *et al.* (2019) Soaking in lactic acid lowers the phytate-phosphorus content and increases the resistant starch in wheat and corn grains. *Anim Feed Sci Technol* **252**, 115-125.

63. Zeller E, Schollenberger M, Kühn I *et al.* (2015) Effect of diets containing enzyme supplements and microwave-treated or untreated wheat on inositol phosphates in the small intestine of broilers. *Anim Feed Sci Technol* **204**, 42-51.

64. Cormick G & Belizán JM (2019) Calcium intake and health. *Nutrients* **11**, 1606.

65. Walk C (2016) The influence of calcium on phytase efficacy in non-ruminant animals. *Anim Prod Sci* **56**, 1345-1349.

66. Selle PH, Cowieson AJ & Ravindran V (2009) Consequences of calcium interactions with phytate and phytase for poultry and pigs. *Livest Sci* **124**, 126-141.

67. Romano N & Kumar V (2018) Phytase in animal feed. In *Enzymes in Human and Animal Nutrition*, pp. 73-88 [Nunes CS & Kumar V, editors]. Cambridge: Academic Press.

68. Brun LR, Traverso A & Rigalli A (2009) Aggregation and inhibition of rat intestinal alkaline phosphatase by high concentrations of calcium. Reversibility of the processes. *J Enzyme Inhib Med Chem* **24**, 691-696.

69. Lagos LV, Lee SA, Fondevila G *et al.* (2019) Influence of the concentration of dietary digestible calcium on growth performance, bone mineralization, plasma calcium, and abundance of genes involved in intestinal absorption of calcium in pigs from 11 to 22 kg fed diets with different concentrations of digestible phosphorus. *J Anim Sci Biotechnol* **10**, 47.

70. Lagos LV, Walk CL, Murphy MR *et al.* (2019) Effects of dietary digestible calcium on growth performance and bone ash concentration in 50-to 85-kg growing pigs fed diets with different concentrations of digestible phosphorus. *Anim Feed Sci Technol* **247**, 262-272.

71. Li T, Xing G, Shao Y *et al.* (2020) Dietary calcium or phosphorus deficiency impairs the bone development by regulating related calcium or phosphorus metabolic utilization parameters of broilers. *Poult Sci* **99**, 3207-3214.

72. Stein HH, Kadzere CT, Kim SW *et al.* (2008) Influence of dietary phosphorus concentration on the digestibility of phosphorus in monocalcium phosphate by growing pigs. *J Anim Sci* **86**, 1861-1867.

73. Kim SW, Li W, Angel R *et al.* (2019) Modification of a limestone solubility method and potential to correlate with *in vivo* limestone calcium digestibility. *Poult Sci* **98**, 6837-6848.

74. Anwar MN, Ravindran V, Morel PC *et al.* (2016) Effect of limestone particle size and calcium to non-phytate phosphorus ratio on true ileal calcium digestibility of limestone for broiler chickens. *Br Poult Sci* **57**, 707-713.

75. Anwar MN, Ravindran V, Morel PC *et al.* (2017) Effect of calcium source and particle size on the true ileal digestibility and total tract retention of calcium in broiler chickens. *Anim Feed Sci Technol* **224**, 39-45.

76. Majeed S, Qudsieh R, Edens FW *et al.* (2020) Limestone particle size, calcium and phosphorus levels, and phytase effects on live performance and nutrients digestibility of broilers. *Poult Sci* **99**, 1502-1514.

77. Lei XG, Weaver JD, Mullaney E *et al.* (2013) Phytase, a new life for an "old" enzyme. *Annu Rev Anim Biosci* **1**, 283-309.

78. Rosenfelder-Kuon P, Siegert W & Rodehutscord M (2020) Effect of microbial phytase supplementation on P digestibility in pigs: a meta-analysis. *Arch Anim Nutr* **74**, 1-18.

79. Wang H, Long W, Chadwick D *et al.* (2020) Can dietary manipulations improve the productivity of pigs with lower environmental and economic cost? a global meta-analysis. *Agric Ecosyst Environ* **289**, 106748.

80. Bougouin A, Appuhamy JA, Kebreab E *et al.* (2014) Effects of phytase supplementation on phosphorus retention in broilers and layers: a meta-analysis. *Poult Sci* **93**, 1981-1992.

81. Li W, Angel R, Plumstead PW *et al.* (2020) Effects of limestone particle size, phytate, calcium source, and phytase on standardized ileal calcium and phosphorus digestibility in broilers. *Poult Sci* **100**, 900-909.

82. Bikker P, Jongbloed AW & Thissen JTNM (2012) Meta-analysis of effects of microbial phytase on digestibility and bioavailability of copper and zinc in growing pigs. *J Anim Sci* **90**, 134-136.

83. Zouaoui M, Létourneau-Montminy MP & Guay F (2018) Effect of phytase on amino acid digestibility in pig: a meta-analysis. *Anim Feed Sci Technol* **238**, 18-28.

84. Dersjant-Li Y & Dusel G (2019) Increasing the dosing of a *Buttiauxella* phytase improves phytate degradation, mineral, energy, and amino acid digestibility in weaned pigs fed a complex diet based on wheat, corn, soybean meal, barley, and rapeseed meal. *J Anim Sci* **97**, 2524-2533.

85. Lee SA, Stein HH, Lagos LV *et al.* (2019) Basal endogenous loss, standardized total tract digestibility of calcium in calcium carbonate, and retention of calcium in gestating sows change during gestation, but microbial phytase reduces basal endogenous loss of calcium. *J Anim Sci* **97**, 1712–1721

86. Gonzalez-Vega JC, Walk CL & Stein HH (2015) Effect of phytate, microbial phytase, fiber, and soybean oil on calculated values for apparent and standardized total tract digestibility of calcium and apparent total tract digestibility of phosphorus in fish meal fed to growing pigs. *J Anim Sci* **93**, 4808-4818.

87. Croze ML & Soulage CO (2013) Potential role and therapeutic interests of myo-inositol in metabolic diseases. *Biochimie* **95**, 1811-1827.

88. Lu H, Cowieson AJ, Wilson JW *et al.* (2019) Extra-phosphoric effects of super dosing phytase on growth performance of pigs is not solely due to release of myo-inositol. *J Anim Sci* **97**, 3898-3906.

89. Lee SA & Bedford MR (2016) Inositol - An effective growth promotor? *Worlds Poult Sci* **72**, 743-760.

90. Hernando N & Wagner CA (2018) Mechanisms and regulation of intestinal phosphate absorption. *Compr Physiol* **8**, 1065-1090.

91. Christakos S, Mady LJ & Dhawan P (2018) Calbindin-D_{28K} and Calbindin-D_{9K} and the epithelial calcium channels TRPV5 and TRPV6. In *Vitamin D (Fourth Edition)*, pp. 343-359 [Feldman D, editor]. Cambridge: Academic Press.

92. Schröder B & Breves G (2006) Mechanisms and regulation of calcium absorption from the gastrointestinal tract in pigs and ruminants: comparative aspects with special emphasis on hypocalcemia in dairy cows. *Anim Health Res Rev* **7**, 31-41.

93. Proszkowiec-Weglarz M & Angel R (2013) Calcium and phosphorus metabolism in broilers: effect of homeostatic mechanism on calcium and phosphorus digestibility. *J Appl Poult Res* **22**, 609-627.

94. Proszkowiec-Weglarz M, Schreier LL, Miska KB *et al.* (2018) Effect of early neonatal development and delayed feeding post-hatch on jejunal and ileal calcium and phosphorus transporter genes expression in broiler chickens. *Poult Sci* **98**, 1861-1871.

95. Gloux A, Le Roy N, Brionne A *et al.* (2019) Candidate genes of the transcellular and paracellular calcium absorption pathways in the small intestine of laying hens. *Poult Sci* **98**, 6005-6018.

96. Michigami T, Kawai M, Yamazaki M *et al.* (2018) Phosphate as a signaling molecule and its sensing mechanism. *Physiol Rev* **98**, 2317-2348.

97. Bon N, Couasnay G, Bourgine A *et al.* (2018) Phosphate (P_i)-regulated heterodimerization of the high-affinity sodium-dependent P_i transporters PiT1/Slc20a1 and PiT2/Slc20a2 underlies extracellular P_i sensing independently of P_i uptake. *J Biol Chem* **293**, 2102-2114.

98. Legati A, Giovannini D, Nicolas G *et al.* (2015) Mutations in XPR1 cause primary familial brain calcification associated with altered phosphate export. *Nat Genet* **47**, 579-581.

99. Hernando N, Pastor-Arroyo EM, Marks J *et al.* (2020) 1,25$(OH)_2$ vitamin D_3 stimulates active phosphate transport but not paracellular phosphate absorption in mouse intestine. *J Physiol* **599**, 1131-1151.

100. Bronner F (2009) Recent developments in intestinal calcium absorption. *Nutr Rev* **67**, 109-113.

101. Hashimoto N, Matsui I, Ishizuka S *et al.* (2020) Lithocholic acid increases intestinal phosphate and calcium absorption in a vitamin D receptor dependent but transcellular pathway independent manner. *Kidney Int* **97**, 1164–1180.

102. Otani T & Furuse M (2020) Tight junction structure and function revisited. *Trends Cell Biol* **30**, 805-817.

103. Fujita H, Sugimoto K, Inatomi S *et al.* (2008) Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca^{2+} absorption between enterocytes. *Mol Biol Cell* **19**, 1912-1921.

104. Van Itallie C, Rahner C & Anderson JM (2001) Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. *J Clin Invest* **107**, 1319-1327.

105. Alexandre MD, Jeansonne BG, Renegar RH *et al.* (2007) The first extracellular domain of claudin-7 affects paracellular Cl^- permeability. *Biochem Biophys Res Commun* **357**, 87-91.

106. Fukumoto S, Takashi Y, Tsoumpra MK *et al.* (2020) How do we sense phosphate to regulate serum phosphate level? *J Bone Miner Res* **38**, 1-6.

107. Torres PAU & De Brauwere DP (2011) Three feedback loops precisely regulating serum phosphate concentration. *Kidney Int* **80**, 443-445.

108. Li X, Zhang D, Yang TY *et al.* (2016) Phosphorus bioavailability: a key aspect for conserving this critical animal feed resource with reference to broiler nutrition. *Agriculture* **6**, 25.

109. Wagner CA, Rubio-Aliaga I & Hernando N (2019) Renal phosphate handling and inherited disorders of phosphate reabsorption: an update. *Pediatr Nephrol* **34**, 549-559.

110. Moor MB & Bonny O (2016) Ways of calcium reabsorption in the kidney. *Am J Physiol Renal Physiol* **310**, F1337-F1350.

111. Hernando N, Gagnon KB & Lederer ED (2020) Phosphate transport in epithelial and nonepithelial tissue. *Physiol Rev* **101**, 1–35.

112. Hou J, Renigunta V, Nie M *et al.* (2019) Phosphorylated claudin-16 interacts with Trpv5 and regulates transcellular calcium transport in the kidney. *Proc Natl Acad Sci U S A* **116**, 19176-19186.

113. Goldstein DL & Braun EJ (1989) Structure and concentrating ability in the avian kidney. *Am J Physiol* **256**, R501-R509.

114. Sherwood L, Klandorf H & Yancey PH (2012) Excretory systems sections. In *Animal physiology: From Genes to Organisms*, pp. 557-610 [Alexander S, editor]. CA: Thomson Brooks Cole.

115. Manangi MK, Clark FD & Coon CN (2007) Improved colostomy technique and excrement (urine) collection device for broilers and broiler breeder hens. *Poult Sci* **86**, 698-704.

116. Rogers K (2011) Chapter 1: Anatomy of the kidneys and renal system. In *The Kidneys and the Renal System*, pp. 9 [Rogers K, editor]. NY: Britannica Educational Publishing.

117. Blaine J, Chonchol M & Levi M (2015) Renal control of calcium, phosphate, and magnesium homeostasis. *Clin J Am Soc Nephrol* **10**, 1257-1272.

118. Romagnani P, Lasagni L & Remuzzi G (2013) Renal progenitors: an evolutionary conserved strategy for kidney regeneration. *Nat Rev Nephrol* **9**, 137-146.

CHAPTER 2

Dietary microbial phytase supplementation enhances the impact of dietary Ca content on P absorption and retention in growing pigs

Y.X. Hu[*†], W.H. Hendriks[†], J. van Baal[†], J.W. Resink[‡], M. Rodehutscord[§], M.M. van Krimpen[*], P. Bikker[*]

[*] Wageningen University & Research, Wageningen Livestock Research, 6700 AH Wageningen, the Netherlands

[†] Wageningen University & Research, Animal Nutrition Group, 6700 AH Wageningen, the Netherlands

[‡] Trouw Nutrition Research and Development, Stationsstraat 77, 3811 MH Amersfoort, the Netherlands

[§] University of Hohenheim, Institute of Animal Science, 70599 Stuttgart, Germany

Submitted to British Journal of Nutrition

Abstract

Sixty growing male pigs were used to test the hypothesis that high dietary Ca content reduces P absorption to a greater extent in microbial phytase-supplemented diets via hampering inositol phosphate (IP) degradation and precipitation of P. Pigs were equally allotted over three Ca content (2.0, 5.8 and 9.6 g/kg) diets supplementation either with or without microbial phytase (0 vs. 500 FTU/kg) in a 2×3 factorial arrangement. Faeces and urine were collected at the end of the 21-day experimental period. At dissection, pigs were euthanized and digesta quantitatively collected from different gastrointestinal tract (GIT) segments. Incremental dietary Ca content reduced apparent P digestibility in all GIT segments posterior to the stomach (P<0.001). Moreover, this negative effect on apparent P digestibility was greater in phytase-supplemented diets in the distal small intestine (P=0.007). Degradation of IP6 in the distal small intestine increased with phytase supplementation (P<0.001) but decreased with dietary Ca content (P=0.014). In addition, the proportion of IP esters in total IP (∑IP) indicated that IP6/∑IP was increased while IP4/∑IP and IP3/∑IP were reduced with incremental dietary Ca content, also with a greater impact in phytase-supplemented diets (P=0.025, 0.018 and 0.009, respectively). In all GIT segments, P solubility was increased with phytase (P<0.001) and tended to be reduced with dietary Ca content (P<0.096). Measurements in GIT segments showed that in pigs incremental dietary Ca content reduced intestinal apparent P digestibility via hampering IP degradation and precipitation of P, with a larger impact in microbial phytase-supplemented diets due to the former.

Key words: calcium, phosphorus, phytase, gastrointestinal tract segments, pigs

Introduction

Phosphorus (P) is currently the third most expensive nutrient in diets for many intensively farmed animals. Most of the P in cereal grains and oil seeds is bound to inositol phosphates (IP) [1], and poorly available for non-ruminant animals such as pigs and poultry. Improving gastrointestinal IP breakdown and reducing P excretion has been intensively studied [2] because of finite global mineral P availability, increasing legal pressure on P output and public concerns regarding environmental P pollution including surface water eutrophication. Dietary reduction of the calcium (Ca) content and inclusion of microbial phytase are widely practiced to improve gastrointestinal P absorption by intensively farmed, non-ruminant animals.

It is generally accepted that a high dietary Ca content reduces phytase efficacy via Ca-IP complexation in the gastrointestinal tract (GIT) [3]. Using growth performance as the main criterion, Lei *et al.* [4] first reported that a normal compared to a low dietary Ca content reduced dietary microbial phytase efficacy. However, their study did not measure intestinal P absorption. Selle *et al.* [5] postulated that microbial phytase activity was not affected by dietary Ca content, because the optimal pH for phytase efficacy was lower than that for Ca-IP or Ca-P complexation, and phytase was active mostly in the stomach while Ca-IP complexation occurred primarily in the small intestine (SI). Poulsen *et al.* [6] and Létourneau-Montminy *et al.* [7] subsequently demonstrated that microbial phytase improved apparent total tract P digestibility independent of dietary Ca content. Wu *et al.* [8] even reported that a high dietary Ca content supported microbial phytase to improve growth performance. Discrepancies among these reported studies may be partly explained by the different phytase activity of the basal diets and criteria used to estimate phytase efficacy. Microbial phytase was added to the basal diets at a low level in the study of Lei *et al.* [4] but not in that of Poulsen *et al.* [6], Létourneau-Montminy *et al.* [7] and Wu *et al.* [8]. Nevertheless, none of these studies investigated the impact of dietary Ca content on IP degradation or P solubility in the digestive tract. It remains unknown if a high dietary Ca content may reduce phytase activity via the generation of Ca-IP and Ca-P complexes or reduce the digestion of other nutrients (e.g. minerals and energy [9]). Moreover, luminal pH gradually increases along the GIT, hence phytase activity seems to be affected more in the distal compared to the proximal GIT. However, none of the aforementioned reports investigated how dietary Ca interacts with phytase in

different GIT segments, and Ca and P absorption along the GIT segments has largely not been investigated.

We hypothesised that a high dietary Ca content reduces P absorption via hampering IP degradation and precipitation of P in the distal SI of pigs, with a greater impact in microbial phytase supplemented diets. As such, a very low Ca diet can be expected to enhance intestinal P absorption but may hamper P deposition and bone formation. Therefore, the objective of this research was to investigate the interactive effect between dietary Ca content and microbial phytase supplementation on apparent Ca and P digestibility and solubility in different GIT segments, IP degradation in the distal SI as well as faecal and urinary Ca and P excretion in growing pigs.

Materials and methods

The experiment was approved by the ethical committee of Wageningen University & Research (2016.D-0065.006) and conducted in the facilities of the Swine Research Centre of Trouw Nutrition (Sint Anthonis, the Netherlands). All procedures agreed with Dutch laws on animal trials in accordance with EU directive 2010/63. Daily monitoring of the animals was conducted by experienced animal caretakers under supervision of veterinarian.

Animals, experimental design and diets

Sixty growing male pigs (Hypor Libra×Maxter, 30.4±1.3 kg) were allocated to a diet containing either 0 or 500 FTU/kg microbial phytase and three levels of dietary Ca (2.0 (low), 5.8 (medium) and 9.6 (high) g/kg) in a 2×3 factorial arrangement. The experiment was replicated over time with two runs of five replicate pigs per treatment per run. Pigs were blocked by their initial body weight (BW) with pigs within a block randomly allocated to one of the six experimental diets for 21 days.

Experimental diets were produced by a feed production plant for research diets (ABZ Diervoeding, Leusden, the Netherlands) using a double mixing procedure. Prior to feed production, a mixture of wheat, barley and soybean meal was heated to 80 °C to deactivate intrinsic phytase activity. Subsequently, a basal diet was made which met or exceeded the minimal requirement of all nutrients

except for Ca and P [10]. No limestone was added to the basal diet, and monosodium phosphate was used to realize the intended digestible P content (1.7 g/kg). Titanium dioxide (TiO_2) was added to the basal diet at 4.0 g/kg as an indigestible marker. The basal diet was thoroughly mixed and divided into six equal portions before the required amount of limestone (Sibelco, Maastricht, the Netherlands) and microbial phytase (Axtra Phy, Danisco Animal Nutrition, Marlborough, United Kingdom) was added at the expense of diamol (Damolin, Kønsborgvej, Denmark) to produce each diet. Diet samples were taken with an automatic sampling device during production. The feeds were pelleted (4 mm) at a maximum temperature of 80 °C to prevent segregation during shipping and storage. The intended and analysed nutrients contents of the diets are shown in **Table 1**.

Table 1. Ingredient composition and analysed nutrient concentrations of the experimental diets

Phytase addition, FTU/kg	0			500		
Ca content, g/kg as-fed	2.0	5.8	9.6	2.0	5.8	9.6
Ingredients, g/kg as-fed						
Wheat	249	249	249	249	249	249
Barley	249	249	249	249	249	249
Maize	173	173	173	173	173	173
Soybean meal	117	117	117	117	117	117
Rapeseed meal	80	80	80	80	80	80
Sunflower seed meal	40	40	40	40	40	40
Soybean oil	32	32	32	32	32	32
Molasses	20	20	20	20	20	20
Premix [1]	5	5	5	5	5	5
Salt	2	2	2	2	2	2
Amino acids [2]	6	6	6	6	6	6
Monosodium phosphate	3	3	3	3	3	3
Phytase [3]	0	0	0	0.1	0.1	0.1
Limestone [4]	0	10	20	0	10	20
Diamol [5]	20	10	0	20	10	0
Titanium dioxide	4	4	4	4	4	4
Calculated composition, g/kg as-fed						
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 [6]	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 [7]	2.9	2.9	2.9	2.9	2.9	2.9
Digestible phosphorus	1.7	1.7	1.7	2.5	2.5	2.5

Table 1 (continued). Ingredient composition and analysed nutrient concentrations of the experimental diets

Phytase addition, FTU/kg	0			500		
Ca content, g/kg as-fed	2.0	5.8	9.6	2.0	5.8	9.6
Analysed composition, g/kg as-fed						
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 [7]	2.3	2.3	2.3	2.3	2.3	2.3
IP5-P [7]	0.2	0.2	0.2	0.2	0.2	0.2
∑IP-P [7]	2.5	2.5	2.5	2.5	2.5	2.5
Phytase activity, FTU/kg as fed	<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] Provided in g per kg of diet: L-Lysine HCl; 4.1; L-Threonine; 0.9; DL-Methionine; 0.7; L-Tryptophan, 0.2.
[3] Axtra Phy, Danisco Animal Nutrition, Marlborough, United Kingdom.
[4] Sibelco, Maastricht, the Netherlands.
[5] Damolin, Kønsborgvej, Denmark.
[6] AID, apparent ileal digestible.
[7] ∑IP-P, sum of inositol phosphate (IP) bound P; IP5-P, inositol pentakisphosphate (IP5) bound P; IP6-P, inositol hexakisphosphate (IP6) bound P; Inositol tetraphosphate (IP4) and triphosphate (IP3) were not detected in the diets.

Animal husbandry and feeding strategy

The experiment lasted 21 days and included a 4-d faeces and urine collection period (d 14-18). Pigs were first individually housed in pens containing a slatted floor (d 0-9) and then transferred to individual metabolism pens (d 10-20). During the collection period, representative samples of faeces were collected in plastic bags connected to the pigs with a Velcro system [(11)] and removed twice a day in the morning and afternoon. Urine was quantitatively collected in plastic buckets via a funnel mounted underneath the metabolism pens. Environmental temperature (23±1 °C) and ventilation were automatically controlled by a climate computer. A dark/light schedule (0600 to 2200 h lights on) was used throughout the entire experimental period, except for the dissection day (d 20) during which

time lights were on from 0200 h in order to feed the pigs according to a frequent feeding regime.

Daily feed allowance was three times net energy requirement for maintenance (293 KJ NE/$BW^{0.75}$) 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 overall daily gain from d 0-10 was used to estimate the ADG from d 10-20 and to calculate the feed allowance accordingly. Pigs received two equal meals per day at 0700 and 1500 h from d 0-17. From d 18 onwards, pigs were fed according to a frequent feeding regime adopted from Schop *et al.* [12] and Martens *et al.* [13] to ensure a constant digesta passage rate in the GIT. Briefly, pigs received 1/6^{th} of their daily allowance in six equal meals from 0700 to 2200 h at 3-h intervals on d 18-19. On d 20, feeding frequency was increased to 1-h intervals and pigs received half of their daily allowance equally distributed over six meals until dissection. Pigs were fed and dissected per block. Liquid feeding without soaking was used (water/feed 2:1, w/w base) throughout the experiment with the Ca content in water (0.034 g/l) included in the Ca intake, apparent digestibility and retention calculations. The P content in the water was very low (0.013 mg/l) hence this was not taken into account in the calculations. Feed refusals were collected, dried and recorded every day.

Sample collection and chemical analysis

On d 20, pigs were sedated with Zoletil® 100 (0.06 ml/kg BW), weighed and then euthanized via a jugular vein injection of Euthasol® (24 mg/kg BW). Blood was collected from the carotid artery before exsanguination thereafter serum was obtained by centrifuging at 3,000×g for 10 min at 4 °C. Subsequently the GIT was exposed, carefully taken out and divided into six segments (enclosed by zip ties) including stomach, proximal and distal half of SI, caeca, proximal and distal half of large intestine (LI). The GIT segments were quantitatively emptied by gentle squeezing. After thorough mixing followed by a pH determination (Mettler Toledo, Schwerzenbach, Switzerland), the digesta were immediately stored at -20 °C before further analysis. The caeca and proximal-LI digesta were pooled after pH

determination. The lower left foreleg was removed for collection of the 3rd metacarpal bone and stored at -20 °C before analysis.

Diets and faeces were analysed for dry matter [14], ash [15], crude protein (CP, N [16]×6.25), crude fat [17], starch [18] and sugar [19] prior to the beginning of the experiment. Because of the number of analyses, two methods were used to determine Ca, P and Ti content. A pilot study using six diets and six faeces samples demonstrated non-significant differences between the two methods in the Ca, P and Ti content for both diet and faeces samples irrespective of dietary treatments (data not shown). In faeces, the Ti [20] and P [21] content was determined using a photometer (Thermoscientific, MA) while the Ca [22] content was determined using an atomic absorption spectrometer (AAS, Varian, CA). For all other samples, the Ca, P and Ti content was determined using ICP-OES (ThermoFisher, MA) [23] after destruction in a microwave (CEM, NC) [24]. After thawing in a cooling chamber (4 °C), individual digesta samples (excl. faeces) were thoroughly mixed and two representative aliquots were taken. One aliquot was freeze-dried, ground to pass a 1-mm sieve (Retsch GmbH, Germany), destructed in a microwave (CEM, NC) and analysed for total Ca, P and Ti (ICP-OES) [23, 24]. The other aliquot was centrifuged at 3,000×g for 5 min at 4 °C, the supernatant harvested and centrifuged again at 10,000×g for 10 min at 4 °C and subsequently analysed for soluble inorganic Ca and P (ICP-OES) [23]. No destruction was applied to the supernatant. Because of the high viscosity, distal-LI digesta were diluted with deionized water before centrifuging (1:1, w/w base).

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].

The IP esters in the diets and distal-SI digesta containing three to six phosphate groups were extracted with 0.2 M EDTA and 0.1 M NaF (pH=10) before being analysed by high-performance ion chromatography and UV detection at 290 nm with an ICS-3000 System (ThermoFisher, MA) as described by Zeller *et al.* [25]. Inositol monophosphate (IP1) and diphosphate (IP2) are not determined using this method.

Calculations and statistical analysis

The apparent Ca and P digestibility coefficient in different GIT segments, inositol hexakisphosphate (IP6) degradation in the distal-SI and apparent total tract digestibility (ATTD) were calculated according to de Vries and Gerrits [26]:

$$ATTD\ (\%)=(1-(X_{digesta}/X_{diet})\times(Ti_{diet}/Ti_{digesta}))\times 100$$

where $X_{digesta}$ and X_{diet} are Ca, P or IP6 content in the digesta/faeces and diet (g/kg), respectively with $Ti_{digesta}$ and Ti_{diet} being the Ti content in the digesta/faeces and diet (g/kg), respectively. The Ca content in the diet included the Ca from drinking water based on the water:feed ratio of 2:1.

The retained Ca and P were calculated as:

$$Retention\ (g/d)=X_{intake}\times ATTD/100-X_{urine}$$

where X_{intake} and X_{urine} are the daily dietary intake and urinary excreted Ca or P (g) during the collection period. The Ca intake included the Ca from water in this calculation based on the water:feed ratio of 2:1.

The Ca and P solubility were calculated as:

$$Solubility\ (\%)=S_{supernatant}/(W_{aliquot}\times DM\times X_{digesta})\times 100$$

where $S_{supernatant}$ is the soluble inorganic Ca or P content in the supernatant (g), $W_{aliquot}$ the weight of fresh digesta used for centrifugation (kg), DM the freeze-dried DM content of the digesta and $X_{digesta}$ the total Ca or P content in the freeze-dried digesta (g/kg).

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

$$IP_n/\sum IP\ (\%)=IP_n/\sum IP_n\times 100$$

where IP_n is the content of individual IP esters (n=3, 4, 5 and 6, mmol/kg) and $\sum IP_n$ the sum of IP_n (n=3, 4, 5 and 6, mmol/kg).

Two sample mean power analysis was conducted with the POWER procedure of SAS (version 9.4, SAS Institute, Cary, NC), using data from an earlier study to investigate a novel phytase supplementation on Ca and P digestibility in pigs [27]. We hypothesized a 3% effect of P digestibility, which required eight replicates to achieve 80% of power for a two-sided significance level (0.05). Pig was the experimental unit. All data were submitted to a two-way ANOVA using the MIXED procedure of SAS using the following model:

$$Y_{ijkmn}=\mu+Ca_i+phytase_j+(Ca\times phytase)_{ij}+B_k+R_m+\varepsilon_{ijkmn}$$

where Y_{ijkmn} is the dependent variable of the n^{th} pig (n=1 to 60), μ the overall mean, Ca_i the fixed effect of dietary Ca content (i=1, 2, 3), $phytase_j$ the fixed effect of dietary phytase level (j=1, 2), and $(Ca\times phytase)_{ij}$ the fixed effect of their interaction, B_k the random effect of block (k=1 to 10), R_m the random effect of run (m=1, 2) and ε_{ijkmn} the residual error term.

The diagnosis of Studentized residual was visually checked by the graphics plotted using the ODS GRAPHICS function. The LSMEANS procedure with a PDIFF option was used to separate means. Probability was considered significant at $P\leq0.05$ and a trend at $0.05<P\leq0.1$.

Results

All animals remained healthy and completed the study with the exception of one pig (5.8 g/kg Ca with phytase supplementation) in the second run that died of volvulus and one pig (9.6 g/kg Ca without phytase supplementation) in the second run requiring a 1-d medical treatment because of diarrhoea. The analysed Ca and P content as well as phytase activity in the diets were in good agreement with the targeted levels (Table 1).

ATTD and retention of Ca and P

The P intake of pigs was similar for all treatment groups (**Table 2**). Overall, incremental dietary Ca reduced intestinal P absorption and urinary P excretion, with a greater impact in the phytase-supplemented compared to phytase-free diets as indicated by the interactive effects ($P_{interaction}$=0.023 and $P_{interaction}$<0.001,

respectively). Increasing dietary Ca content from 2.0 to 9.6 g/kg reduced ATTD of P by 6 vs. 15% units in the phytase-free and phytase-supplemented diets, respectively with ATTD P values of the phytase-free diets being approximately half of the corresponding values of the phytase-supplemented diets. As a result, P retention was also affected by the interactive effect between dietary Ca content and phytase inclusion ($P_{interaction}$=0.002 and $P_{interaction}$<0.001 for retained P (g/d) and retained P/digested P (%), respectively). Specifically increasing dietary Ca content from 2.0 to 5.8 g/kg increased P retention in the phytase-supplemented but not the phytase-free diets. Overall, phytase inclusion consistently enhanced the ATTD and retention of P with a magnitude depending on the dietary Ca content.

Table 2. Mean intake, faecal and urinary excretion, retention, and apparent total tract digestibility (ATTD) of phosphorus (P) of growing pigs as affected by dietary calcium (Ca) content and microbial phytase supplementation [1,2]

Ca	Phytase	P intake	Faecal P	Urinary P	rP	rP/dP	rP/P intake	ATTD
g/kg	FTU/kg	g/d	g/d	g/d	g/d	%	%	%
2.0	0	6.9	4.9^{b}	0.32^{b}	1.7^{d}	84.2^{c}	25.0^{cd}	29.7^{d}
5.8	0	6.9	5.0^{ab}	0.04^{c}	1.8^{d}	98.0^{a}	26.4^{c}	27.0^{d}
9.6	0	6.8	5.2^{a}	0.02^{c}	1.6^{d}	98.7^{a}	23.2^{d}	23.4^{e}
2.0	500	6.9	2.7^{e}	1.41^{a}	2.7^{c}	65.5^{d}	39.5^{b}	60.1^{a}
5.8	500	7.0	3.3^{d}	0.22^{b}	3.4^{a}	93.9^{b}	48.8^{a}	52.0^{b}
9.6	500	6.9	3.7^{c}	0.02^{c}	3.1^{b}	99.4^{a}	45.5^{a}	45.8^{c}
Pooled SEM		0.06	0.10	0.066	0.12	1.82	1.60	1.37
Ca								
2.0		6.9	3.8	0.86	2.2	74.9	32.2	44.9
5.8		6.9	4.2	0.12	2.6	96.1	37.1	39.5
9.6		6.8	4.5	0.02	2.5	99.1	36.2	34.6
Pooled SEM		0.05	0.07	0.046	0.09	1.32	1.10	0.98
Phytase								
0		6.9	5.1	0.13	1.8	93.4	25.4	26.7
500		6.9	3.3	0.55	3.1	86.0	44.5	52.7
Pooled SEM		0.04	0.06	0.037	0.07	1.08	0.90	0.80
P-value								
Ca		0.116	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Phytase		0.570	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Ca × phytase		0.258	0.006	<0.001	0.002	<0.001	0.001	0.023

[1] Dietary P content was fixed at 4.7 g/kg of diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[a-e] Values without common superscript within a column differ significantly ($P \leq 0.05$).
rP, retained P (g/d); dP, apparently digested P (g/d).

The Ca intake increased (P<0.001) with incremental dietary Ca content and was not affected (P=0.290) by phytase supplementation of the diets (**Table 3**). Phytase increased ATTD of Ca with a greater effect in diets with a lower Ca content ($P_{interaction}$<0.001). Incremental dietary Ca content increased and reduced ATTD of Ca in the phytase-free and phytase-supplemented diets, respectively ($P_{interaction}$<0.001). Both faecal and urinary Ca excretion were reduced by phytase inclusion and increased with incremental dietary Ca content (P<0.001). The impact of incremental dietary Ca content on urinary Ca excretion was greater for the phytase-free compared to phytase-supplemented diets ($P_{interaction}$<0.001). Incremental dietary Ca content increased retained Ca (g/d) but reduced retained Ca as percentage of digestible (retained Ca/dCa) or total (retained Ca/Ca) Ca intake. The magnitude of these effects depended on the phytase supplementation of the diets ($P_{interaction}$<0.001).

Table 3. Mean calcium (Ca) intake, faecal and urinary excretion, retention and apparent total tract digestibility (ATTD) of growing pigs as affected by dietary Ca content and microbial phytase supplementation [1,2]

Ca	Phytase	Ca intake	Faecal Ca	Urinary Ca	rCa	rCa/dCa	rCa/Ca intake	ATTD
g/kg	FTU/kg	g/d	g/d	g/d	g/d	%	%	%
2.0	0	2.6	1.6	0.08^{d}	0.9^{d}	91.0^{b}	33.7^{d}	36.9^{e}
5.8	0	7.6	4.4	1.17^{c}	2.0^{c}	63.2^{d}	26.6^{e}	42.0^{d}
9.6	0	12.4	7.4	2.59^{a}	2.4^{b}	48.3^{e}	19.6^{f}	40.6^{de}
2.0	500	2.6	0.7	0.05^{d}	1.8^{c}	97.4^{a}	69.5^{a}	71.3^{a}
5.8	500	7.6	3.0	0.10^{d}	4.5^{a}	97.8^{a}	58.8^{b}	60.1^{b}
9.6	500	12.5	6.2	1.60^{b}	4.7^{a}	74.2^{c}	37.4^{c}	50.2^{c}
Pooled SEM		0.08	0.17	0.132	0.18	3.11	2.01	1.85
Ca								
2.0		2.6^{c}	1.2^{c}	0.06	1.3	94.2	51.6	54.1
5.8		7.6^{b}	3.8^{b}	0.66	3.2	79.6	41.9	50.6
9.6		12.4^{a}	6.8^{a}	2.10	3.6	61.3	28.5	45.4
Pooled SEM		0.06	0.12	0.092	0.13	2.23	1.40	1.32
Phytase								
0		7.5	4.5	1.28	1.8	67.5	26.7	39.9
500		7.6	3.4	0.60	3.6	89.5	55.1	60.6
Pooled SEM		0.05	0.10	0.074	0.10	1.81	1.14	1.08
P-value								
Ca		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Phytase		0.290	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Ca × phytase		0.474	0.227	<0.001	<0.001	<0.001	<0.001	<0.001

[1] Dietary P content was fixed at 4.7 g/kg of diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[a-f] Values without common superscript within a column differ significantly (P≤0.05).
rCa, retained Ca (g/d); dCa, apparently digested Ca (g/d).

Apparent Ca and P digestibility in different GIT segments

The apparent P digestibility was improved by phytase inclusion in all GIT segments investigated and was reduced by incremental dietary Ca content posterior to the stomach (**Table 4**). A significant interaction between phytase and dietary Ca content on apparent P digestibility was observed in the distal ileum ($P_{interaction}$=0.007), where a dietary Ca content of 9.6 compared to 2.0 g/kg reduced apparent P digestibility by 5 vs. 15% units in the phytase-free and phytase-supplemented diets, respectively. Apparent digestibility of P was low in the stomach, gradually increased along SI followed by a reduction in the proximal-LI. The apparent P digestibility was much higher in the distal compared to proximal LI, particularly for the phytase-free diets.

Table 4. Dietary phosphorus (P) and calcium (Ca) apparent digestibility (%) in difference gastrointestinal tract segments in growing pigs as affected by dietary Ca content and microbial phytase supplementation [1,2]

Ca	Phytase	P					Ca				
			Small intestine		Large intestine			Small intestine		Large intestine	
g/kg	FTU/kg	Stomach	Proximal	Distal	Proximal	Distal	Stomach	Proximal	Distal	Proximal	Distal
2.0	0	1.0	13.6	25.8^{c}	16.4	28.6	-15.9	23.9^{c}	11.9^{d}	24.6^{c}	29.5^{c}
5.8	0	5.2	11.8	25.0^{c}	0.68	18.5	-2.9	34.8^{b}	28.9^{c}	23.1^{c}	37.5^{c}
9.6	0	3.8	-0.7	20.9^{c}	3.22	18.0	0.7	36.1^{b}	28.8^{c}	44.6^{b}	53.6^{b}
2.0	500	10.7	43.4	62.9^{a}	56.2	66.2	-14.2	60.1^{a}	64.3^{a}	62.3^{a}	72.9^{a}
5.8	500	13.4	36.1	50.9^{b}	46.7	55.5	3.9	51.4^{a}	55.9^{b}	54.2^{ab}	62.4^{b}
9.6	500	5.1	23.9	47.7^{b}	34.5	47.0	0.6	40.1^{b}	43.7^{b}	40.9^{b}	55.9^{b}
Pooled SEM		3.78	5.37	2.65	4.74	3.31	5.51	5.27	5.06	6.41	4.59
Ca											
2.0		5.9	28.5^{a}	44.4	36.3^{a}	47.4^{a}	-15.1^{b}	42.0	38.1	43.4	51.2
5.8		9.1	23.3^{a}	37.3	22.5^{b}	36.0^{b}	0.33^{a}	42.7	41.7	37.8	49.3
9.6		4.4	11.6^{b}	34.3	18.8^{b}	32.5^{b}	0.66^{a}	38.1	36.2	42.7	54.7
Pooled SEM		2.72	3.85	1.90	3.30	2.37	3.85	3.67	3.52	4.46	3.29
Phytase											
0		3.3	8.2	23.9	6.8	21.7	-6.0	31.6	23.2	30.8	40.2
500		9.6	34.4	53.9	45.7	56.2	-3.5	50.5	54.6	52.4	63.8
Pooled SEM		2.21	3.13	1.54	2.68	1.93	3.13	2.99	2.86	3.62	2.67
P-value											
Ca		0.232	<0.001	<0.001	<0.001	<0.001	<0.001	0.367	0.428	0.463	0.210
Phytase		0.007	<0.001	<0.001	<0.001	<0.001	0.384	<0.001	<0.001	<0.001	<0.001
Ca × phytase		0.273	0.717	0.007	0.137	0.157	0.649	<0.001	<0.001	<0.001	<0.001

[1] Dietary P content was fixed at 4.7 g/kg of diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[a-d] Values without common superscript within a column differ significantly ($P \leq 0.05$).

In all GIT segments posterior to the stomach, apparent Ca digestibility was affected by the interaction between dietary Ca content and microbial phytase addition. Incremental dietary Ca content increased apparent Ca digestibility posterior to the stomach in phytase-free diets but reduced it in phytase-supplemented diets ($P_{interaction}$<0.001, Table 4). Phytase inclusion increased apparent Ca digestibility (P<0.001) in all GIT segments posterior to the stomach. The apparent Ca digestibility was negative in the stomach for the diets with the lowest Ca content, and substantially increased in the proximal-SI followed by a reduction in the distal-SI for the phytase-free but not the phytase-supplemented diets. Moreover, apparent digestibility of Ca was much higher in the distal-LI compared to distal-SI, especially for the phytase-free diets.

Ca and P solubility

Overall, the solubility of inorganic P gradually decreased along the GIT segments, with a sharp reduction in the distal compared to proximal SI (**Table 5**). No interaction between dietary Ca content and phytase inclusion on P solubility was observed for any of the GIT segments. In all GIT segments, incremental dietary Ca content reduced or tended to reduce P solubility while phytase increased P solubility.

The Ca solubility was high in the stomach, lower in the proximal-SI and decreased sharply in the distal-SI (Table 5). Incremental dietary Ca content increased Ca solubility in the stomach and distal-SI, with less consistent effects in the other segments. Phytase tended to increase the Ca solubility in the stomach, distal SI and distal LI with less consistent effects in the other GIT segments.

Table 5. Mean solubility (%) of inorganic phosphorus (P) and calcium (Ca) in digesta of difference gastrointestinal tract segments in growing pigs as affected by dietary Ca content and microbial phytase supplementation [1,2]

Ca	Phytase	P					Ca				
			Small intestine		Large intestine			Small intestine		Large intestine	
g/kg	FTU/kg	Stomach	Proximal	Distal	Proximal	Distal	Stomach	Proximal	Distal	Proximal	Distal
2.0	0	43.7	56.8	15.0	20.2	21.7	43.6	43.8	3.8	9.3[a]	5.4
5.8	0	47.3	28.9	12.2	15.6	15.9	49.9	21.6	5.8	4.4[b]	2.7
9.6	0	39.5	32.4	14.6	14.0	16.1	48.7	41.2	9.1	4.8[b]	4.2
2.0	500	55.4	70.8	44.0	26.3	29.0	43.1	30.2	6.8	6.9[b]	5.3
5.8	500	57.8	51.3	31.8	24.0	28.7	56.1	32.7	9.2	9.4[a]	5.2
9.6	500	55.1	54.5	30.6	18.1	19.1	54.4	50.0	11.1	5.7[b]	5.2
Pooled SEM		2.94	7.15	5.18	1.72	3.39	3.42	10.69	2.62	1.12	1.09
Ca											
2.0		49.6	63.8[a]	29.5	23.3[a]	25.4[a]	43.4[b]	37.0	5.3[b]	8.1	5.3
5.8		52.3	39.5[b]	21.5	19.6[b]	21.9[ab]	52.8[a]	26.8	7.4[ab]	6.8	3.9
9.6		47.3	43.4[b]	22.6	16.0[c]	17.6[b]	51.5[a]	45.6	10.1[a]	5.2	4.7
Pooled SEM		2.11	5.13	3.72	1.24	2.43	2.46	7.67	1.88	0.80	0.78
Phytase											
0		43.5	39.4	13.9	16.6	17.9	47.4	35.5	6.2	6.2	4.1
500		56.1	59.1	35.6	22.8	25.5	51.0	37.8	9.0	7.3	5.2
Pooled SEM		1.71	4.17	3.02	1.00	1.98	2.00	6.23	1.53	0.65	0.64
P-value											
Ca		0.052	0.001	0.096	<0.001	0.009	<0.001	0.095	0.045	0.003	0.219
Phytase		<0.001	0.001	<0.001	<0.001	<0.001	0.057	0.633	0.072	0.089	0.082
Ca × phytase		0.454	0.631	0.206	0.244	0.149	0.304	0.170	0.936	<0.001	0.289

[1] Dietary P content was fixed at 4.7 g/kg of diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[a-c] Values without common superscript within a column differ significantly ($P \leq 0.05$).

Inositol phosphate esters in the distal-SI digesta

Microbial phytase supplementation significantly increased dietary IP6 degradation from an average of 5.8 to 78.6% and reduced ∑IP content in the freeze-dried digesta of the distal-SI (**Table 6**). The IP6 was largely degraded in the phytase-supplemented diets, with an average proportion of individual IP in ∑IP of 25.8, 7.2, 37.3 and 29.7% for IP6, IP5, IP4 and IP3, respectively. In contrast, in the phytase-free diets, IP6 was largely intact and represented 76.5% of ∑IP. Dietary Ca addition significantly reduced IP6 degradation but not ∑IP in the digesta (P=0.014 and 0.137, respectively). Moreover, increasing dietary Ca content from 2.0 to 9.6 g/kg increased IP6/∑IP to a greater extent in the phytase-supplemented

compared to phytase-free diets (16 vs. 4% units, $P_{interaction}$=0.025). Incremental dietary Ca content also reduced IP4/∑IP and IP3/∑IP but only for the phytase-supplemented diets ($P_{interaction}$=0.018 and 0.009, respectively). The IP5/∑IP was inconsistently affected by dietary Ca content ($P_{interaction}$=0.001).

Table 6. Mean inositol phosphate (IP) degradation, total IP content (∑IP) and percentage of different IP esters in freeze-dried distal small intestinal digesta of growing pigs as affected by dietary calcium (Ca) content and microbial phytase supplementation [1,2]

Ca	Phytase	IP6 degradation	∑IP content	IP3/∑IP	IP4/∑IP	IP5/∑IP	IP6/∑IP
g/kg	FTU/kg	%	mmol/kg	%	%	%	%
2.0	0	8.4	46.3	2.6^{c}	6.2^{c}	18.2^{a}	72.9^{b}
5.8	0	3.0	45.0	1.0^{c}	4.2^{c}	15.5^{b}	79.3^{a}
9.6	0	6.1	41.2	1.0^{c}	5.5^{c}	16.4^{ab}	77.2^{ab}
2.0	500	88.9	26.7	37.5^{a}	39.4^{a}	5.8^{d}	17.4^{e}
5.8	500	73.9	36.1	24.0^{b}	39.4^{a}	9.6^{c}	26.9^{d}
9.6	500	73.1	28.6	26.9^{b}	33.5^{b}	6.4^{d}	33.3^{c}
Pooled SEM		5.08	3.89	2.71	1.82	1.19	2.99
Ca							
2.0		48.6^{a}	36.5	20.1	22.8	12.0	45.2
5.8		38.4^{b}	40.6	12.5	21.8	12.6	53.1
9.6		39.6^{b}	34.9	13.9	19.5	11.4	55.2
Pooled SEM		3.65	2.79	1.95	1.27	0.86	2.14
Phytase							
0		5.8	44.2	1.5	5.3	16.7	76.5
500		78.6	30.3	29.7	37.3	7.2	25.8
Pooled SEM		2.96	2.27	1.58	1.03	0.70	1.74
P-value							
Ca		0.014	0.137	0.001	0.033	0.387	<0.001
Phytase		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Ca × phytase		0.168	0.170	0.009	0.018	0.001	0.025

[1] Dietary P content was fixed at 4.7 g/kg of diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[a-c] Values without common superscript within a column differ significantly (P≤0.05).

Composition of the 3rd metacarpus

The defatted mass of the 3rd metacarpus was increased by both dietary Ca content and phytase (P=0.001, **Table 7**). A significant interactive effect between dietary Ca content and phytase supplementation was observed on ash content ($P_{interaction}$=0.034). Increasing dietary Ca content from 2.0 to 5.8 g/kg increased ash content in phytase-supplemented but not in phytase-free diets; further increasing dietary Ca content from 5.8 to 9.6 g/kg resulted in a reduced ash

content in the phytase-free but not in phytase-supplemented diets. In addition, the metacarpal Ca mass was enhanced with incremental dietary Ca content irrespective of dietary phytase addition (P<0.001), whereas the P mass was enhanced with dietary Ca only in phytase-supplemented diets ($P_{interaction}$=0.034). The Ca/P in the metacarpus ash was not significantly affected by dietary phytase and Ca content, although a tendency was observed (P=0.056) for an increase with incremental dietary Ca content.

Table 7. Mean defatted mass, ash content, calcium (Ca) and phosphorus (P) mass and Ca/P in ash of the 3rd metacarpus in growing pigs as affected by dietary Ca content and microbial phytase supplementation [1,2]

Ca g/kg	Phytase FTU/kg	Defatted mass g	Ash content %	Ca mass g	P mass g	Ca/P in ash
2.0	0	3.4	56.9^{cd}	0.378	0.183^{b}	2.06
5.8	0	3.6	57.5^{bc}	0.405	0.190^{b}	2.13
9.6	0	3.7	56.7^{d}	0.434	0.195^{b}	2.27
2.0	500	3.6	57.0^{cd}	0.408	0.194^{b}	2.10
5.8	500	4.2	58.5^{a}	0.492	0.240^{a}	2.06
9.6	500	4.0	58.1^{ab}	0.485	0.228^{a}	2.12
Pooled SEM		0.15	0.03	0.021	0.009	0.07
Ca						
2.0		3.5^{b}	56.9	0.393^{c}	0.189	2.08
5.8		3.9^{a}	58.0	0.447^{b}	0.214	2.09
9.6		3.9^{a}	57.5	0.460^{a}	0.212	2.19
Pooled SEM		0.11	0.02	0.015	0.007	0.05
Phytase						
0		3.6	57.0	0.406	0.189	2.15
500		3.9	57.9	0.462	0.221	2.09
Pooled SEM		0.09	0.02	0.012	0.005	0.04
P-value						
Ca		0.001	<0.001	<0.001	0.001	0.056
Phytase		<0.001	<0.001	<0.001	<0.001	0.173
Ca × phytase		0.329	0.034	0.219	0.034	0.189

[1] Dietary P content was fixed at 4.7 g/kg of diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[a-d] Values without common superscript within a column differ significantly (P≤0.05).

Growth performance and ATTD of proximate components

Dietary Ca content reduced (P=0.016) whereas phytase increased (P=0.025) ADG from d 0-21 (**Table 8**). Average daily feed intake was not affected by dietary Ca or phytase. The feed to gain ratio was increased by an incremental dietary Ca

content and reduced by dietary phytase addition with a tendency for an interaction ($P_{interaction}$=0.067). Dietary Ca supplementation reduced the ATTD of crude fat (P<0.001, Table 8). The ATTD of ash was increased by both dietary Ca and phytase inclusion (P<0.001), while the ATTD of CP and organic matter was not affected by the dietary treatments.

Table 8. Mean growth performance parameters from d 0-20 of growing pigs and apparent total tract digestibility (ATTD) of proximate components as affected by dietary calcium (Ca) content and microbial phytase supplementation [1,2]

Ca	Phytase	Growth performance			ATTD of proximate components, %				
g/kg	FTU/kg	ADG g/d	ADFI kg/d	F/G g/g	DM	CP	Ash	OM	CFat
2.0	0	736	1.29	1.77	81.1	79.3	22.6[e]	85.1	81.6
5.8	0	700	1.30	1.86	82.2	80.8	32.5[d]	85.6	81.2
9.6	0	642	1.27	2.01	81.9	80.9	38.2[c]	85.0	78.3
2.0	500	729	1.27	1.74	82.0	78.9	33.5[d]	85.3	81.4
5.8	500	752	1.31	1.75	82.8	81.1	39.9[b]	85.7	80.8
9.6	500	712	1.28	1.80	82.3	80.6	45.9[a]	84.8	78.7
Pooled SEM		28.1	0.023	0.05	0.47	1.32	0.74	0.50	0.97
Ca									
2.0		732[a]	1.28	1.76[b]	81.5[b]	79.1	28.0	85.2	81.5[a]
5.8		725[a]	1.30	1.81[b]	82.4[a]	80.9	36.0	85.6	81.0[a]
9.6		677[b]	1.27	1.90[a]	82.1[a]	80.7	42.0	84.9	78.5[b]
Pooled SEM		20.2	0.016	0.04	0.34	0.95	0.53	0.35	0.70
Phytase									
0		693	1.29	1.88	81.7	80.3	31.1	85.2	80.4
500		730	1.28	1.77	82.3	80.2	39.7	85.3	80.3
Pooled SEM		16.5	0.013	0.03	0.27	0.77	0.43	0.28	0.57
P-value									
Ca		0.016	0.208	0.002	0.026	0.123	<0.001	0.104	<0.001
Phytase		0.025	0.742	0.001	0.034	0.834	<0.001	0.891	0.852
Ca × phytase		0.132	0.463	0.067	0.687	0.932	0.002	0.841	0.800

[1] Dietary P content was fixed at 4.7 g/kg of diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[a-e] Values without common superscript within a column differ significantly (P≤0.05).
ADFI, average daily feed intake; ADG, average daily gain; CFat, crude fat, CP, crude protein; DM, dry matter; F/G, feed to gain ratio; OM, organic matter.

Discussion

The hypothesis that a high dietary Ca content reduces P absorption to a greater extent in the phytase-supplemented diets via hampering IP degradation and precipitation of P in pigs was supported by the results of this study. Increasing dietary Ca content from 2.0 to 9.6 g/kg reduced ATTD of P by 6 vs. 15% units in the phytase-free and phytase-supplemented diets, respectively (Table 2). This phytase dependent reduction in apparent P digestibility with incremental dietary Ca content was present from the distal-SI (Table 4), confirming the hypothesis of the study. The profile of IP esters in the distal-SI further indicated that incremental dietary Ca content reduced apparent P digestibility via reducing IP6 degradation, with a greater impact on IP6/∑IP in the phytase-supplemented compared to phytase-free diets (16 vs. 4% units, Table 6). It can be concluded that a high dietary Ca content reduced apparent P digestibility via reduction of IP degradation and increased precipitation of P, with a greater impact for the phytase-supplemented diets.

Ca and phytase interaction

A high dietary Ca content might inhibit phytase efficacy via Ca-IP complexation and pH buffering. Both Ca-IP complexation and phytase efficacy are pH dependent. Digesta pH gradually increased along the GIT with a mean pH observed of 3.6, 5.9 and 6.4 in the stomach, proximal-SI and distal-SI, respectively (**Supplementary Table 1**). Complexation of Ca-IP at a pH below 5 is negligible [(5)], hence Ca-IP complexation can be expected to predominantly have occurred in the SI but not in the stomach. In addition, digesta pH was increased with incremental dietary Ca content in the stomach, caeca and proximal-LI (Supplementary Table 1), while IP is better soluble at a low compared to high pH [(28)]. 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.

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 [29]. 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 real-time quantitative PCR (RT-qPCR) technology. The candidate genes were multiple inositol polyphosphate phosphatase 1 [30] and intestinal alkaline phosphatase (IAP) [31]. The expression of both phosphatases was not affected by dietary Ca content or phytase supplementation in the jejunal and colonic mucosa (Chapter 3). 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 [32]. This might explain why effects of Ca supplementation were exerted in the aforementioned broiler study [29] but not in the present study. Brun *et al.* [33] reported that luminal Ca binds and aggregates IAP but has no impact on the expression level of IAP in the small intestine of rats; results which agree those found in the present study in pigs. As such a high dietary Ca content does not have an impact on the expression level of mucosal phosphatases but might inhibit their activity to hydrolyse IP in the GIT of pigs.

In contrast to our study, Poulsen *et al.* [6] and Létourneau-Montminy *et al.* [7] reported no detrimental impact of a high dietary Ca content on phytase efficacy to improve apparent P digestibility. Dietary Ca content was 4 vs. 8 g/kg and 7 vs. 10 g/kg in Poulsen *et al.* [6] and Létourneau-Montminy *et al.* [7], 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.* [6] 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 efficacy (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.* [7] 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. As the impact of dietary Ca content on microbial phytase activity is dependent on diet type, our conclusion that a high dietary Ca content reduces

microbial phytase efficacy is valid in the condition of a low intrinsic phytase activity and dietary P content being marginally deficient.

Complexation of Ca and inorganic P might be another mechanism by which dietary Ca can reduce phytase efficacy. However, this was not supported by our results, because on average the P disappearance represented 94% of the IP-P disappearance in the distal-SI in the presence of microbial phytase in this study. Thus, the released IP-P appeared to have been readily and effectively absorbed in the GIT of the pigs. Phytase supplementation caused an increase in mean IP6 disappearance in the distal SI from 6 to 79% (Table 6), while the apparent P digestibility in this section was only increased from 24 to 54% (Table 4). However, phytase supplementation did not completely dephosphorylate the inositol ring as shown by the enrichment of IP3 and IP4 in the presence of phytase compared to the diets without added phytase. Phosphate bound in these IP esters, probably extended by IP2 and IP1 which were not analysed herein, remained undigested and caused the responses in apparent P digestibility to be smaller than IP6 degradation. This is consistent with results by Rosenfelder-Kuon *et al.* [34] and Lu *et al.* [35] who reported an increased concentration of IP3 and IP4 in the ileal digesta of pigs when phytase was supplemented to the feed, even at activities (3000 FTU/kg) that were higher than in the present study.

Ca and P absorption along the GIT

Calcium was primarily absorbed in the proximal-SI and LI. Recently, the stomach was suggested to be responsible for approximately 30% of the apparent total tract Ca absorption [36; 37]. In contrast, our results showed that gastric apparent Ca absorption was minor. The discrepancy may be due to surgical preparation of the pigs with T-cannulas in the duodenum and distal ileum in the latter two studies but not in the present study. Cannulation might have affected GIT motility and digesta mean retention in their studies hence resulting in a greater Ca absorption in the stomach comparing to pigs under normal physiological conditions. Similar to our finding, Rutherfurd *et al.* [38] reported that apparent Ca absorption was minor in the stomach, substantial in the jejunum and negligible in the ileum. The negligible apparent Ca absorption in the distal-SI was probably caused by the low Ca solubility under relatively high pH conditions. Apparent Ca digestibility was even

lower in the distal compared to proximal SI in the phytase-free but not in the phytase-supplemented diets (Table 4). The reduction of Ca digestibility might indicate higher intestinal Ca secretion in the absence of microbial phytase, hence dietary phytase inclusion appeared to reduce endogenous Ca loss. This assumption is supported by Lee *et al.* [39] who reported that microbial phytase addition reduced basal endogenous Ca losses using Ca-free diets in gestating sows. Interestingly, substantial Ca absorption was observed in the colon in the present study with colonic Ca absorption being higher in the phytase-free compared to the phytase-supplemented diets (17 vs. 6% units, Table 4). The mechanism behind this effect might be that in phytase-free diets colonic microbiota degraded IP more than in phytase supplemented diets, with subsequent higher release and absorption of IP bound Ca. Alternatively, although unlikely, the data might be influenced by segregation between the marker (Ti) and the nutrients of interest (see below).

Phosphorus was primarily absorbed in the SI and partly in the distal-LI. As mentioned before, microbial phytase might be most active in the stomach and improved apparent P digestibility in the proximal SI (Table 4). Thus, the P absorption site seemed to be affected by microbial phytase inclusion. A negative P absorption, i.e. P secretion was observed between the distal-SI and the proximal-LI, with subsequent P absorption in the distal-LI (Table 4). Colonic P secretion is supported by data reported by Larsen and Sandstrom [40] who demonstrated a higher ileal compared to faecal apparent P digestibility in cannulated pigs. In contrast, Mesina *et al.* [36] and Rosenfelder-Kuon *et al.* [34] demonstrated a similar ileal and faecal apparent P digestibility. Nonetheless, none of these studies including the present, can exclude secretion and reabsorption of P in the caeca and colon. Gonzalez-Vega *et al.* [37] reported that colonic P absorption was minor in IP free but substantial in IP enriched semi-purified diets. Their finding is in line with our results, as in the present study apparent P digestibility in the distal-LI was 20 vs. 7% in the phytase-free and phytase-supplemented diets, respectively (Table 3 and 4). Inositol phosphates were hardly detected in the faeces of pigs even when remarkable amounts of IP entered the LI, indicating intense microbial IP degradation in the LI [34; 41]. As mentioned earlier, colonic microbiota also in the present study may have degraded IP and released IP bound Ca and P more for the phytase-free diets hence a higher colonic P absorption was observed. Existence of colonic P absorption was proven by Liu *et al.* [42, 43], who reported that P absorption

in the colon was substantial (10%) from diets containing maize distiller's dried grains with solubles, while in diets containing soybean meal and canola meal, colonic P absorption was less (3-4%). They concluded that colonic P absorption was not affected by dietary P content but was highly dependent on dietary P sources. The colon, therefore, can have a significant capacity to absorb P depending on the diet. Mechanisms for colonic P absorption in pigs, however, remain largely unknown, i.e. whether it is mediated via active transcellular routes or paracellular permeation. The latter is addressed in our companion study (Chapter 3). Another possible explanation for the fluctuation of apparent P digestibility in the colon might be that Ti transiently segregated from the digesta, which was observed in the gizzard and caeca in our previous broiler study [44]. However, this assumption is less likely under steady-state conditions with a constant passage rate of digesta in the GIT. Moreover, DM digestibility gradually increased along the GIT as expected (**Supplementary Table 2**) hence the marker (Ti) appeared to function well.

Ca and P retention and excretion

An adequate dietary Ca/P is essential for P deposition in bone. Dietary phytase inclusion enhanced both absorption and retention of Ca and P, with a larger effect in medium and high Ca supplemented diets. In the low Ca diets, the Ca supply limited P retention as indicated by the relatively high urinary P excretion. Despite a reduction in ATTD of P, the increase in dietary Ca from 2.0 to 5.8 increased the P retention, rP/dP, bone ash content and bone mineral mass, in particular in the phytase supplemented diets as indicated by the interaction. The high retention of both ingested P (94%) and Ca (98%) at this treatment indicates that the digestible Ca to P ratio was close to optimum in this treatment. The highest retention of ingested P was realised at the medium dietary Ca content. The increase to the highest Ca content reduced the P retention because of a reduction in ATTD of P. These results indicate that for optimal dietary P utilisation the effects of Ca on absorption and retention of P must be taken into account.

Conclusions

A high dietary Ca content reduces small intestinal apparent P digestibility to a greater extent in phytase-supplemented diets via reducing IP degradation and precipitation of P. Dietary Ca is primarily absorbed in the proximal-SI and to a lesser extent in the LI in pigs, while P is mostly absorbed in the proximal and distal SI with minor net absorption occurring in the LI. An adequate dietary Ca/P is essential for optimal P absorption and post-absorptive utilisation.

Acknowledgement

This research was conducted by Wageningen Livestock Research, within the framework of the public private partnership "Feed4Foodure" and partially funded by the Ministry of Economic Affairs (Policy Support Research project number BO-31.03-005-00). The authors acknowledge the Chinese Scholarship Council (CSC) for the scholarship of Y.X. Hu to study at Wageningen University & Research.

Author contributions

P. Bikker, J.W. Resink and Y.X. Hu designed and conducted the study; Y.X. Hu conducted sample and data analysis; Y.X. Hu, P. Bikker, W.H. Hendriks, J. van Baal, M.M. van Krimpen and M. Rodehutscord critically discussed and interpreted data, and wrote the paper. All authors with the exception of the late M.M. van Krimpen have read and approved the final manuscript. J.W. Resink is an employee of Trouw Nutrition, all other authors declare no conflict of interest.

References

1. Rodehutscord M, Ruckert C, Maurer HP *et al.* (2016) Variation in chemical composition and physical characteristics of cereal grains from different genotypes. *Arch Anim Nutr* **70**, 87-107.

2. Misiura MM, Filipe JAN, Walk CL *et al.* (2018) Do not neglect calcium: a systematic review and meta-analysis (meta-regression) of its digestibility and utilisation in growing and finishing pigs. *Br J Nutr* **119**, 1207-1219.

3. Dersjant-Li Y, Awati A, Schulze H *et al.* (2015) Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. *J Sci Food Agric* **95**, 878-896.

4. Lei XG, Ku PK, Miller ER *et al.* (1994) Calcium level affects the efficacy of supplemental microbial phytase in corn-soybean meal diets of weanling pigs. *J Anim Sci* **72**, 139-143.

5. Selle PH, Cowieson AJ & Ravindran V (2009) Consequences of calcium interactions with phytate and phytase for poultry and pigs. *Livest Sci* **124**, 126-141.

6. Poulsen HD, Carlson D, Nørgaard JV *et al.* (2010) Phosphorus digestibility is highly influenced by phytase but slightly by calcium in growing pigs. *Livest Sci* **134**, 100-102.

7. Létourneau-Montminy M-P, Narcy A, Magnin M *et al.* (2010) Effect of reduced dietary calcium concentration and phytase supplementation on calcium and phosphorus utilization in weanling pigs with modified mineral status. *J Anim Sci* **88**, 1706-1717.

8. Wu F, Tokach MD, Dritz SS *et al.* (2018) Effects of dietary calcium to phosphorus ratio and addition of phytase on growth performance of nursery pigs. *J Anim Sci* **96**, 1825-1837.

9. Kies AK, Gerrits WJJ, Schrama JW *et al.* (2005) Mineral absorption and excretion as affected by microbial phytase, and their effect on energy metabolism in young piglets. *J Nutr* **135**, 1131-1138.

10. CVB (2016) *Voedernormen Varkens en Voederwaarden Voedermiddelen voor Varkens (Feeding Standards, Feeding Advices and Nutritional Values of Feeding Ingredients of Pigs)*. Wageningen, the Netherlands: Federatie Nederlandse Diervoederketen.

11. van Kleef D, Deuring K & van Leeuwen P (1994) A new method of faeces collection in the pig. *Lab Anim* **28**, 78-79.

12. Schop M, Jansman AJM, de Vries S *et al.* (2019) Increasing intake of dietary soluble nutrients affects digesta passage rate in the stomach of growing pigs. *Br J Nutr* **121**, 529-537.

13. Martens BMJ, Flécher T, de Vries S *et al.* (2019) Starch digestion kinetics and mechanisms of hydrolysing enzymes in growing pigs fed processed and native cereal based diets. *Br J Nutr* **121**, 1124-1136.

14. ISO (1999) 6496: *Animal Feeding Stuffs - Determination of Moisture and Other Volatile Matter Content*. Geneva: International Organization for Standardization.

15. ISO (2002) 5984: *Animal Feeding Stuffs - Determination of Crude Ash*. Geneva: International Organization for Standardization.

16. ISO (2005) 5983-1: *Animal Feeding Stuffs - Determination of Nitrogen Content and Calculation of Crude Protein Content - Part 1: Kjeldahl Method*. Geneva: International Organization for Standardization.

17. ISO (1999) 6492: *Animal Feeding Stuffs - Determination of Fat Content*. Geneva: International Organization for Standardization.

18. ISO (2004) 15914: *Animal Feeding Stuffs - Enzymatic Determination of Total Starch Content*. Geneva: International Organization for Standardization.

19. van Vuuren A, van der Koelen C, Valk H *et al.* (1993) Effects of partial replacement of ryegrass by low protein feeds on rumen fermentation and nitrogen loss by dairy cows. *J Dairy Sci* **76**, 2982-2993.

20. Myers WD, Ludden PA, Nayigihugu V *et al.* (2004) A procedure for the preparation and quantitative analysis of samples for titanium dioxide. *J Anim Sci* **82**, 179-183.

21. ISO (2000) 6869: *Animal Feeding Stuffs - Determination of The Contents of Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Sodium and Zinc - Method Using Atomic Absorption Spectrometry*. Geneva: International Organization for Standardization.

22. ISO (1998) 6491: *Animal Feeding Stuffs - Determination of Phosphorus Content - Spectrometric Method*. Geneva: International Organization for Standardization.

23. ISO (2009) 27085: *Animal Feeding Stuffs - Determination of Calcium, Sodium, Phosphorus, Magnesium, Potassium, Iron, Zinc, Copper, Manganese, Cobalt, Molybdenum, Arsenic, Lead and Cadmium by ICP-AES*. Geneva: International Organization for Standardization.

24. Wang J, Nakazato T, Sakanishi K *et al.* (2004) Microwave digestion with HNO_3/H_2O_2 mixture at high temperatures for determination of trace elements in coal by ICP-OES and ICP-MS. *Anal Chim Acta* **514**, 115-124.

25. Zeller E, Schollenberger M, Kühn I *et al.* (2015) Hydrolysis of phytate and formation of inositol phosphate isomers without or with supplemented phytases in different segments of the digestive tract of broilers. *J Nutr Sci* **4**, e1.

26. de Vries S & Gerrits WJJ (2018) The use of tracers or markers in digestion studies. In *Feed Evaluation Science*, pp. 275–294 [Moughan PJ and Hendriks WH, editors]. Wageningen, the Netherlands: Wageningen Academic Publishers.

27. Bento MHL, Pedersen C, Plumstead PW *et al.* (2012) Dose response of a new phytase on dry matter, calcium, and phosphorus digestibility in weaned piglets. *J Anim Sci* **90**, 245-247.

28. Siener R, Heynck H & Hesse A (2001) Calcium-binding capacities of different brans under simulated gastrointestinal pH conditions. *In vitro* study with ^{45}Ca. *J Agric Food Chem* **49**, 4397-4401.

29. Applegate TJ, Angel R & Classen HL (2003) Effect of dietary calcium, 25-hydroxycholecalciferol, or bird strain on small intestinal phytase activity in broiler chickens. *Poult Sci* **82**, 1140-1148.

30. Windhorst S, Lin H, Blechner C *et al.* (2013) Tumour cells can employ extracellular Ins(1,2,3,4,5,6)P_6 and multiple inositol-polyphosphate phosphatase 1 (MINPP1) dephosphorylation to improve their proliferation. *Biochem J* **450**, 115-125.

31. Schlemmer U, Jany KD, Berk A *et al.* (2001) Degradation of phytate in the gut of pigs-pathway of gastrointestinal inositol phosphate hydrolysis and enzymes involved. *Arch Anim Nutr* **55**, 255-280.

32. Rodehutscord M & Rosenfelder P (2016) Chapter 1 Update on phytate degradation pattern in the gastrointestinal tract of pigs and broiler chickens. In *Phytate Destruction-Consequences for Precision Animal Nutrition*, pp. 15-28 [Walk CL, Kühn I, Stein HH, Kidd MT and Rodehutscord M, editors]. Wageningen, the Netherlands: Wageningen Academic Publishers.

33. Brun LR, Traverso A & Rigalli A (2009) Aggregation and inhibition of rat intestinal alkaline phosphatase by high concentrations of calcium. Reversibility of the processes. *J Enzyme Inhib Med Chem* **24**, 691-696.

34. Rosenfelder-Kuon P, Klein N, Zegowitz B *et al.* (2020) Phytate degradation cascade in pigs as affected by phytase supplementation and rapeseed cake inclusion in corn-soybean meal-based diets. *J Anim Sci* **98**, skaa053.

35. Lu H, Shin S, Kuehn I *et al.* (2020) Effect of phytase on nutrient digestibility and expression of intestinal tight junction and nutrient transporter genes in pigs. *J Anim Sci* **98**, skaa206.

36. Mesina VGR, Lagos LV, Sulabo RC *et al.* (2018) Effects of microbial phytase on mucin synthesis, gastric protein hydrolysis, and degradation of phytate along the gastrointestinal tract of growing pigs. *J Anim Sci* **97**, 756-767.

37. Gonzalez-Vega JC, Walk CL, Liu Y *et al.* (2014) The site of net absorption of Ca from the intestinal tract of growing pigs and effect of phytic acid, Ca level and Ca source on Ca digestibility. *Arch Anim Nutr* **68**, 126-142.

38. Rutherfurd SM, Chung TK & Moughan PJ (2014) Effect of microbial phytase on phytate P degradation and apparent digestibility of total P and Ca throughout the gastrointestinal tract of the growing pig. *J Anim Sci* **92**, 189-197.

39. Lee SA, Stein HH, Lagos LV *et al.* (2019) Basal endogenous loss, standardized total tract digestibility of calcium in calcium carbonate, and retention of calcium in gestating sows change during gestation, but microbial phytase reduces basal endogenous loss of calcium. *J Anim Sci* **97**, 1712–1721.

40. Larsen T & Sandstrom B (1993) Effect of dietary calcium level on mineral and trace element utilization from a rapeseed (*Brassica napus* L.) diet fed to ileum-fistulated pigs. *Br J Nutr* **69**, 211-224.

41. Baumgärtel T, Metzler BU, Mosenthin R *et al.* (2008) Precaecal and postileal metabolism of P, Ca and N in pigs as affected by different carbohydrate sources fed at low level of P intake. *Arch Anim Nutr* **62**, 169-181.

42. Liu JB, Shen XY, Zhai HX *et al.* (2017) Dietary sources of phosphorus affect postileal phosphorus digestion in growing pigs. *J Anim Sci* **95**, 4490-4498.

43. Liu JB, Xue PC, Cao SC *et al.* (2018) Effects of dietary phosphorus concentration and body weight on postileal phosphorus digestion in pigs. *Anim Feed Sci Technol* **242**, 86-94.

44. Hu YX, Bikker P, Duijster M *et al.* (2020) Coarse limestone does not alleviate the negative effect of a low Ca/P ratio diet on characteristics of tibia strength and growth performance in broilers. *Poult Sci* **99**, 4978-4989.

Supplementary Table 1. Mean digesta pH of growing pigs as affected by dietary calcium (Ca) content and microbial phytase supplementation [1,2]

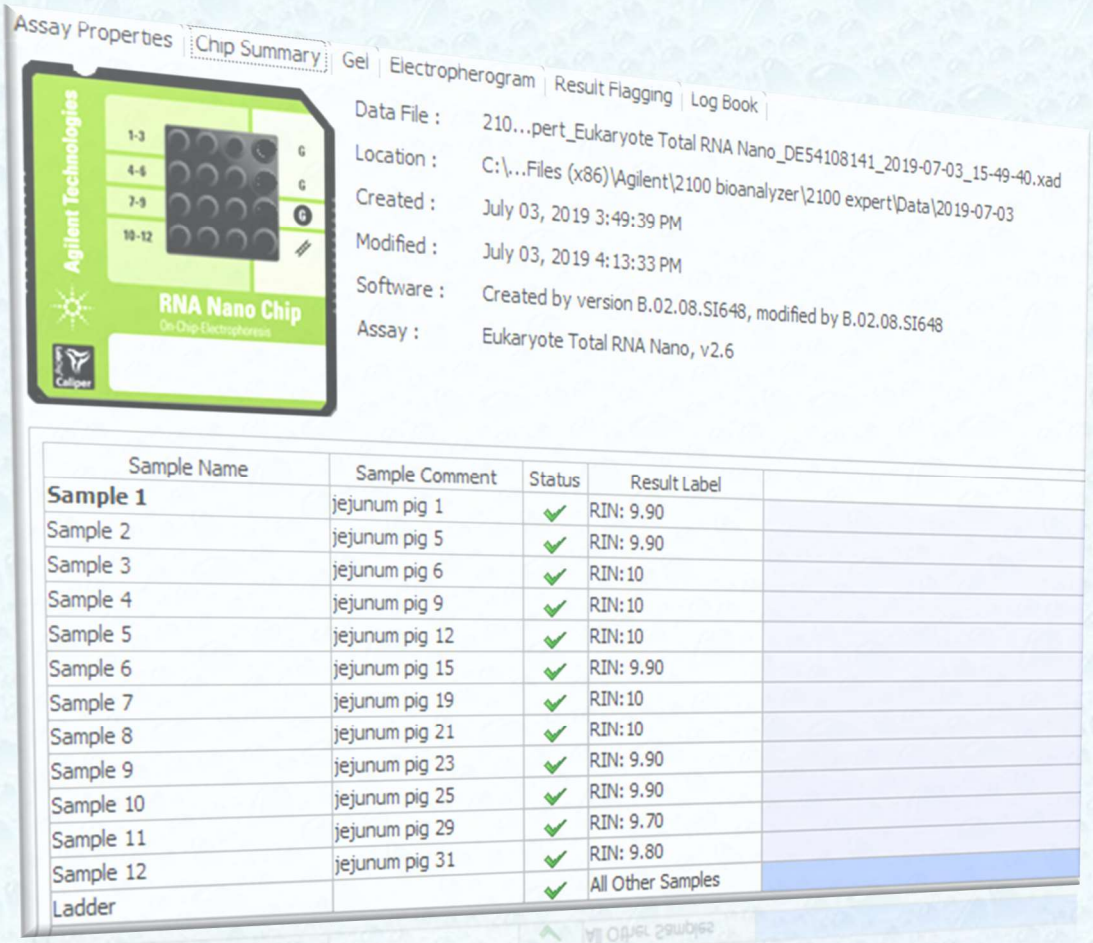
Ca	Phytase	Stomach	Small intestine		Caeca	Large intestine	
g/kg	FTU/kg		Proximal	Distal		Proximal	Distal
2.0	0	3.2	5.8	6.6[a]	5.5	5.4	5.8
5.8	0	4.1	6.0	6.2[bc]	5.5	5.5	5.9
9.6	0	4.1	5.7	6.2[c]	5.8	5.7	6.0
2.0	500	3.0	6.0	6.5[a]	5.4	5.4	5.8
5.8	500	3.5	6.0	6.5[ab]	5.4	5.5	6.0
9.6	500	4.0	5.8	6.6[a]	5.6	5.7	6.1
Pooled SEM		0.21	0.11	0.11	0.09	0.07	0.10
Ca							
2.0		3.1[c]	5.9[ab]	6.5	5.4[b]	5.4[b]	5.8
5.8		3.8[b]	6.0[a]	6.4	5.5[b]	5.5[b]	6.0
9.6		4.1[a]	5.8[b]	6.4	5.7[a]	5.7[a]	6.0
Pooled SEM		0.15	0.08	0.08	0.07	0.05	0.08
Phytase							
0		3.8	5.8	6.3	5.6	5.5	5.9
500		3.5	5.9	6.5	5.5	5.5	6.0
Pooled 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

[1] Dietary P content was fixed at 4.7 g/kg of diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[a-c] Values without common superscript within a column differ significantly ($P \leq 0.05$).

Supplementary Table 2. Mean apparent digestibility (%) of dry matter in different gastrointestinal tract of growing pigs as affected by dietary calcium (Ca) content and microbial phytase supplementation [1,2]

Ca	Phytase	Stomach	Small intestine		Large intestine	
g/kg	FTU/kg		Proximal	Distal	Proximal	Distal
2.0	0	-75.2	27.1	59.7	76.4	78.4
5.8	0	-41.0	35.8	59.4	75.2	77.6
9.6	0	-47.7	21.4	55.9	77.5	78.0
2.0	500	-81.3	40.4	65.6	75.8	79.7
5.8	500	-64.2	38.5	61.3	77.8	78.8
9.6	500	-52.0	26.5	58.3	76.2	79.1
Pooled SEM		16.5	7.43	2.47	1.35	0.94
Ca						
2.0		-78.2^{b}	33.8^{ab}	62.6^{a}	76.1	79.1
5.8		-52.6^{a}	37.1^{a}	60.4^{ab}	76.5	78.2
9.6		-49.9^{a}	24.0^{b}	57.1^{b}	76.9	78.6
Pooled SEM		11.8	5.32	1.77	0.97	0.67
Phytase						
0		-54.6	28.1	58.3	76.3	78.0
500		-65.9	35.0	61.8	76.6	79.2
Pooled SEM		9.60	4.33	1.44	0.79	0.55
P-value						
Ca		0.041	0.050	0.011	0.716	0.406
Phytase		0.213	0.123	0.034	0.784	0.035
Ca × phytase		0.603	0.549	0.379	0.146	0.988

[1] Dietary P content was fixed at 4.7 g/kg of diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[a,b] Values without common superscript within a column differ significantly ($P \leq 0.05$).



Sample Name	Sample Comment	Status	Result Label
Sample 1	jejunum pig 1	✓	RIN: 9.90
Sample 2	jejunum pig 5	✓	RIN: 9.90
Sample 3	jejunum pig 6	✓	RIN: 10
Sample 4	jejunum pig 9	✓	RIN: 10
Sample 5	jejunum pig 12	✓	RIN: 10
Sample 6	jejunum pig 15	✓	RIN: 9.90
Sample 7	jejunum pig 19	✓	RIN: 10
Sample 8	jejunum pig 21	✓	RIN: 10
Sample 9	jejunum pig 23	✓	RIN: 9.90
Sample 10	jejunum pig 25	✓	RIN: 9.90
Sample 11	jejunum pig 29	✓	RIN: 9.70
Sample 12	jejunum pig 31	✓	RIN: 9.80
Ladder		✓	All Other Samples

CHAPTER 3

High dietary Ca and microbial phytase reduce expression of Ca transporters while enhancing claudins involved in paracellular Ca absorption in the porcine jejunum and colon

Y.X. Hu[*†], J. van Baal[†], W.H. Hendriks[†], J.W. Resink[‡], A. Liesegang[ℓ], M.M. van Krimpen[*], P. Bikker[*]

[*] Wageningen University & Research, Wageningen Livestock Research, 6700 AH Wageningen, the Netherlands

[†] Wageningen University & Research, Animal Nutrition Group, 6700 AH Wageningen, the Netherlands

[‡] Trouw Nutrition Research and Development, Veerstraat 38, 5831 JN Boxmeer, the Netherlands

[ℓ] University of Zurich, Institute of Animal Nutrition, Vetsuisse Faculty, 8057 Zürich, Switzerland

To be submitted

Abstract

Colonic and jejunal expression levels of genes related to Ca and P homeostasis (transporters and claudins (CLDN)) was investigated in pigs. Sixty growing pigs received a low, medium and high Ca content (2.0, 5.8, 9.6 g/kg, respectively) diet with or without microbial phytase (500 FTU/kg) for 21 days. Expression of genes (RT-qPCR) related to Ca and P absorption were determined in jejunal and colonic mucosa. Dietary Ca intake affected serum Ca and P, $1,25(OH)_2D_3$, alkaline phosphatase and parathyroid hormone concentration. Expression of TRPV5 mRNA was reduced in colonic mucosa by dietary Ca (34%) and phytase (44%). In jejunal mucosa, expression of TRPV5 mRNA was decreased (32%) with phytase inclusion only. TRPV6 mRNA expression was reduced (30%) with microbial phytase in both mucosae. Unlike jejunal mucosa, calbindin-D9k mRNA expression was lower in colonic mucosa with high dietary Ca (59%) and microbial phytase (37%). None of the mRNAs encoding the Na-P cotransporters (NaPi-IIc, PiT-1, PiT-2) were affected. Expression of the phosphate transporter XPR1, was slightly downregulated with dietary Ca in jejunal, but not colonic mucosa. Dietary Ca downregulated colonic CLDN-4 (20%) and -10 (40%) expression while CLDN-7 was reduced by phytase inclusion in pigs fed low dietary Ca. Expression of colonic CLDN-12 tended to be increased by phytase. In jejunal mucosa, dietary Ca increased CLDN-2 expression (48%) and decreased CLDN-10 (49%) expression, while phytase slightly upregulated CLDN-12 expression. High dietary Ca and phytase intake in pigs downregulate jejunal and colonic genes related to transcellular Ca absorption and upregulate Ca pore forming claudins.

Keywords: jejunum and colon, metabolism, calcium and phosphorus transporters, claudins, pig

Introduction

The two essential macro minerals calcium (Ca) and phosphorus (P) regulate a wide range of biochemical and cell signalling processes and are the principal constituents of bone in terrestrial organisms [1]. Intestinal absorption of these minerals from the diet plays an important role in the regulation of whole body Ca and P homeostasis. Ionized Ca is absorbed predominantly in the small intestine and partly in the colon, accounting for approximately 80 and 20% of the total tract Ca absorption in pigs, respectively (Chapter 2). The uptake of these minerals occurs either transcellular or paracellular with the former involving Ca transport proteins calbindin-D9k/-D28k (CaBP-D9k/-D28k), sodium (Na)-Ca exchanger (NCX1) in concert with plasma membrane Ca-ATPase 1 (PMCA1) [2]. The components responsible for transcellular P uptake are less clear. Experimental data showed that the apical step is mediated mainly by members of the Na-dependent inorganic phosphate cotransporter type II (NaPi-II) family [3]. Based on studies in rats and mice, phosphate inorganic transporter 1 (PiT-1) and 2 (PiT-2) also appear to play a role in active intestinal P absorption [4]. In addition, it has been proposed that the basolateral step could be mediated by xenotropic and polytropic retrovirus receptor 1 (XPR1/SLC53A1) [5]. While transcellular transport is an active, ATP consuming process, paracellular transport is dependent on passive diffusion. Diffusion of Ca and P occurs through a complex of tight junction proteins, that enclose the apical and lateral membranes of enterocytes. In this context, claudins (CLDN) are the most important transmembrane components of the tight junction complex, comprising barrier (i.e. tightening) and permeability-mediating members [6]. The latter group includes cation-selective CLDN-2 and -12, which build pores highly permeable for Ca [7]. In addition, CLDN-10b [8] and -15 [9] can modulate small intestinal Na permeability. The anion-selective CLDN members, on the other hand, are largely unknown as inconsistent results have been reported. Depending on cell-type, CLDN-4 [10] and -7 [11] have been shown to form pores permeable for chloride (Cl) but a barrier to Na. Another tight junction protein is zonula occludens protein 1 (ZO-1/TJP1), which ensures a stabile connection to the cytoskeleton of the epithelial cell [12]. Understanding the molecular mechanisms underlying the modulation of intestinal Ca and P transporters through dietary intervention is an important topic in livestock in order to further improve performance and health

status of animals, assure efficient utilization of Ca and P, alleviate legal pressure on P output and minimize environmental pollution.

Recently, we reported that growing pigs fed microbial phytase supplemented diets displayed improved inositol phosphate (IP) degradation and enhanced apparent absorption of Ca and P in the small intestine (Chapter 2). Strikingly, the phytase free diets resulted in increased passage of substantial amounts of P towards the large intestine, where it was absorbed three times more compared to phytase supplemented diets. Furthermore, apparent absorption of Ca in the colon was approximately 2-fold greater with phytase free diets. Similar findings have been reported by Gonzalez-Vega *et al.* [13], who investigated Ca and P absorption in various gut segments in pigs receiving semi-purified diets. They demonstrated that apparent P absorption in the colon was substantial in the IP supplemented but not in IP free diets. These observations imply that colonic Ca and P absorption is dependent on luminal microbial phytase activity and its IP content. It is conceivable that phytase supplementation enhanced IP degradation in the distal region of the small intestine in our previous study (Chapter 2). Consequently, less IP would be available for the microbiota residing in the colon to release Ca and P ions and inositol from the IP complex for colonic absorption. In addition, mucosal phosphatases (e.g. multiple inositol polyphosphate phosphatase 1 (MINPP1) and intestinal alkaline phosphatase (IAP)) might also be reduced because less substrate was available in the colon. However, molecular mechanisms underlying the regulation of colonic Ca and P absorption remain largely unknown. Because of the high viscosity of digesta and the lower permeability of the colon to defend against pathogens and fermentation toxins (e.g. phenol, cresol), colonic Ca and P absorption is more likely to be mediated via the more controllable, active transcellular pathway. The aim of the research described in this paper was to assess the effect of dietary Ca level and microbial phytase supplementation on the expression of genes related to Ca, P and inositol absorption as well as mucosal phosphatases in the jejunum and colon. We hypothesized that the expression level of genes involved in transcellular and paracellular transport in the colon of pigs would be greater when feeding diets not supplemented with phytase to favour the absorption of these two minerals.

Materials and methods

The work described here is a follow-up of our recent *in vivo* pig study in which the interactive effects of dietary Ca level and microbial phytase supplementation on Ca and P absorption and solubility along the intestine in growing pigs was investigated (Chapter 2). This study was approved by the ethical committee of Wageningen University & Research (2016.D-0065.006) and conducted in the facilities of the Swine Research Centre of Trouw Nutrition (Sint Anthonis, the Netherlands). All procedures were in agreement with the Dutch laws on animal experiments.

Experimental design and diets

Details regarding the experimental design, feed composition, animal husbandry, feeding regime, as well as sample collection, are described in our previous study (Chapter 2). Briefly, sixty young growing pigs (Hypor Libra×Maxter), weighing 30.4±1.3 kg, were allotted to two dietary levels of microbial phytase (0 vs. 500 FTU/kg) and three levels of Ca (2.0, 5.8 and 9.6 g/kg) in a 2×3 factorial arrangement. The experiment was replicated over time with two runs of five replicate pigs per treatment per run. Pigs were blocked by their initial body weight (BW) with pigs within a block randomly allocated to one of the six experimental diets for 21 days. The analysed Ca, P and IP content as well as phytase activity in the diets were in good agreement with the targeted values for all treatment groups.

Sample collection and treatment selection

At the end of the experiment, pigs were sedated with Zoletil® 100 (0.06 ml/kg BW) and then euthanized via jugular vein injection of Euthasol® 100 (24 mg/kg BW). Blood was collected from the carotid artery before exsanguination, centrifuged at 3,000×g for 10 min at 4 °C to harvest serum. Subsequently, the gastrointestinal tract (GIT) was dissected out and emptied by gentle squeezing before the mucosa was cleansed with water and scraped from the middle of jejunum and colon, frozen in liquid nitrogen and stored at -80 °C until further analysis. In the present study, only tissue samples from the low (2.0 g/kg) and high (9.6 g/kg) Ca level with (500

FTU/kg) or without dietary phytase groups were used for the gene expression analysis described hereafter.

Serum analysis

Serum Ca and P were analysed using a Cobas 8000 modular analyser with C701 Photometric measuring unit (Roche Diagnostics Limited, Rotkreuz, Switzerland). Commercially available test kits were used to analyse serum parathyroid hormone (PTH, Immunotopics, San Clemente, USA), 25-hydroxycholecalciferol ($25(OH)D_3$) and 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$, Immunodiagnostic Systems GmbH, Germany), and alkaline phosphatase (ALP, Diatools AG, Villmergen, Switzerland).

Real-time quantitative PCR analysis

The RNA isolation and gene expression determination were conducted following the standard protocol in our laboratory. Briefly, deep-frozen jejunum and colon mucosa were ground in liquid nitrogen and used for total RNA isolation using TRIzol (ThermoFisher Scientific). The 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. The RNA integrity number values ranged from 9.7 to 10. A total of 500 ng RNA was reverse transcribed with Superscript III kit (ThermoFisher Scientific) and mRNA levels were assessed by real-time 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 s and 60 °C for 30 s for 40 cycles. The used primer sequences were designed with Primer Express Software (Life Technologies, Bleiswijk, the Netherlands), and where possible recommended primer sets that span an intron were selected and presented in **Table 1**. Absolute mRNA levels of genes of interest were normalized to the endogenous expression level of importin 8 (IPO8), since Normfinder [(14)] pointed out that IPO8 was the most stable gene compared to that of eukaryotic elongation factor 2 (EEF2) and beta actin (ACTB).

Table 1. Primers used for RT-qPCR determination

Genes	Accession number	Forward (5'-3')	Reverse (5'-3')
Ca transporters			
CaSR	XM_021068447	TTGCTCATGCCCTGCAAGAT	AAGTGCCGTAGGTGCTTCAG
CaBP-D9k	NM_214140	TATGCAGCCAAAGAAGGGGAT	CTAGGGTTCTCGGACCTTTCAG
CaBP-D28k	NM_001130226	GGCTTCATTTCGACGCTGAC	TCGGGTGATAACTCCAATCCAG
NCX1	XM_021088333	GTTTGTGGCGCTTGGAACTT	TGCCCGTGACGTTACCTATG
TRPM7	XM_013993003	AGCACCATCTTGGACTCTTGC	CCCAGGACCACAGATTTCACG
TRPV5	XM_021078896	GCTGCAACAGAAGAGGATTCG	CCGAAAGTCACAGGTTCGGT
TRPV6	XM_021078898	AGCAGAAGCGGATCTGGG	GGGCTCCTTTCTGGTGGACA
P transporters			
NaPi-IIa	XM_021082353	GACAGGACTGACTTCCGGC	CACGGATGTTGAAGGAGGCT
NaPi-IIb	NM_001256772	CTCTGTAGCTGCCGGGTCCTAA	GGTCAGAGTCGACGAGAACAC
NaPi-IIc	XM_021081190	GCATCCTGTTGTGGTACGTG	AGAAGCCGAGCAACAGGTAG
PiT-1	XM_021087023	GATGTTTGGTTCTGCTGTGTG	AGACCACTTGACACCCTCCTG
PiT-2	XM_021078067	TCACGAACCAAGCCACGAA	GATCTCACCCCCATCACACC
XPR1	XM_003130352	GGTGGCCTCTTGCCAAATGA	GCACTGGATAAAGCGAAGCC
Tight junctions			
CLDN-2	NM_001161638	CTGCCCACTGCAAGGAAATC	ACTCACTCTTGGCTTTGGGT
CLDN-4	XM_021085910	ATGGGTGCCTCGCTCTACAT	GAGTAGGGCTTGTCGGTACG
CLDN-7	XM_021066034	GCAGGTCTTTGTGCGTTGAT	AAGATGGCAGGGCCAAACTC
CLDN-10	XM_021065095	GACCGGGTGTTCCCTGTATG	GGCTCCTGCCCATCCAATAA
CLDN-12	NM_001160079	ATGACGTCCGTTCTGCTCTT	TACGTATGCATGCTGGGAGG
CLDN-16	NM_001161644	TTTTTGGCTGGTGCTGTCCT	CATACGACTTGGCCATCGAAAC
ZO-1	XM_021098891	GTTGGACAACCAGACGTGGA	ACTAACTTCATGCTGGGCCG
Other proteins			
CYP24	NM_214075	GGGCGGAGGATTTGAGGAAT	ATCAACACGGTTCCTTTGGGT
IAP	XM_003133725	CTAAAGGGGCAGATGAATGG	CACCTGTCTGTCCACGTTGT
MINPP1	XM_001927672	AGAAGCAAAGTTCTCAGCCAGTT	GGGGCTCCTTGTCTTTGAAGTA
SMIT	XM_005657149	CAAGGGGCCTTCTATGGTGG	CTGGCCTCTCATCAGGTTGG
VDR	NM_001097414	CTCTCCAGACACGATGGAGC	TTGGCAAAGCCGATGACCTT
Reference genes			
ACTB	XM_021086047	CGTGAGAAGATGACCCAGATCA	TCTCCGGAGTCCATCACGAT
EEF2	XM_003354002	AGTCCACTCTGACGGACTCA	AGAGGGAAATGGCCGTTGAT
IPO8	XM_003126400	TGCCATGGTATTTCTCCTCAAA	GCAGAAGAGGCATCATGTCTGT

ACTB, beta actin; CaBP-D9k, calbindin-D9k; CaBP-D28k, calbindin-D28k; CaSR, Ca sensing receptor; CYP24, vitamin D_3 24-hydroxylase; EEF2, eukaryotic elongation factor 2; IAP, intestinal alkaline phosphatase; IPO8, importin 8; MINPP1, multiple inositol polyphosphate phosphatase 1; NaPi-IIa (SLC34A1), sodium-dependent phosphate transporter type IIa; NaPi-IIb (SLC34A2), sodium-dependent phosphate transporter type IIb; NaPi-IIc (SLC34A3), sodium-dependent phosphate transporter type IIc; NCX1(SLC8A1), sodium-Ca exchanger; PiT-1 (SLC20A1), inorganic phosphate transporter 1; PiT-2 (SLC20A2), inorganic phosphate transporter 2; SMIT (SLC5A3), sodium/myo-inositol cotransporter; 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_3 receptor; XPR1 (SLC53A1), xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1.

Data analysis

Pig was the experimental unit for all analysis. Serum and gene expression data were analysed by two-way ANOVA using the MIXED procedure of SAS (version 9.4, SAS Institute, Cary, NC) with dietary phytase, Ca level and their interaction as fixed effects and batch and block as random effects. Distribution and homogeneity of variance of the Studentized residuals were visually checked via graphics plotted by the ODS GRAPHICS procedure. The LSMEANS procedure with a PDIFF option was used to separate means. Probability was considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.1$.

Results

Serum Ca, P, ALP, PTH and vitamin D_3

As expected, incremental dietary Ca content gradually increased the serum Ca content of the pigs (**Table 2**). This increase was dampened with inclusion of microbial phytase ($P_{interaction} < 0.001$). Moreover, incremental dietary Ca reduced serum P content, with a greater effect in diets without phytase supplementation ($P_{interaction} < 0.001$). Serum ALP concentration increased with increasing dietary Ca ($P < 0.001$) but reduced with phytase supplementation ($P = 0.030$). Incremental dietary Ca level drastically decreased ($P < 0.001$) serum PTH concentration, and this inhibitory effect was significantly impeded with phytase inclusion in the medium Ca group (73 vs. 5 pg/ml, $P_{interaction} = 0.011$). The serum concentration of the active form of vitamin D_3, $1,25(OH)_2D_3$ was, however, reduced by incremental dietary Ca level but only in combination with phytase supplementation ($P_{interaction} = 0.010$).

Table 2. Least squares means of various serum components as affected by dietary calcium (Ca) level and microbial phytase supplementation in growing pigs [1,2]

Item	Dietary treatment							*P*-value		
Phytase, FTU/kg	0			500			Pooled SEM	Ca	Phytase	Ca × Phytase
Ca, g/kg	2.0	5.8	9.6	2.0	5.8	9.6				
Ca, mM	2.38^{cd}	2.78^{b}	3.38^{a}	2.35^{d}	2.48^{c}	2.82^{b}	0.072	<0.001	<0.001	<0.001
P, mM	2.90^{b}	2.19^{d}	1.85^{e}	3.23^{a}	3.09^{ab}	2.63^{c}	0.098	<0.001	<0.001	<0.001
ALP, U/l	100	113	141	98	99	116	10.0	<0.001	0.030	0.280
PTH, pg/ml	118^{a}	5^{c}	1^{c}	127^{a}	73^{b}	4^{c}	15.9	<0.001	0.006	0.011
$25(OH)D_3$, nM	70	86	89	84	102	110	6.4	<0.001	<0.001	0.710
$1,25(OH)_2D_3$, pM	1048^{a}	1007^{a}	1003^{a}	1013^{a}	873^{b}	860^{b}	26.3	<0.001	<0.001	0.010

[1] Dietary P content was fixed at 4.7 g/kg diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[a-e] Values lacking a common superscript within a row differ ($P \leq 0.05$).
ALP, alkaline phosphatase; PTH, parathyroid hormone; $25(OH)D_3$, 25-hydroxycalciferol; $1,25(OH)_2D_3$, 1,25-dihydroxycalciferol.

Colonic expression levels

The effects of dietary Ca and microbial phytase supplementation on the gene expression levels of Ca and P transporters and tight junction proteins in the colonic and jejunal mucosa of the pigs are shown in **Tables 3** and **4**. Expression of colonic TRPV5 mRNA was reduced by 34 and 44% with high Ca intake and phytase inclusion (P=0.030 and 0.004, respectively; Table 3). Similarly, high dietary Ca intake reduced TRPV6 and CaBP-D9k mRNA expression by approximately 55% (P<0.001), and phytase reduced their expression to a less extent (approximately 35% reduction, P<0.002). Neither the expression of NCX1 nor that of the none-selective cation channel, transient receptor potential cation channel subfamily M member 7 (TRPM7), were affected by the dietary treatments. Colonic expression of CaBP-D28k and the extracellular Ca sensing receptor (CaSR) mRNA were beyond the limit of detection.

Table 3. Least squares means of mRNA levels of calcium (Ca) and phosphorus (P) transporter and claudins (CLDN) as affected by dietary Ca level and microbial phytase supplementation in the colonic mucosa of growing pigs [1,2,3]

Item	Dietary treatments					*P*-value		
Phytase, FTU/kg	0		500		Pooled SEM	Ca	Phytase	Ca × Phytase
Ca, g/kg	2.0	9.6	2.0	9.6				
Ca transporters								
CaBP-D9k	215	108	159	44	26.1	<0.001	<0.001	0.738
NCX1	1.77	1.56	1.72	1.74	0.139	0.350	0.521	0.258
TRPM7	20.9	20.1	20.5	20.4	0.74	0.421	0.896	0.556
TRPV5	0.034	0.025	0.022	0.011	0.008	0.030	0.004	0.778
TRPV6	1.85	1.03	1.43	0.49	0.216	<0.001	0.002	0.572
P transporters								
PiT-1	17.0	17.5	16.1	18.2	1.03	0.055	0.827	0.211
PiT-2	122	115	134	119	15.1	0.452	0.610	0.781
XPR1	9.9	10.4	10.7	10.5	0.71	0.714	0.385	0.534
Tight junctions								
CLDN-2	12.5	12.0	12.0	12.6	1.08	0.891	0.962	0.511
CLDN-4	62.5	43.8	57.6	53.8	6.82	0.027	0.603	0.136
CLDN-7	32.5[a]	29.4[b]	29.7[b]	30.3[ab]	1.20	0.153	0.279	0.046
CLDN-10	1.90	1.19	2.17	1.24	0.264	<0.001	0.406	0.555
CLDN-12	22.8	24.4	25.2	26.0	1.50	0.246	0.072	0.711
ZO-1	5.19	5.00	5.33	5.30	0.185	0.390	0.101	0.551
Other proteins								
VDR	7.06	6.74	7.85	7.60	0.406	0.498	0.052	0.935
MINPP1	9.27	9.40	9.47	9.27	0.474	0.913	0.914	0.622
SMIT	11.9	10.8	11.8	10.7	0.65	0.026	0.893	0.957

[1] Dietary P content was fixed at 4.7 g/kg diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[3] Determined using absolute quantification normalized by IPO8.
[a-b] Values lacking a common superscript within a row differ ($P \leq 0.05$).
CaBP-D9k, calbindin-D9k; IPO8, importin 8; MINPP1, multiple inositol polyphosphate phosphatase 1; NCX1, sodium-Ca exchanger; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; SMIT, sodium/myo-inositol cotransporter; 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_3 receptor; XPR1, xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1. Of note, transcript levels of Ca sensing receptor, calbindin-D28k, sodium-dependent phosphate transporters type IIa, IIb and IIc, CLDN-15 and alkaline phosphatase in the porcine colonic mucosa were beyond the limit of detection.

All mRNA encoding members of type II Na-Pi transporters (i.e. NaPi-IIa, NaPi-IIb and NaPi-IIc) were beyond the limit of detection in the colonic mucosa. Expression of PiT-1 tended (P=0.055) to be enhanced by 8% with intake of high Ca but not with phytase (P=0.827). Transcript levels of XPR1 and PiT-2 were unaltered by the treatments.

Colonic CLDN-4 and -10 mRNA were reduced by 20 and 40%, respectively with high Ca intake (P=0.027 and <0.001, respectively), but not with phytase (P=0.603 and 0.406, respectively). Phytase tended (P=0.072) to enhance the expression of CLDN-12 by 8%. Furthermore, CLDN-7 mRNA was approximately 10% lower after high Ca intake only when no phytase was supplied ($P_{interaction}$=0.046). In contrast, the treatments did not affect CLDN-2 and ZO-1 mRNA levels in the colon. CLDN-15, -16 and -17 mRNA in this segment were not detectable.

Phytase tended (P=0.052) to enhance colonic expression of vitamin D_3 receptor (VDR) by 12% while high Ca intake had no impact (P=0.498). Moreover, high dietary Ca intake reduced expression of inositol transporter by 9% (Na/myo-inositol cotransporter (SMIT), P=0.026). Neither dietary Ca level nor phytase impacted colonic expression of a candidate mucosal phosphatase (MINPP1). The other mucosal phosphatase (IAP), as well as vitamin-D_3 24 hydroxylase (CYP24) were not expressed.

Jejunal expression levels

The jejunal level of CaBP-D9k mRNA was more than 4000-times higher compared to CaBP-D28k mRNA (Table 4). Dietary treatments, however, did not alter their expression levels, nor these of NCX1. Jejunal mRNA levels of TRPV6, on the contrary, were reduced (P<0.003) with high Ca and phytase intake (56% and 30% reduction, respectively). Also, TRPV5 mRNA was reduced by 32% with phytase, but not with dietary Ca intake (P=0.003 and 0.746, respectively). Expression of TRPM7 was elevated by 7% with high dietary Ca (P=0.040), but not with phytase intake (P=0.285).

Table 4. Least squares means of mRNA levels of calcium (Ca) and phosphorus (P) transporter and claudins (CLDN) as affected by dietary Ca content and microbial phytase supplementation in the jejunal mucosa of growing pigs [1,2,3]

Item	Dietary treatments					*P*-value		
Phytase, FTU/kg	0		500		Pooled SEM	Ca	Phytase	Ca × Phytase
Ca, g/kg	2.0	9.6	2.0	9.6				
Ca transporters								
CaBP-D9k	261	308	229	254	60.6	0.179	0.112	0.671
CaBP-D28k	0.099	0.064	0.071	0.060	0.028	0.333	0.486	0.605
NCX1	1.57	1.68	1.56	1.69	0.099	0.105	0.992	0.879
TRPM7	15.5	16.0	14.5	16.0	0.65	0.040	0.285	0.302
TRPV5	0.025	0.029	0.020	0.017	0.004	0.746	0.003	0.207
TRPV6	1.15	0.61	0.93	0.31	0.160	<0.001	0.003	0.630
P transporters								
NaPi-IIc	0.34	0.38	0.34	0.34	0.056	0.407	0.397	0.411
PiT-1	4.49	4.89	4.33	4.35	0.229	0.367	0.135	0.401
PiT-2	28.6	28.8	30.2	22.5	4.76	0.276	0.492	0.250
XPR1	7.90	7.17	8.10	7.20	0.480	0.024	0.734	0.797
Tight junctions								
CLDN-2	18.7	24.8	17.3	28.4	2.89	<0.001	0.598	0.233
CLDN-4	66.9	69.4	69.3	61.9	6.98	0.622	0.606	0.323
CLDN-7	23.5	23.9	24.2	24.3	1.30	0.811	0.558	0.910
CLDN-10	0.87	0.40	0.71	0.40	0.141	<0.001	0.408	0.417
CLDN-12	9.69	10.67	11.21	11.62	0.661	0.150	0.013	0.544
CLDN-15	17.1	17.4	17.8	18.5	1.60	0.665	0.433	0.851
ZO-1	3.61	3.64	3.51	3.49	0.097	0.949	0.086	0.695
Other proteins								
IAP	34.3	35.1	37.5	37.9	3.76	0.828	0.248	0.934
MINPP1	7.79	7.38	7.37	7.71	0.422	0.915	0.869	0.221
SMIT	5.69	5.60	5.21	5.51	0.412	0.719	0.339	0.511
VDR	9.1	19.5	10.1	21.1	1.52	<0.001	0.390	0.862

[1] Dietary P content was fixed at 4.7 g/kg diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[3] Determined using absolute quantification normalized by IPO8.
CaBP-D9k, calbindin-D9k; CaBP-D28k, calbindin-D28k; IAP, intestinal alkaline phosphatase; IPO8, importin 8; MINPP1, multiple inositol polyphosphate phosphatase 1; NaPi-IIc, sodium-dependent phosphate transporter type IIc; NCX1, sodium-Ca exchanger; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; SMIT, sodium/myo-inositol cotransporter; 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_3 receptor; XPR1, xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1. Of note, transcript levels of Ca sensing receptor, sodium-dependent phosphate transporter type IIa and IIb were beyond the limit of detection.

In the jejunal mucosa, expression of NaPi-IIc, PiT-1 and PiT-2 remained unaffected by the treatments. High Ca but not phytase intake decreased XPR1 mRNA (10% reduction with high Ca; P=0.024 and 0.734, respectively). Noteworthy, jejunal PiT-1 and PiT-2 mRNA levels were approximately three times lower than those in colonic mucosa (compare Table 3). Transcripts encoding NaPi-IIa and NaPi-IIb were undetectable.

Pigs receiving high dietary Ca had approximately 50% greater (P<0.001) and 50% lower (P<0.001) expression of CLDN-2 and -10, respectively. Phytase inclusion elevated jejunal CLDN-12 expression by 12% (P=0.013). Expression of ZO-1 tended (P=0.086) to be marginally reduced with phytase. The dietary treatments did not influence the transcript levels of CLDN-4, -7 and -15.

Intake of high Ca, but not phytase, increased VDR mRNA by two-fold (P<0.001 and 0.390, respectively). Expression of MINPP1, IAP and SMIT were not affected by the dietary treatments.

Discussion

The accompanying data reported previously showed that Ca and P absorption in the colon were higher in growing pigs fed a phytase free compared to a phytase supplemented diet (Chapter 2). We hypothesized that the phytase-induced reduction in colonic Ca and P absorption would be accompanied by a reduced expression level of Ca and P transporters in the colon. The data reported here fully supported the hypothesis: microbial phytase and high Ca intake reduced mRNA expression of TRPV5, TRPV6 and CaBP-D9k in the colonic mucosa (Table 3). A similar inhibitory effect of these two dietary factors was observed regarding the expression of TRPV6 in the porcine jejunal mucosa (Table 4). We conclude that the expression level of Ca transporters in both the jejunal (TRPV6) and colonic (TRPV5, TRPV6 and CaBP-D9k) mucosa are regulable by dietary Ca and phytase intake and that their mRNA expression level negatively correlates with the amount of absorbed Ca in the intestine.

Effect on colonic Ca transporters, phosphatases and CLDN

In line with our previous study (Chapter 2), post-ileal Ca absorption in pigs was also observed by others (e.g. Liu *et al.* [15]), but the molecular mechanisms clarifying this observation remained to be elucidated. Here, we demonstrate that phytase inclusion tended to elevate the expression of CLDN-12 mRNA in the colonic mucosa of pigs. A previous study in mice and Caco-2 cell line demonstrated that CLDN-2 and -12 form pores permitting Ca ions to pass through, and their expression was upregulated by $1,25(OH)_2D_3$ exposure [16]. This indicates that the enhanced expression of CLDN-12 in response to phytase inclusion might improve the colonic mucosal permeability to Ca.

Contrary to CLDN-12, expression of TRPV5, TRPV6 and CaBP-D9k was reduced with phytase and high Ca intake. Expression of Ca transporters in the colon of pigs has not been reported previously. *In vivo* studies in mice demonstrated that a high dietary Ca level reduced mRNA levels of TRPV6 in the proximal colon [17]. Using the Ussing chamber technique, the researchers in the same study further demonstrated that application of high extracellular Ca concentration inhibited transcellular Ca absorption in proximal colon tissues of mice. Their results indicate that colonic Ca absorption, particularly active transcellular Ca absorption, is reduced by a high dietary Ca intake, which was in line with our observations in pigs. This modulation of Ca transport might be mediated via the action of PTH and vitamin D_3 [18], as we observed that the inhibition of expression of TRPV5, TRPV6 and CaBP-D9k in response to high dietary Ca intake was accompanied by a lower serum PTH content. We propose that the elevated serum Ca content in response to high dietary Ca intake inhibits PTH secretion by stimulating CaSR expressed in the parathyroid gland [19]. A reduction of PTH, in turn, decreases circulatory $1,25(OH)_2D_3$ to limit renal Ca reabsorption by the upregulation of renal expression of CYP24 mRNA, encoding a mitochondrial monooxygenase responsible for the modification and inactivation of bioactive $1,25(OH)_2D_3$. The observed downregulation of TRPV5 and TRPV6 mRNA expression in the colon could be ascribed to the reduced concentration of serum $1,25(OH)_2D_3$ since the genes of these transporters contain a VDR-responsive element in their promoter region [20]. The upregulated expression of VDR in both jejunal and colonic mucosa in response of high Ca and/or phytase intake supports this idea. In mice, Ca-sensing by CaSR located in the intestine may decrease expression of TRPV6

and CaBP-D9k directly [17]. However, this mechanism seems to be unlikely in pigs since CaSR mRNA was beyond the limit of detection in both jejunal and colonic mucosa in the present study (data not shown).

We determined the mRNA expression of mucosal phosphatases (MINPP1 and IAP) and inositol transporters (SMIT) assuming that their expression level would be affected by our dietary treatments. However, IAP mRNA was beyond the limit of detection in colon and not affected by dietary treatments in jejunum. Moreover, MINPP1 mRNA was altered neither by phytase inclusion nor dietary Ca intake in both intestinal segments. The MINPP1 is an intercellular phosphatase located in endoplasmic reticulum. Recent *in vitro* studies in the Caco-2 [21] and H1299 cell line [22] indicate that MINPP1 is secreted into the medium and can catalyse dephosphorylation of extracellular IP. It is, therefore, possible that MINPP1 is involved in the degradation of IP in the intestine. Previous studies in broilers indicated that high Ca intake reduced mucosal phosphatase efficacy to degrade IP in the brush-border vesicles of the small intestine [23]. Brun *et al.* [24, 25] also reported that high Ca intake modified activity of IAP without affecting its protein expression level in the small intestine of rats. These studies support our results and indicate that dietary Ca intake or phytase does not affect the level of IAP expression to regulate the efficacy of IAP. Our results also demonstrated that colonic expression of SMIT was significantly reduced with high dietary Ca intake although this effect was minor. We observed that high Ca intake reduced the degradation of IP in the small intestine in pigs (Chapter 2), which subsequently promotes the available of IP for degradation into inositol in the colon.

The colon plays an important role in mineral and water absorption in pigs [26]. The reduction of mRNA expression of CLDN-4, -7 and -10 in the colon indicates that high Ca intake impaired paracellular permeability for anions and cations in the porcine colon. Unfortunately, the ion permeability of these CLDN members is still debated. Van Itallie *et al.* [27] reported that CLDN-4 can mediate Cl permeation, while Hou *et al.* [28] argued that it builds pores for Na. Experiments to unravel the ion-selectivity of CLDN-7 led to controversial results; the protein may mediate transepithelial permeation of magnesium [29] or Cl [30]. Other studies (e.g. Van Itallie *et al.* [31]) demonstrated the existence of two splice variants of CLDN-10, designated CLDN-10a and CLDN-10b, each with different physiological functions in mice. By contrast, Gunzel *et al.* [32] reported the existence of six different

isoforms of CLDN-10 as a result of alternative splicing in mice and humans. We performed an intensive Blast search (Build Sscrofa11.1) and analysed two porcine CLDN-10 homologues: CLDN-10a (accession no. XM_021065094) and CLDN-10b (accession no. XM_021065095). In the present study, we used a primer set that hybridizes to a completely identical region, shared by both isoforms in order to determine the sum expression level. Taken together, downregulation of CLDN-4, -7 and -10 may alter the mucosal paracellular permeability to traverse certain minerals across the colonic epithelial layer. It remains unknown which specific minerals are involved.

Effect on jejunal Ca transporters and CLDN

Similar to the colon, high Ca and phytase intake reduced TRPV6 expression in jejunal mucosa. Gonzalez-Vega *et al.* [33] reported that incremental dietary Ca intake linearly reduced jejunal expression of TRPV6 and CaBP-D9k, which is in agreement with our results. Noteworthy, in our study jejunal TRPV5 expression was not affected by dietary Ca level, whereas it was not reported by Gonzalez-Vega *et al.* [33]. An explanation may be that TRPV5 in mice is primarily expressed in the kidney [33], while TRPV6 is ubiquitously expressed [2]. Indeed, we found that TRPV6 expression was approximately 30 and 50 time higher than TRPV5 in porcine jejunal and colonic mucosa, respectively. By contrast, renal expression of TRPV5 in pigs was approximately 6 times higher than TRPV6 (Chapter 7). Collectively, it appears that TRPV5 is less involved in intestinal Ca absorption compared to TRPV6. We also observed an upregulation of TRPM7 with dietary Ca intake in the jejunal mucosa. However, unlike TRPV5 and TRPV6, two channels highly selective for Ca [34], TRPM7 seems to be less selective with higher affinity for zinc, magnesium and manganese than Ca [35]. An upregulation of TRPM7 might be indicative of an antagonistic impact of a high dietary Ca against other cations.

Contrary to TRPV5 and TRPV6, CLDN-2 and -12 mRNA expression in the jejunal mucosa was enhanced by high Ca and phytase intake, respectively. This regulation pattern of CLDN-2 and -12 was in line with the soluble inorganic Ca content in the distal small intestine, which indicated that Ca was transported in the direction of the electrochemical gradient across the epithelial layer with high Ca intake but against it with low Ca intake (**Supplementary Table1**). It could be

that low dietary Ca intake reduced CLDN-2 expression in order to prevent the backflow of Ca to the luminal side. By contrast, high dietary Ca intake enhanced CLDN-2 expression probably to increase the capacity of passive Ca uptake as an energy-saving process. Taken together, the inhibitory action on the expression of TRPV6, and also TRPV5 and CaBP-D9k in the colon, and stimulatory effect on Ca permeable CLDN-2 and CLDN-12 with high Ca intake, indicates that paracellular Ca permeation was minor at a low but more pronounced with high Ca intake. Thus, Ca absorption shifts from transcellular to paracellular pathway with high dietary Ca and phytase intake.

Compared to CaBP-D9k, transcript levels of CaBP-D28k were very low in the jejunal mucosa, even absent in the colonic mucosa. This indicates that CaBP-D9k may play a greater role in shuttling Ca across cytoplasm in the GIT of pigs. Christakos *et al.* [36] reviewed that CaBP-D28k was expressed highest in the avian intestine and mammalian kidney, while CaBP-D9k was observed only in mammals and was abundantly expressed in the mammalian intestine. Indeed, we observed a much higher expression of CaBP-D28k compared to CaBP-D9k in the kidney of pigs (Chapter 7). Thus, distribution of CaBP-D28k and CaBP-D9k appears tissue-specific in pigs. Moreover, despite the similar names, the physiological role of CaBP-D28k may differ from CaBP-D9k. A previous *in vivo* study using transgenic mice [37] indicated that mice lacking CaBP-D28k but with normal expression of CaBP-D9k developed little calcaemic abnormalities; by contrast, mice expressing only 10% of CaBP-D9k but with normal expression level of CaBP-D28k developed hypocalcaemia and rickets. It seems that the Ca buffering function of CaBP-D28k can be largely compensated by CaBP-D9k, but not *vice versa*. It is, therefore, possible that CaBP-D9k plays a greater role in the transit of Ca across the intestinal mucosa to serve whole body Ca homeostasis.

Effect on P transporters

Instead of NaPi-IIb, we detected NaPi-IIc in the porcine jejunum. Moreover, all type II transporters were absent in the colonic mucosa. Apparently, NaPi-IIc is the most important Na-P cotransporter in the small intestine of pigs. Wubuli *et al.* [38] investigated the tissue-wide mRNA expression status of all currently annotated P transporters. Their results combined with our observation indicate that NaPi-IIc is

the only type II P transporter abundantly expressed in the GIT of pigs and that this isotype contributes to intestinal P absorption. We demonstrated that high Ca uptake reduced intestinal P absorption in the small intestine of these pigs (Chapter 2). Here, we found that the level of NaPi-IIc mRNA was neither affected by dietary Ca level nor phytase inclusion, suggesting that the reduction of P absorption was attributed to a lower NaPi-IIc activity which was regulated at the posttranscription level, e.g. vesicle trafficking of NaPi-IIc to-and-from the apical membrane.

We observed a nominally (P=0.055) elevated expression of PiT-1 mRNA in the colon when pigs were fed a high Ca compared to low Ca diet. Previous transgenic studies showed that overexpression of PiT-1 enhanced serum P and reduced serum Ca in both rats [39] and mice [40]. These data raise the possibility that high dietary Ca led to insoluble Ca-P complexes impairing intestinal P absorption and serum P content. A higher expression of PiT-1 may increase the capacity of P absorption in the pig's colon to support systemic P homeostasis.

Paracellular P permeation might also substantially contribute to intestinal P absorption in pigs. Soluble inorganic P content was much lower in the serum than in the digesta in all GIT segments (Supplementary Table 1), which generated a wide electrochemical gradient difference of P across the epithelium cell. To the knowledge of the authors, paracellular P absorption in pigs has not been reported before. Using the *in situ* ligated intestinal loop technique, Marks *et al.* [41] demonstrated in rats that only 30% of the intestinal P absorption was Na dependent, irrespective of P content in the buffer solution instilled into the GIT segments. Their results suggest that P absorption might be primarily mediated via paracellular permeation in the GIT. This assumption is in line with an *in vivo* study in pigs [42] which demonstrated that incremental dietary P level linearly enhanced total tract P digestibility. Thus, P absorption is highly dependent on luminal P content, which suggested that paracellular P absorption may be substantial in pigs. However, the mechanism for P permeation remains unknown.

In conclusion, Ca absorption shifts from transcellular to paracellular pathway with high dietary Ca and phytase intake, as evidenced by downregulation of jejunal and colonic genes related to transcellular Ca absorption and upregulation of Ca pore forming claudins (CLDN-2 and -12). Paracellular P absorption may be substantial in pigs as expression of genes related to transcellular P absorption remain mostly unaffected by dietary Ca and phytase content.

Acknowledgement

This research was conducted by Wageningen Livestock Research, within the framework of the public private partnership "Feed4Foodure" and partially funded by the Ministry of Economic Affairs (Policy Support Research project number BO-31.03-005-00). Mr L. Kruijt for assistance in RT-PCR analysis, and the Chinese Scholarship Council (CSC) for the scholarship of Y.X. Hu to study at Wageningen University & Research.

Author contributions

P. Bikker, J.W. Resink, M.M. van Krimpen and Y.X. Hu designed and conducted the research; Y.X. Hu and A. Liesegang analysed the samples and data; J. van Baal designed and validated the RT-qPCR primers. Y.X. Hu, P. Bikker, J. van Baal and W.H. Hendriks interpreted the data and wrote the manuscript. All authors with the exception of the late M.M. van Krimpen have read and approved the final manuscript. Mr J.W. Resink is an employee of Trouw Nutrition, all other authors declare no conflict of interest.

References

1. Takeda E, Yamamoto H, Yamanaka-Okumura H *et al.* (2012) Dietary phosphorus in bone health and quality of life. *Nutr Rev* **70**, 311-321.

2. Khanal RC & Nemere I (2008) Regulation of intestinal calcium transport. *Annu Rev Nutr* **28**, 179-196.

3. Forster IC, Hernando N, Biber J *et al.* (2013) Phosphate transporters of the SLC20 and SLC34 families. *Mol Aspects Med* **34**, 386-395.

4. Biber J, Hernando N & Forster I (2013) Phosphate transporters and their function. *Annu Rev Physiol* **75**, 535-550.

5. Giovannini D, Touhami J, Charnet P *et al.* (2013) Inorganic phosphate export by the retrovirus receptor XPR1 in metazoans. *Cell Rep* **3**, 1866-1873.

6. Tsukita S, Tanaka H & Tamura A (2019) The claudins: from tight junctions to biological systems. *Trends Biochem Sci* **44**, 141-152.

7. Fujita H, Chiba H, Yokozaki H *et al.* (2006) Differential expression and subcellular localization of claudin-7, -8, -12, -13, and -15 along the mouse intestine. *J Histochem Cytochem* **54**, 933-944.

8. Breiderhoff T, Himmerkus N, Stuiver M *et al.* (2012) Deletion of claudin-10 (Cldn10) in the thick ascending limb impairs paracellular sodium permeability and leads to hypermagnesemia and nephrocalcinosis. *Proc Natl Acad Sci U S A* **109**, 14241-14246.

9. Tamura A, Hayashi H, Imasato M *et al.* (2011) Loss of claudin-15, but not claudin-2, causes Na^+ deficiency and glucose malabsorption in mouse small intestine. *Gastroenterol* **140**, 913-923.

10. Van Itallie C, Rahner C & Anderson JM (2001) Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. *J Clin Invest* **107**, 1319-1327.

11. Alexandre MD, Jeansonne BG, Renegar RH *et al.* (2007) The first extracellular domain of claudin-7 affects paracellular Cl^- permeability. *Biochem Biophys Res Commun* **357**, 87-91.

12. Beutel O, Maraspini R, Pombo-García K *et al.* (2019) Phase separation of zonula occludens proteins drives formation of tight junctions. *Cell* **179**, 923-936.

13. Gonzalez-Vega JC, Walk CL, Liu Y *et al.* (2014) The site of net absorption of Ca from the intestinal tract of growing pigs and effect of phytic acid, Ca level and Ca source on Ca digestibility. *Arch Anim Nutr* **68**, 126-142.

14. Andersen CL, Jensen JL & Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* **64**, 5245-5250.

15. Liu J, Bollinger DW, Ledoux DR *et al.* (2000) Effects of dietary calcium:phosphorus ratios on apparent absorption of calcium and phosphorus in the small intestine, cecum, and colon of pigs. *J Anim Sci* **78**, 106-109.

16. Fujita H, Sugimoto K, Inatomi S *et al.* (2008) Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca^{2+} absorption between enterocytes. *Mol Biol Cell* **19**, 1912-1921.

17. Lee JJ, Liu X, O'Neill D *et al.* (2019) Activation of the calcium-sensing receptor attenuates TRPV6-dependent intestinal calcium absorption. *JCI insight* **4**, e128013.

18. Sassi F, Tamone C & D'Amelio P (2018) Vitamin D: nutrient, hormone, and immunomodulator. *Nutrients* **10**, 1656.

19. Chen RA & Goodman WG (2004) Role of the calcium-sensing receptor in parathyroid gland physiology. *Am J Physiol Renal Physiol* **286**, F1005-F1011.

20. Hoenderop JGJ, Nilius B & Bindels RJM (2005) Calcium absorption across epithelia. *Physiol Rev* **85**, 373-422.

21. Briviba K, Schollenberger M, Rodehutscord M *et al.* (2018) Dephosphorylation of myo-inositol phosphates in the *in vitro* intestinal Caco-2 cell model. *Int J Food Sci Nutr* **69**, 46-51.

22. Windhorst S, Lin H, Blechner C *et al.* (2013) Tumour cells can employ extracellular Ins(1,2,3,4,5,6)P_6 and multiple inositol-polyphosphate phosphatase 1 (MINPP1) dephosphorylation to improve their proliferation. *Biochem J* **450**, 115-125.

23. Applegate T, Angel R & Classen H (2003) Effect of dietary calcium, 25-hydroxycholecalciferol, or bird strain on small intestinal phytase activity in broiler chickens. *Poult Sci* **82**, 1140-1148.

24. Brun LR, Traverso A & Rigalli A (2009) Aggregation and inhibition of rat intestinal alkaline phosphatase by high concentrations of calcium. Reversibility of the processes. *J Enzyme Inhib Med Chem* **24**, 691-696.

25. Brun LR, Brance ML & Rigalli A (2012) Luminal calcium concentration controls intestinal calcium absorption by modification of intestinal alkaline phosphatase activity. *Br J Nutr* **108**, 229-233.

26. Breves G, Kock J & Schröder B (2007) Transport of nutrients and electrolytes across the intestinal wall in pigs. *Livest Sci* **109**, 4-13.

27. Van Itallie C, Rahner C & Anderson JM (2001) Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. *J Clin Invest* **107**, 1319-1327.

28. Hou J, Renigunta A, Yang J *et al.* (2010) Claudin-4 forms paracellular chloride channel in the kidney and requires claudin-8 for tight junction localization. *Proc Natl Acad Sci U S A* **107**, 18010-18015.

29. Thongon N & Krishnamra N (2012) Apical acidity decreases inhibitory effect of omeprazole on Mg^{2+} absorption and claudin-7 and -12 expression in Caco-2 monolayers. *Exp Mol Med* **44**, 684-693.

30. Fan J, Tatum R, Hogard J *et al.* (2019) Claudin-7 modulates Cl^- and Na^+ homeostasis and WNK4 expression in renal collecting duct cells. *Int J Mol Sci* **20**, 3798.

31. Van Itallie CM, Rogan S, Yu A *et al.* (2006) Two splice variants of claudin-10 in the kidney create paracellular pores with different ion selectivities. *Am J Physiol Renal Physiol* **291**, F1288-F1299.

32. Gunzel D, Stuiver M, Kausalya PJ *et al.* (2009) Claudin-10 exists in six alternatively spliced isoforms that exhibit distinct localization and function. *J Cell Sci* **122**, 1507-1517.

33. Gonzalez-Vega JC, Liu Y, McCann JC *et al.* (2016) Requirement for digestible calcium by eleven- to twenty-five-kilogram pigs as determined by growth performance, bone ash concentration, calcium and phosphorus balances, and expression of genes involved in transport of calcium in intestinal and kidney cells. *J Anim Sci* **94**, 3321-3334.

34. den Dekker E, Hoenderop JGJ, Nilius B *et al.* (2003) The epithelial calcium channels, TRPV5 & TRPV6: from identification towards regulation. *Cell Calcium* **33**, 497-507.

35. Bates-Withers C, Sah R & Clapham DE (2011) TRPM7, the Mg^{2+} inhibited channel and kinase. *Adv Exp Med Biol* **704**, 173-183.

36. Christakos S, Mady LJ & Dhawan P (2018) Chapter 21 - Calbindin-D_{28K} and calbindin-D_{9K} and the epithelial calcium channels TRPV5 and TRPV6. In *Vitamin D (Fourth Edition)*, pp. 343-359 [Feldman D, Pike JW, Bouillon R, Giovannucci E, Goltzman D and Hewison M, editors]. London: Academic Press.

37. Zheng W, Xie Y, Li G *et al.* (2004) Critical role of calbindin-D28k in calcium homeostasis revealed by mice lacking both vitamin D receptor and calbindin-D28k. *J Biol Chem* **279**, 52406-52413.

38. Wubuli A, Reyer H, Murani E *et al.* (2019) Tissue-wide gene expression analysis of sodium/phosphate co-transporters in pigs. *Int J Mol Sci* **20**, 5576.

39. Suzuki A, Ammann P, Nishiwaki-Yasuda K *et al.* (2010) Effects of transgenic Pit-1 overexpression on calcium phosphate and bone metabolism. *J Bone Miner Metab* **28**, 139-148.

40. Chande S, Ho B, Fetene J *et al.* (2019) Transgenic mouse model for conditional expression of influenza hemagglutinin-tagged human SLC20A1/PIT1. *PLoS One* **14**, e0223052.

41. Marks J, Lee GJ, Nadaraja SP *et al.* (2015) Experimental and regional variations in Na^+-dependent and Na^+-independent phosphate transport along the rat small intestine and colon. *Physiol Rep* **3**, e12281.

42. Stein HH, Kadzere CT, Kim SW *et al.* (2008) Influence of dietary phosphorus concentration on the digestibility of phosphorus in monocalcium phosphate by growing pigs. *J Anim Sci* **86**, 1861-1867.

Supplementary Table 1. Least squares means of soluble inorganic calcium (Ca) and phosphorus (P) content (mM) along the intestine and serum inorganic Ca and P as affected by dietary Ca level and microbial phytase supplementation in growing pigs [1,2,3].

Item	Dietary treatment					
Phytase, FTU/kg	0			500		
Ca, g/kg	2.0	5.8	9.6	2.0	5.8	9.6
Inorganic Ca						
Stomach	6.8	23.9	37.2	6.8	21.6	36.0
Proximal-SI	3.9	5.3	12.5	1.6	6.0	13.6
Distal-SI	0.6	2.6	4.6	0.6	2.7	4.9
Proximal-LI	5.4	7.0	7.0	2.0	9.2	9.6
Distal-LI	1.3	1.7	2.6	0.5	1.8	3.0
Serum	2.38	2.78	3.38	2.35	2.48	2.82
Inorganic P						
Stomach	20.9	24.4	21.2	23.9	23.8	25
Proximal-SI	20.6	12.0	12.3	19.4	17.1	15.8
Distal-SI	7.4	7.2	5.9	15.1	12.5	9.7
Proximal-LI	45.8	38.7	31.6	28.8	34.0	27.2
Distal-LI	18.5	16.9	15.8	12.6	13.9	11.2
Serum	2.90	2.19	1.85	3.23	3.09	2.63

[1] Dietary P content was fixed at 4.7 g/kg diet.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[3] Statistically significant effects of dietary Ca level and phytase inclusion are reported in the previous study (Chapter 2).
SI, small intestine; LI, large intestine.

CHAPTER 4

Coarse limestone does not alleviate the negative effect of a low Ca/P ratio diet on characteristics of tibia strength and growth performance in broilers

Y.X. Hu[*†], P. Bikker[*], M. Duijster[‡], W.H. Hendriks[†], J. van Baal[†], M.M. van Krimpen[*]

[*] Wageningen University & Research, Wageningen Livestock Research, 6700 AH Wageningen, the Netherlands

[†] Wageningen University & Research, Animal Nutrition Group, 6700 AH Wageningen, the Netherlands

[‡] De Heus Animal Nutrition B.V., 6710 BJ Ede, the Netherlands

Poultry Science (2020) 99:4978–4989

https://doi.org/10.1016/j.psj.2020.06.037

Abstract

The hypothesis was tested that an increased digestion of coarse compared to fine limestone can alleviate the negative effects of a low dietary Ca/P ratio on the growth performance and characteristics of tibia strength (CTS) in broilers. A total of 1152 Ross 308 broiler chickens received a standard commercial starter feed from d 0-13. From d 14 onwards, birds received one of 12 diets containing one of six Ca/P ratios (0.50, 0.75, 1.00, 1.25, 1.50 and 1.75) and one of two limestone particle sizes (<500 (fine) and 500-2,000 (coarse) μm) in a study with a 6×2 factorial arrangement of treatments. Total P content was fixed at 5.5 g/kg for all treatment diets. Each treatment was replicated six times with 16 birds per replicate pen. On d 20 and 21, 12 birds per pen were randomly selected from four of the six replicate pens for tibia analysis and digesta collection from different gut segments. The apparent Ca digestibility was higher for fine than coarse limestone in the jejunum (P=0.043). However, this difference in Ca digestibility disappeared for the low while it remained for the high Ca/P ratios in the proximal ($P_{interaction}$=0.067) and distal ($P_{interaction}$=0.052) ileum. In addition, coarse limestone improved apparent P digestibility in the proximal and distal ileum (P<0.001) but not in the jejunum (P=0.305). Regardless of limestone particle size, reducing dietary Ca/P ratio linearly improved apparent Ca and P digestibility in the proximal and distal ileum (P<0.001). Moreover, decreasing dietary Ca/P ratio linearly (P<0.001) and quadratically (P<0.046) reduced the CTS. Reducing dietary Ca/P ratio linearly (P<0.001) and quadratically (P≤0.006) decreased body weight gain and feed conversion ratio. For both fine and coarse limestone, the optimal Ca/P ratio was 1.00-1.25 to optimize apparent Ca and P digestibility while maintaining growth performance and CTS. Reducing Ca/P ratio from 1.75 to 1.00 improved apparent distal ileal Ca and P digestibility from 36.6 to 53.7% and 48.0 to 58.3%, respectively. In conclusion, coarse limestone is equally digestible with fine limestone at a low Ca/P ratio but is less digestible at a high Ca/P ratio, and the optimal Ca/P ratio in the diet is 1.00-1.25 for both fine and coarse limestone.

Key words: particle size of limestone, Ca/P ratio, phosphorus and calcium digestibility, broilers

Introduction

Phosphorus (P) and calcium (Ca) are essential macro nutrients for all animals and play an important role in numerous physiological processes including bone formation [1]. Improving P utilization helps to alleviate the global mineral P depletion, save feed costs as well as reduce environmental pollution of P. Calcium plays a pivotal role in P utilization as it hampers P absorption via Ca-P complexation in the gut, but it is essential to bind P to form hydroxyapatite in bone [2]. Reducing the dietary Ca/P ratio is an effective way to improve P digestibility [3], but over-reduction of the dietary Ca/P ratio compromises growth performance and characteristics of tibia strength (CTS) [4].

Compared to fine limestone (particle size <500 µm), coarse limestone (particle size 1000-2000 µm) has been shown to be more digestible in broilers, with a true ileal digestibility of 70 vs 40% [5; 6]. It, therefore, seems possible to reduce the dietary Ca/P ratio through the use of coarse limestone while maintaining growth performance and bone development. In addition, in the presence of microbial phytase, ileal P digestibility was reduced by pulverised limestone (particle size <75 µm), while it was not affected by the particulate limestone (particle size 402 µm) [7]. The P digestion seems to be related to the particle size of limestone, and the coarser limestone may have a less negative impact on P digestion. It is hypothesized that coarse limestone with its higher digestibility in broilers, can alleviate the negative effect of a low Ca/P ratio on growth performance and bone development and may improve P digestibility, compared to fine limestone. The objective of the present study was to determine the impact of limestone particle size and dietary Ca/P ratio on apparent Ca and P digestibility in different gut segments, CTS as well as growth performance in broilers.

Materials and methods

The experiment was conducted in the broiler research accommodation of De Heus (Eerde, the Netherlands). All procedures complied with the Dutch law on animal experiments and study was approved by the Ethical Committee of Wageningen University & Research, the Netherlands (no. 2016.D-0065.004).

Animal housing and management

A total of 1152 0-d-old Ross 308 male broilers, housed in 72 pens (1 m^2, 16 birds per pen) on wood shavings (0.9-1.0 kg/m^2) were used. The barn was mechanically ventilated and the temperature was controlled by a climate computer. The room temperature was set at 35 °C on the day of arrival, and thereafter gradually decreased by 1 °C per day to 20-18 °C. A light: dark schedule of 18:6 was used in the barn with a light intensity of 20 lux. Continuous lighting (24L:0D) was used on the first 2 days of the experiment (d 0-1), as well as the 4 d before and during dissection (d 18-21). Dead birds or birds with visible malfunction (e.g. scissor beak, ascites, torticollis) were removed and weighed at the time of removal. Birds had *ad libitum* access to water and feed throughout the experiment (d 0-39). All birds received a normal commercial starter feed from d 0-13 (2973 kcal/kg ME, 212 g/kg CP, 8.6 g/kg Ca, 5.5 g/kg P). Experimental diets were provided to the animals from d 14 onwards.

Experimental treatments and diets

On d 14, birds were weighed and randomly allotted to one of 12 treatments in a 6×2 factorial arrangements of treatments including six dietary Ca/P ratios (0.50, 0.75, 1.00, 1.25, 1.50 and 1.75) and two limestone particle sizes (<500 (fine) and 500-2000 µm (coarse)). Each treatment was replicated six times. The coarse and fine limestone were obtained from the same limestone product (Sibelco, Maastricht, the Netherlands) via sieving through a 500 µm screen (Retsch, GmbH, Germany).

A basal diet was made and then split into 12 equal portions, to which the required amount of coarse or fine limestone was added at the expense of diamol (Damolin, Kønsborgvej, Denmark) according to the experimental design. The basal diet met or exceeded the minimum requirement of all nutrients except Ca [8] and included titanium dioxide at 5 g/kg as an indigestible marker. The composition of the grower (d 14-29) and finisher (d 30-39) diets is shown in **Table 1** and **Supplementary Table 1**, respectively. The P content was 5.5 and 5.1 g/kg in all grower and finisher diets, respectively. The Ca content was 2.7-9.6 g/kg for the grower, and 2.5-8.8 g/kg for the finisher diets. The Ca content was below the minimum requirement of 7.0 and 6.0 g/kg for grower and finisher birds,

respectively (Ca/P ratio 1.25 [8]) in the three low Ca/P ratios while exceeding the requirement in the two high Ca/P ratios. The experimental diets were made in increasing order of Ca/P ratio. Experimental diets were produced by a feed production plant for research diets (Research Diet Services in Wijk bij Duurstede, the Netherlands) using a double mixing procedure to assure equal composition of the experimental diets. All experimental diets were given in pelleted form to prevent segregation.

Table 1. Composition and nutrient content (g/kg as-fed basis, d 14-29) of grower diets with fine or coarse limestone and incremental Ca/P ratios

Particle size limestone	Fine [1]						Coarse [2]					
Ca/P ratio	0.50	0.75	1.00	1.25	1.50	1.75	0.50	0.75	1.00	1.25	1.50	1.75
Ingredients												
Corn	354	354	354	354	354	354	354	354	354	354	354	354
Wheat	300	300	300	300	300	300	300	300	300	300	300	300
Soybean meal, extracted	255	255	255	255	255	255	255	255	255	255	255	255
Soybean oil	36.5	36.5	36.5	36.5	36.5	36.5	36.5	36.5	36.5	36.5	36.5	36.5
Rapeseed meal, extracted	10.1	10.1	10.1	10.1	10.1	10.1	10.1	10.1	10.1	10.1	10.1	10.1
Monosodium phosphate	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Monocalcium phosphate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Limestone (fine) [1]	3.6	7.2	10.8	14.4	18.0	21.6	0.0	0.0	0.0	0.0	0.0	0.0
Limestone (coarse) [2]	0.0	0.0	0.0	0.0	0.0	0.0	3.6	7.2	10.8	14.4	18.0	21.6
Salt	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Val (98%)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Met (99%)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
L-Lys (79%)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Thr (88%)	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Salinocox [3]	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Diamol [4]	18.1	14.5	10.9	7.3	3.7	0.0	18.1	14.5	10.9	7.3	3.7	0.0
Titanium dioxide (TiO_2)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Premix [5]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Calculated nutrients												
Dry matter	878	878	878	878	878	878	878	878	878	878	878	878
ME, kcal/kg	3029	3029	3029	3029	3029	3029	3029	3029	3029	3029	3029	3029
Crude protein	194	194	194	194	194	194	194	194	194	194	194	194
Lys	1.02	1.02	1.02	1.02	1.02	1.02	1.02	1.02	1.02	1.02	1.02	1.02
Met	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49
Met+Cys	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6
Thr	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Ca	2.7	4.1	5.5	6.9	8.2	9.6	2.7	4.1	5.5	6.9	8.2	9.6
Total P (P)	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Available P (aP)	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Ca/P	0.50	0.75	1.00	1.25	1.50	1.75	0.50	0.75	1.00	1.25	1.50	1.75

Table 1 (continued). Composition and nutrient content (g/kg as-fed basis, d 14-29) of grower diets with fine or coarse limestone and incremental Ca/P ratios

Particle size limestone	Fine [1]						Coarse [2]					
Ca/P ratio	0.50	0.75	1.00	1.25	1.50	1.75	0.50	0.75	1.00	1.25	1.50	1.75
Analysed nutrients												
Dry matter	888	895	896	895	896	891	891	898	898	896	895	890
Crude protein	187	191	188	187	189	192	188	189	186	190	189	192
Crude fat	60.7	61.4	59.8	59.4	60.1	60.0	60.6	59.9	60.7	61.3	61.2	59.6
Ca	3.1	4.4	6.3	7.2	9.3	10.5	3.1	4.6	5.8	7.8	8.7	10.1
P	5.8	5.8	5.8	5.8	5.9	5.9	5.8	5.9	5.9	5.9	5.9	5.8
Ca/P	0.54	0.76	1.08	1.23	1.59	1.78	0.53	0.78	0.99	1.32	1.47	1.73

[1] Analysed Ca content: 41.1%. Particle size distribution: >2000 µm, 0.0%; 1000-2000 µm, 0.0%; 500-1000 µm, 0.2%; 250-500 µm; 33.6%; <250 µm, 66.2%. Geometric mean diameter 160 µm, geometric standard deviation 96 µm.
[2] Analysed Ca content: 41.1%. Particle size distribution: >2000 µm, 0.0%; 1000-2000 µm, 59.3%; 500-1000 µm, 40.4%; 250-500 µm, 0.1%; <250 µm, 0.2%. Geometric mean diameter 1062 µm, geometric standard deviation 387 µm.
[3] Sacox, Antwerp, Belgium.
[4] Damolin, Kønsborgvej, Denmark.
[5] Provided per kg of diet: 12,000 IE retinol, 2,400 IE cholecalciferol, 50 mg dl-a-tocopherol, 1.5 mg menadione, 2.0 mg thiamine, 7.5 mg riboflavin, 3.5 mg pyridoxine, 20 mg cyanocobalamins, 35 mg niacin, 12 mg D-pantothenic acid, 460 mg choline chloride, 1.0 mg folic acid, 0.2 mg biotin, 80 mg iron, 12 mg copper, 85 mg manganese, 60 mg zinc, 0.4 mg cobalt, 0.8 mg iodine, 0.1 mg selenium, 125 mg antioxidant mixture.

Sample collection

Feed samples were collected in the feed mill during production. Birds were weighed on day of arrival (d 0), experimental diet allotment (d 14), dissection and sample collection (d 20-21), the end of the grower period (d 29) and the end of the experiment (d 39). On d 20 and 21, 12 birds per pen were randomly selected from four of the six replicate pens per treatment and euthanized by electrocution before blood was collected from the carotid artery of two dissected birds per pen. A number of four replicate pens per treatment was considered adequate since the focus was not on comparison of individual treatments but on determination of linear and quadratic effects of Ca/P ratio and the interactions, based on six treatment groups, including 24 pens. Serum was harvested after centrifuging at 3,000×g for 10 min at 4 °C. After exsanguination, the chest cavity and the abdomen were opened. The gastrointestinal tract (GIT) was ligated by tie wraps into eight segments including crop, proventriculus plus gizzard, duodenum, jejunum, the first half of ileum (proximal ileum), the second half of ileum (distal ileum), ceca and colon, to prevent digesta flowing between gut compartments. The proventriculus and gizzard were not separated but merged as one GIT segment, because the proventriculus did not contain any substantial amount of digesta. The jejunum was defined as the GIT segment from the end of the duodenal loop to the Meckel's diverticulum. The ileum was divided into two equal

parts in order to determine the apparent precaecal or distal ileal digestibility of P and Ca [9]. After ligating, the GIT was removed from the birds. The digesta were quantitatively collected per segment by flushing with deionized water. The ceca were emptied by gently squeezing because of the viscosity of the cecal digesta. The digesta samples were pooled per segment per pen, and the pH was determined using a pH meter (Seven2Go, Mettler Toledo, Schwerzenbach, Switzerland). Because of the interference with urine excretion, Ca and P digestibility in the colon was not measured. All digesta samples were stored in the freezer (-20 °C) until further analysis. The right tibia was removed from six of the 12 dissected birds per pen, and stored (-20 °C) prior to a bone breaking test. The remaining birds were kept until d 39 to determine the effect of dietary treatments on growth performance in the overall experimental period from d 14-39.

Observations and chemical analysis

The particle size distribution of the pelleted diets was determined using wet sieving, and the geometric mean diameter (GMD) and geometric standard deviation (GSD) were calculated [10]. To examine the contrast of limestone particle size in pelleted diets, the four diets of the lowest and highest Ca/P ratio for both fine and coarse limestone were incinerated at 550 °C. Particle size distribution of the feed ash was subsequently determined using dry sieving. Diets were also analysed for dry matter by drying at 103 °C [11], N was analysed using the Kjeldahl method and crude protein content was calculated as N×6.25 (FOSS, Hillerod, Denmark) [12]. Particle size distribution of the coarse and fine limestone was determined using dry sieve analysis. The digesta samples were freeze-dried and ground to pass a 1-mm sieve using a Retsch ZM 100 mill (Retsch GmbH, Germany). The ground digesta and feeds were incinerated at 550 °C [13], P content was determined spectrophotometrically (Evolution 201; Thermo scientific, MA) [14] and Ca was determined using atomic absorption spectrometry (AA240 FS; Varian, CA) [15]. Titanium content was determined using a spectrophotometer (Evolution 201; Thermo Scientific, MA) after destruction by H_2SO_4 (FOSS, Hillerod, Denmark), according to Myers *et al.* [16]. Serum Ca and P were analysed using a Cobas 8000 modular analyser for clinical analysis with C701 Photometric measuring unit (Roche Diagnostics Limited, Rotkreuz, Switzerland). Commercially

available kits were used to analyse the serum alkaline phosphatase (ALP, Diatools AG, Villmergen, Switzerland), 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$, Immunodiagnostic Systems GmbH, Germany) and parathyroid hormone (PTH, Immunotopics, San Clemente, CA). The CTS was determined by a bone breaking test using an Instron Texture Analyzer (type 5564, MA). Energy to fracture, maximum compressive load, stiffness and diameter were determined as CTS, as described by Guz *et al.* [17].

Solubility of P was determined in the freeze-dried digesta of the crop, gizzard and jejunum of the lowest and highest Ca/P ratio diet for both coarse and fine limestone. The dried digesta were incubated in a buffer solution to mimic *in vivo* digesta conditions (pH and dry matter) as determined in the subsequent gut segment. This was done to determine the amount of potentially digestible P entering to the next gut segment. Specifically, 1 g of the ground crop digesta was incubated in 2 ml buffer solution to mimic *in vivo* gizzard digesta conditions (pH 3.2, DM 33%), and 1 g of ground gizzard or jejunum sample was incubated in 6 ml buffer solution to mimic *in vivo* jejunum/ileum digesta condition (pH 5.8, DM 14%). The soluble P was extracted by mixing an aliquot of digesta on a horizontal shaker at 150 rpm and 42 °C for 30 (crop digesta) or 40 (gizzard and jejunum digesta) min. Thereafter, sample were centrifuged at 3000×g for 15 min, supernatant with soluble P was discarded, the residue with insoluble was dried and the P content was subsequently determined.

Calculations and statistical analysis

The following equation was used to calculate the nutrient digestibility in gut segments:

$$\text{Digestibility coefficient}, \% = (1 - \frac{\text{X}_{\text{digesta}}}{\text{X}_{\text{diet}}} \times \frac{\text{Ti}_{\text{diet}}}{\text{Ti}_{\text{digesta}}}) \times 100$$

where $X_{digesta}$ and $Ti_{digesta}$ are the nutrient (Ca or P) and Ti content in the freeze-dried digesta (g/kg), respectively and X_{diet} and Ti_{diet} the nutrient (Ca or P) and Ti content in the diet (g/kg), respectively.

The digesta mean retention time (MRT) in different gut segments was calculated according to de Vries and Gerrits [18] as:

$$\mathrm{MRT, min} = \frac{1440 \times \mathrm{Ti_{digesta}} \times \mathrm{W_{digesta}}}{\mathrm{FI} \times \mathrm{Ti_{diet}}}$$

where 1440 are the minutes per day (min/d), $Ti_{digesta}$ the Ti content in the freeze-dried digesta (g/kg), $W_{digesta}$ the quantitative weight of the dried digesta (kg) in each of the respective segments, FI the feed intake over 24 h (kg/d) and Ti_{diet} the Ti content in the diet (g/kg).

The P solubility was calculated using the equation:

$$\mathrm{P\ solubility, \%} = \frac{W_{\mathrm{digesta}} \times \mathrm{P_{digesta}} - \mathrm{W_{residue}} \times \mathrm{P_{residue}}}{\mathrm{W_{digesta}} \times \mathrm{P_{digesta}}} \times 100$$

where $W_{digesta}$ is the weight of freeze-dried digesta used for this test (g), $P_{digesta}$ the P content in the freeze-dried digesta (g/kg), $W_{residue}$ the dried weight of digesta residue after discarding the supernatant (g) and $P_{residue}$ the P content in the dried digesta residue (g/kg).

The CTS was determined on individual tibia (6 tibias per pen), but for all other measurements, samples were pooled per pen and determined at a pen level. Pen was the experimental unit for data analysis. All data except the CTS were subjected to a 2-way ANOVA using the GLM procedure of SAS 9.4 (SAS Institute, Cary, NC). The limestone particle size, Ca/P ratio and their interaction were used as fixed effects. The CTS were subjected to a 2-way ANOVA using the MIXED procedure with limestone particle size, Ca/P ratio and their interaction as fixed effects and pen as random effect. The distribution, variance and homogeneity of Studentized residuals were visually checked via graphics plotted using the ODS GRAPHICS function. The LSMEANS procedure with a PDIFF option was used to estimate the difference between means if a significant interaction was obtained. A CONTRAST procedure was used to estimate the linear and quadratic effect of dietary Ca/P ratio regardless of the limestone particle size. The coefficients for the CONTRAST procedure were obtained using the IML procedure. Probability was considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.1$.

Results

The analysed Ca and P contents in the diets were slightly higher than the calculated levels, but the analysed Ca/P ratios were in good agreement with the designed ratios (Table 1). The GMD and GSD for the coarse limestone were 1062 and 387 µm, respectively and 160 and 96 µm for the fine limestone. As for the diets, wet sieve analysis indicated that the GMD and GSD were increased with the incremental Ca/P ratio for the coarse limestone, and were approximately 174 (163-183) and 261 (243-280) µm for the 6 diets, respectively. In the ash fraction of the lowest and highest Ca/P ratio diets, more coarse particles (>500 µm) were observed for coarse than fine limestone diets. Accordingly, the GMD of feed ashes was greater for coarse than the fine limestone (116 vs 101 at a Ca/P ratio of 0.50 and 196 vs 116 at a Ca/P ratio of 1.75; **Table 2**). 76% of the coarse limestone particles included in the diet were recovered in the feed ashes.

During the whole experimental period, the birds realized a high feed intake and growth rate, and their average body weight at the end of the experiment met or exceeded the performance objectives of the breeding company [19].

Table 2. Dry sieve analysis of ashes of pelleted diets with the highest (1.75) and lowest (0.50) Ca/P ratio for both fine and coarse limestone, % unless otherwise specified [1]

Particle size limestone	Fine		Coarse	
Ca/P ratio	0.50	1.75	0.50	1.75
Sieve diameter, µm				
>2500	0.07	0.88	0.24	0.23
1250-2500	0.07	0.67	0.59	2.60
1000-1250	0.07	0.82	1.42	6.29
630-1000	0.34	0.72	2.43	18.9
320-630	1.28	3.92	2.08	4.85
160-320	6.40	12.6	5.63	5.89
63-160	78.8	64.2	80.8	49.1
<63	13.0	16.1	6.82	12.1
GMD, µm	101	116	116	196
GSD, µm	41	85	73	255

[1] The Ca content and particle size distribution of the coarse and fine limestone are provided in Table 1.
GMD, geometric mean diameter; GSD, geometric standard deviation.

Ca and P apparent digestibility and solubility

No significant interaction was observed between limestone particle size and Ca/P ratio on apparent P or Ca digestibility for any of the gut segments (**Table 3**). For apparent Ca digestibility, a trend for an interactive effect was observed in the proximal ($P_{interaction}$=0.067) and distal ($P_{interaction}$=0.052) ileum. The apparent Ca digestibility was not different between the fine and coarse limestone at the low Ca/P ratio, while it was higher for the fine than the coarse limestone at the high Ca/P ratio. In the jejunum, the apparent Ca digestibility was higher for the fine than for the coarse limestone (P=0.043) irrespective of the Ca/P ratio. Regardless of limestone particle size, the incremental Ca/P ratio linearly (P<0.001) reduced apparent Ca digestibility in the proximal and distal ileum, but not in the jejunum (P=0.246). The apparent P digestibility was higher for the treatments with coarse limestone (P<0.001) in the proximal and distal ileum, but not in the jejunum (P=0.305). Regardless of limestone particle size, increasing the Ca/P ratio, linearly (P<0.001) and quadratically (P<0.001) decreased apparent P digestibility in the proximal and distal ileum. The incremental Ca/P ratio also linearly (P<0.001) decreased and tended to quadratically (P=0.088) decrease apparent P digestibility in the jejunum. The P solubility was not affected by the limestone particle size, Ca/P ratio or their interactions in the crop, gizzard or jejunum (**Table 4**).

Table 3. Effect of dietary Ca/P ratio and particle size of limestone on apparent P and Ca digestibility (%) in the jejunum and ileum in broilers [1,2,3]

Particle size	Ca/P ratio	P digestibility			Ca digestibility		
		Jejunum	Proximal ileum	Distal ileum	Jejunum	Proximal ileum	Distal ileum
Fine	0.50	61.8	72.6	71.8	49.9	59.0	61.9
	0.75	55.6	63.4	65.0	45.5	55.1	57.2
	1.00	49.3	55.4	55.2	49.8	52.2	54.1
	1.25	45.4	50.2	51.9	47.1	42.3	42.9
	1.50	45.6	50.3	50.9	46.2	44.7	49.9
	1.75	40.6	46.8	47.5	39.0	41.5	42.7
Coarse	0.50	62.7	75.5	75.8	48.0	61.6	66.0
	0.75	55.4	66.6	67.8	35.9	58.9	60.9
	1.00	53.8	60.5	61.3	43.0	52.4	53.3
	1.25	48.0	54.4	56.8	42.7	37.1	45.4
	1.50	43.6	51.4	53.0	33.1	42.5	39.1
	1.75	45.0	50.2	48.4	42.0	29.4	30.5
Pooled SEM		2.84	1.36	1.67	4.51	2.74	3.27
Particle size							
Fine		49.7	56.4	57.0	46.2	49.2	51.5
Coarse		51.4	59.8	60.5	40.8	47.0	49.2
Pooled SEM		1.16	0.56	0.68	1.84	1.12	1.34
Ca/P ratio							
0.50		62.3	74.1	73.8	49.0	60.3	64.0
0.75		55.5	65.0	66.4	40.7	57.0	59.1
1.00		51.6	58.0	58.3	46.4	52.3	53.7
1.25		46.7	52.3	54.4	44.9	39.7	44.2
1.50		44.6	50.9	52.0	39.7	43.6	44.5
1.75		42.8	48.5	48.0	40.5	35.5	36.6
Pooled SEM		2.00	0.96	1.18	3.19	1.94	2.31
P-value							
Particle size		0.305	<0.001	<0.001	0.043	0.178	0.250
Ca/P ratio		<0.001	<0.001	<0.001	0.246	<0.001	<0.001
Particle size × Ca/P ratio		0.832	0.785	0.673	0.556	0.067	0.052
Linear (Ca/P ratio)		<0.001	<0.001	<0.001	0.089	<0.001	<0.001
Quadratic (Ca/P ratio)		0.088	<0.001	0.001	0.951	0.568	0.718

[1] Dietary P content was fixed at 5.5 g/kg diet.
[2] Data are presented as treatment means, 4 replicate pens per treatment (n=4).
[3] Ca content and particle size distribution of the fine and coarse limestone are shown in Table 1.

Table 4. Effect of Ca/P ratio and particle size of limestone on P solubility in freeze dried digesta, solubilised under the conditions (pH and dry matter content) of the digesta in the subsequent segment of the digestive tract in broilers [1,2,3]

Particle size	Ca/P ratio	Crop	Gizzard	Jejunum
Fine	0.50	30.0	56.5	80.0
	1.75	31.1	55.7	72.8
Coarse	0.50	26.3	73.2	67.1
	1.75	27.7	56.1	78.3
Pooled SEM		3.63	5.88	6.26
P-value				
Particle size		0.386	0.172	0.569
Ca/P ratio		0.750	0.153	0.760
Particle size × Ca/P ratio		0.965	0.192	0.166

[1] Dietary P content was fixed at 5.5 g/kg diet.
[2] Data are presented as treatment means, 4 replicate pens per treatment (n=4).
[3] The Ca content and particle distribution of the coarse and fine limestone are shown in Table 1.

Tibia and serum characteristics

No interaction was observed between particle size of the limestone and Ca/P ratio on CTS (**Table 5**) or serum characteristics (**Table 6**). Limestone particle size did not affect CTS or serum characteristics either. Regardless of limestone particle size, tibia maximum compressive load, fracture energy, and stiffness were linearly (P<0.001) and quadratically (P<0.046) increased with an incremental Ca/P ratio, whereas tibia diameter was not affected. Increasing dietary Ca/P ratio linearly (P<0.001) increased serum Ca content, and tended to linearly decrease (P=0.073) serum $1,25(OH)_2D_3$ content. The incremental Ca/P ratio linearly (P<0.001) decreased serum P content. However, the Ca/P ratio had no impact on the serum ALP or PTH.

Table 5. Effect of dietary Ca/P ratio and particle size of limestone on characteristics of tibia strength in broilers determined by a bone breaking test as described by Guz *et al.* [17] [1,2,3]

Particle size	Ca/P ratio	Maximum compressive load	Energy to fracture	Stiffness	Thickness
		N	N·mm	N/mm	mm
Fine	0.50	139	185	105	5.44
	0.75	179	206	138	5.57
	1.00	216	264	145	5.69
	1.25	203	233	156	5.42
	1.50	235	274	181	5.73
	1.75	212	244	171	5.60
Coarse	0.50	144	189	106	5.50
	0.75	154	181	126	5.42
	1.00	216	263	160	5.73
	1.25	210	240	168	5.66
	1.50	203	216	162	5.76
	1.75	224	249	163	5.59
Pooled SEM		13.0	20.7	10.4	0.111
Particle size					
Fine		197	234	149	5.58
Coarse		192	223	148	5.61
Pooled SEM		5.31	8.45	4.26	0.045
Ca/P ratio					
0.50		142	187	106	5.47
0.75		167	194	132	5.50
1.00		216	264	153	5.71
1.25		207	237	162	5.54
1.50		219	245	172	5.75
1.75		218	247	167	5.60
Pooled SEM		9.23	14.7	7.39	0.078
P-value					
Particle size		0.459	0.345	0.722	0.610
Ca/P ratio		<0.001	0.001	<0.001	0.066
Particle size × Ca/P ratio		0.413	0.567	0.522	0.663
Linear (Ca/P ratio)		<0.001	0.001	<0.001	0.063
Quadratic (Ca/P ratio)		0.001	0.046	0.004	0.201

[1] Dietary P content was fixed at 5.5 g/kg diet.
[2] Data are presented as treatment means, 6 tibias per replicate pen and 4 replicate pens per treatment (n=24).
[3] The Ca content and particle distribution of the coarse and fine limestone are shown in Table 1.

Table 6. Effect of dietary Ca/P ratio and particle size of limestone on serum characteristics in broilers [1,2,3]

Particle size	Ca/P ratio	Ca mmol/L	P mmol/L	ALP U/L	PTH pg/mL	$1,25(OH)_2D_3$ pg/mL
Fine	0.50	2.28	2.53	6293	210	228
	0.75	2.63	2.50	6040	121	202
	1.00	2.43	2.08	5737	105	218
	1.25	2.53	2.03	6247	198	201
	1.50	2.60	2.18	6766	48	188
	1.75	2.83	1.80	6053	206	173
Coarse	0.50	2.30	2.63	5664	246	229
	0.75	2.58	2.55	5895	91	199
	1.00	2.53	2.30	4492	184	210
	1.25	2.58	2.28	5595	91	170
	1.50	2.43	2.15	6139	238	252
	1.75	2.70	1.83	7157	188	180
Pooled SEM		0.097	0.148	823	55.0	21.0
Particle size						
Fine		2.55	2.19	6189	148	202
Coarse		2.52	2.29	5824	173	207
Pooled SEM		0.040	0.060	336	23.1	8.57
Ca/P ratio						
0.50		2.29	2.58	5979	228	229
0.75		2.61	2.53	5968	106	201
1.00		2.48	2.19	5115	145	214
1.25		2.56	2.16	5921	145	186
1.50		2.52	2.17	6453	143	220
1.75		2.77	1.82	6605	197	177
Pooled SEM		0.069	0.105	582	38.9	14.8
P-value						
Particle size		0.607	0.231	0.447	0.449	0.692
Ca/P ratio		0.001	<0.001	0.554	0.302	0.124
Particle size × Ca/P ratio		0.702	0.920	0.794	0.186	0.368
Linear (Ca/P ratio)		0.001	<0.001	0.276	0.903	0.073
Quadratic (Ca/P ratio)		0.953	0.918	0.242	0.056	0.950

[1] Dietary P content was fixed at 5.5 g/kg diet.
[2] Data are presented as treatment means, 4 replicate pens per treatment (n=4).
[3] The Ca content and particle distribution of the coarse and fine limestone are shown in Table 1.
ALP, alkaline phosphatase; PTH, parathyroid hormone; $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol.

Growth performance

Limestone particle size did not impact growth performance, except that coarse limestone reduced feed conversion ratio (FCR) from d 14-20 (*P*=0.010, **Table 7**). Overall, incremental dietary Ca/P ratio improved body weight gain (BWG) from d 14-20, and this positive effect of dietary Ca/P ratio on BWG was also observed in the total grower (d 14-29) and overall experimental period (d 14-39, **Supplementary Table**

2). From d 14-20 and d 14-29, the FCR was linearly (*P*<0.001) and quadratically (*P*<0.004) reduced by incremental dietary Ca/P ratio. However, this effect of dietary Ca/P ratio on FCR disappeared in the finisher (d 29-39) and overall (d 14-39) period. The feed intake (FI) was linearly (d 14-29, *P*=0.041) or quadratically (d 14-20, *P*=0.045) increased by incremental Ca/P ratio in the grower period, and continued to be increased by dietary Ca/P ratio in the finisher and overall experimental period.

Table 7. Effect of Ca/P ratio and particle size of limestone on growth performance in the grower period in broilers [1,2,3,4]

Particle size	Ca/P ratio	d 14-20			d 14-29		
		BWG, g	FI, g	FCR, g/g	BWG, g	FI, g	FCR, g/g
Fine	0.50	462[bc]	664	1.44	1271	1824	1.43
	0.75	482[ab]	678	1.41	1429	2020	1.41
	1.00	480[ab]	657	1.37	1397	1921	1.38
	1.25	485[a]	662	1.36	1444	1999	1.38
	1.50	490[a]	673	1.37	1489	2049	1.38
	1.75	457[c]	631	1.38	1383	1919	1.39
Coarse	0.50	444[c]	638	1.44	1237	1800	1.46
	0.75	478[ab]	662	1.39	1382	1949	1.41
	1.00	490[a]	671	1.37	1399	1943	1.39
	1.25	489[a]	659	1.35	1414	1948	1.38
	1.50	487[a]	665	1.37	1384	1912	1.38
	1.75	493[a]	667	1.35	1471	2015	1.37
Pooled SEM		7.4	10.2	0.008	39.1	64.8	0.01
Particle size							
Fine		476	661	1.39	1402	1955	1.40
Coarse		478	661	1.38	1381	1928	1.40
Pooled SEM		3.1	4.2	0.003	15.9	26.5	0.01
Ca/P ratio							
0.50		453	651	1.44	1254	1812	1.45
0.75		480	670	1.40	1406	1985	1.41
1.00		485	665	1.37	1398	1932	1.39
1.25		487	661	1.36	1429	1974	1.38
1.50		488	669	1.37	1437	1981	1.38
1.75		475	649	1.37	1427	1967	1.38
Pooled SEM		5.2	7.2	0.006	27.6	45.8	0.01
P-value							
Particle size		0.346	0.937	0.010	0.391	0.476	0.901
Ca/P ratio		<0.001	0.201	<0.001	<0.001	0.081	<0.001
Particle size × Ca/P ratio		0.020	0.055	0.404	0.302	0.601	0.738
Linear (Ca/P ratio)		0.003	0.779	<0.001	<0.001	0.041	<0.001
Quadratic (Ca/P ratio)		<0.001	0.045	<0.001	0.006	0.107	0.004

[1] Dietary P content was fixed at 5.5 g/kg diet.
[2] Data are presented as treatment means, 6 replicate pens per treatment (n=6).
[3] Each treatment had an equal number of 6 pens with 16 birds per pen before d 20, and an unequal number of 4 pens with 4 birds per pen and 2 pens with 16 birds per pen after d 21.
[4] The Ca content and particle distribution of the coarse and fine limestone are shown in Table 1.
BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.

Digesta pH and MRT

No interaction was found between particle size of the limestone and Ca/P ratio on digesta pH in gut segments except for the duodenum ($P_{interaction}$=0.026, **Table 8**). No other effects of limestone particle size on digesta pH was observed in either of the segments, except in the ceca (P=0.026) where digesta pH was higher for the fine than the coarse limestone. Increasing dietary Ca/P ratio linearly increased (P<0.011) digesta pH in the proventriculus plus gizzard, ceca and colon, while it linearly decreased (P=0.002) digesta pH in the crop. Digesta pH was not affected by the Ca/P ratio in jejunum, proximal or distal ileum.

Table 8. Effect of Ca/P ratio and particle size of limestone on digesta pH in different intestinal segments in broilers [1,2,3]

Particle size	Ca/P ratio	Crop	Prov.+gizzard	Duodenum	Jejunum	Prox. ileum	Distal ileum	Ceca	Colon
Fine	0.50	5.51	3.09	5.95^{abc}	5.77	5.94	5.71	6.07	5.70
	0.75	5.21	2.96	5.89^{abc}	5.73	5.74	5.67	6.06	5.60
	1.00	5.01	3.03	5.89^{abc}	5.73	5.93	6.14	6.17	5.83
	1.25	4.93	3.53	5.74^{c}	5.73	5.69	6.42	6.55	5.83
	1.50	5.06	3.52	5.94^{abc}	5.81	6.00	6.15	6.59	6.12
	1.75	4.80	3.52	6.05^{a}	5.79	5.88	6.49	6.69	6.41
Coarse	0.50	5.32	3.07	5.78^{c}	5.76	5.78	5.66	5.96	5.55
	0.75	4.99	2.91	5.95^{abc}	5.75	5.95	5.82	5.96	5.70
	1.00	5.06	3.15	5.89^{abc}	5.75	5.99	6.20	5.99	6.03
	1.25	5.08	3.15	5.99^{ab}	5.77	6.18	6.07	6.34	6.14
	1.50	5.02	3.48	5.84^{bc}	5.78	6.11	6.17	6.35	6.22
	1.75	5.00	3.39	5.82^{bc}	5.74	5.76	5.94	6.34	5.98
Pooled SEM		0.135	0.098	0.073	0.044	0.150	0.249	0.148	0.121
Particle size									
Fine		5.09	3.28	5.91	5.76	5.86	6.10	6.36	5.92
Coarse		5.08	3.19	5.88	5.76	5.96	5.98	6.16	5.94
Pooled SEM		0.057	0.040	0.030	0.018	0.061	0.102	0.060	0.051
Ca/P ratio									
0.50		5.42	3.08	5.87	5.77	5.86	5.69	6.02	5.63
0.75		5.10	2.94	5.92	5.74	5.85	5.75	6.01	5.65
1.00		5.04	3.09	5.89	5.74	5.96	6.17	6.08	5.93
1.25		5.01	3.34	5.87	5.75	5.94	6.25	6.45	5.99
1.50		5.04	3.50	5.89	5.80	6.06	6.16	6.47	6.17
1.75		4.90	3.46	5.94	5.77	5.82	6.22	6.52	6.20
Pooled SEM		0.096	0.069	0.051	0.031	0.107	0.176	0.105	0.085
P-value									
Particle size		0.889	0.142	0.455	0.922	0.268	0.415	0.026	0.812
Ca/P ratio		0.035	<0.001	0.884	0.803	0.637	0.098	0.001	<0.001
Partide size × Ca/P ratio		0.573	0.238	0.026	0.918	0.305	0.711	0.960	0.052
Linear (Ca/P ratio)		0.002	<0.001	0.526	0.528	0.647	0.011	<0.001	<0.001
Quadratic (Ca/P ratio)		0.179	0.428	0.722	0.539	0.272	0.212	0.921	0.643

[1] Dietary P content was fixed at 5.5 g/kg diet.
[2] Data are presented as treatment means, 4 replicate pens per treatment (n=4).
[3] The Ca content and particle distribution of the coarse and fine limestone are shown in Table 1.
Prov.+gizzard, proventriculus plus gizzard; Prox. Ileum, proximal ileum, the first half of ileum.

No interaction was found between particle size of the limestone and Ca/P ratio on the digesta MRT (**Table 9**). Limestone particle size did not affect digesta MRT in the gut segments, except that MRT was greater for the coarse limestone in the proventriculus plus gizzard (P=0.004) and ceca (P=0.034). Increasing Ca/P ratio linearly increased (P=0.004) the digesta MRT in the crop, but it linearly decreased (P<0.001) MRT in the proventriculus plus gizzard and ceca. The MRT was not affected by the Ca/P ratio in the duodenum, jejunum or ileum.

Table 9. Effect of Ca/P ratio and particle size of limestone on MRT (min) in different intestinal segments in broilers [1,2,3]

Particle size	Ca/P ratio	Crop	Prov.+gizzard	Duodenum	Jejunum	Prox. ileum	Distal ileum	Ceca
Fine	0.50	15.6	30.1	1.36	43.6	39.0	44.9	2.41
	0.75	23.7	26.2	2.04	45.8	37.3	49.6	1.47
	1.00	24.5	23.0	1.26	44.8	39.3	43.9	1.31
	1.25	28.3	20.5	1.64	50.1	46.7	47.9	0.87
	1.50	30.6	22.1	1.22	43.2	41.9	50.4	0.81
	1.75	32.9	23.8	1.26	43.6	40.2	48.0	0.60
Coarse	0.50	20.6	30.1	1.70	48.9	39.0	52.1	2.32
	0.75	27.3	32.7	1.26	47.1	39.5	52.7	2.44
	1.00	34.9	34.2	1.18	45.7	38.9	53.9	1.82
	1.25	27.6	28.6	1.02	46.4	38.1	44.0	1.23
	1.50	23.8	22.8	1.46	56.1	43.6	52.9	0.94
	1.75	32.3	24.4	1.40	47.5	39.5	48.5	0.83
Pooled SEM		4.52	2.49	0.28	3.68	2.89	3.48	0.28
Particle size								
Fine		25.9	24.3	1.46	45.2	40.7	47.5	1.25
Coarse		27.8	28.8	1.34	48.6	39.8	50.7	1.60
Pooled SEM		1.64	1.02	0.11	1.50	1.18	1.42	0.11
Ca/P ratio								
0.50		18.1	30.1	1.53	46.3	39.0	48.5	2.37
0.75		25.5	29.5	1.65	46.5	38.4	51.2	1.96
1.00		29.7	28.6	1.22	45.3	39.1	48.9	1.57
1.25		28.0	24.6	1.33	48.3	42.4	46.0	1.05
1.50		27.2	22.5	1.34	49.7	42.8	51.7	0.88
1.75		32.6	24.1	1.33	45.6	39.9	48.3	0.72
Pooled SEM		2.76	1.76	0.20	2.60	2.04	2.46	0.19
P-value								
Particle size		0.441	0.004	0.441	0.113	0.564	0.618	0.034
Ca/P ratio		0.042	0.014	0.682	0.824	0.550	0.116	<0.001
Partide size × Ca/P ratio		0.410	0.141	0.266	0.343	0.484	0.439	0.512
Linear (Ca/P ratio)		0.004	0.001	0.281	0.684	0.244	0.899	<0.001
Quadratic (Ca/P ratio)		0.271	0.682	0.551	0.641	0.501	0.942	0.240

[1] Dietary P content was fixed at 5.5 g/kg diet.
[2] Data are presented as treatment means, 4 replicate pens per treatment (n=4).
[3] The Ca content and particle distribution of the coarse and fine limestone are shown in Table 1.
MRT, mean retention time; Prov.+gizzard, proventriculus plus gizzard; Prox. ileum, proximal ileum, the first half of ileum.

Discussion

The pelleting process was found to retain the dietary particle size treatment imposed by limestone addition. In the feed ash of the coarse limestone diets, 76% of the added particles could be recovered. Full recovery is unlikely in this respect due to an expected (limited) negative effect of pelleting or incineration on limestone particle size. In addition, the GMD of both the coarse and fine limestone diets linearly increase with incremental Ca/P ratio. The increase for the coarse limestone diets was close to the theoretical increase where the slope for the analysed was 16 vs. 15 for the theoretical linear regression line. In addition, it should be noted that the treatments aimed to impact the Ca and P digestion by affecting the solubility and/or interaction of Ca and P but not gizzard development or surface area available to the digestive enzymes. As such, masking of the limestone particle size difference, given that the diets had less than 2% limestone, by the particles in the diet is integral to the design of the study reported here in order to only test the effect of limestone particle size and not overall diet particle size. However, it can be ruled out that the observed effects are only related to solubility and/or interaction of Ca and P.

The hypothesis that coarse limestone was more digestible to the broilers was not proven in the present study. The jejunal apparent Ca digestibility was higher for fine than coarse limestone (46.2 vs. 40.8%), but the apparent distal ileal Ca digestibility was similar for coarse and fine limestone (51.5 vs. 49.2%). Therefore, the experimental results did not support the possibility to reduce the Ca/P ratio while maintaining the digestible Ca supply through the use of coarse rather than fine limestone. Compared to fine limestone, coarse limestone slightly improved the distal ileal P digestibility (60.5 vs. 57.0%). Nevertheless, the coarse limestone could not alleviate the negative effect of a low Ca/P ratio on the CTS or growth performance. As observed by Díaz-Alonso *et al.* [20], broilers required more Ca and P to ensure adequate bone mineralization than maximum growth performance. In the current study, both growth performance and CTS were compromised by a low dietary Ca/P ratio, regardless of limestone particle size.

Coarse and fine limestone had a similar Ca digestibility at a low inclusion level in the distal ileum, while fine limestone had a higher Ca digestibility at a high dietary Ca/P ratio. Compared to fine limestone (particle size <500 µm), coarse

limestone (particle size 1000-2000 µm) has been reported to have a higher ileal Ca digestibility (70 vs 40%; Anwar *et al.* [5; 6]). The latter authors used coarser limestone compared to the present study (1000-2000 vs 500-2000 µm). Moreover, mash feed was used by Anwar *et al.* [5; 6], while pelleted feed was used in the present study. Adaptation time to the diets might be another reason for the discrepancy between Anwar *et al.* [5; 6] and the present study (3 vs. 6 days, respectively). David *et al.* [21] reported that observed distal ileal Ca digestibility decreased linearly with increasing dietary adaptation time, with higher digestibility obtained at 1-d adaptation than 3 or 5 days of adaptation (limestone particle size 370 µm). Limestone transiently accumulated in the crop and gizzard, with a greater concentration effect for coarse limestone (**Supplementary Table 3**), hence a longer adaption time might be required for coarse than fine limestone to achieve a steady passage rate of digesta. Due to the adaptation of broilers to the changes of diets, short-term experiments may not represent the response of broilers over a longer period. In a 6-week study in broiler breeder hens [22], the large-particle-size limestone decreased the P but not the Ca content in the excreta (average particle size was 185 and 3490 µm for fine and coarse limestone, respectively). These results agree with our finding, indicating that particle size of limestone probably had an impact on P digestion while it had less impact on Ca digestion. In an *in vitro* study to mimic the Ca solubilization in the GIT [23], fine limestone initially released more Ca than coarse limestone; however, the same amount of Ca was released after 20 min (particle size <75 and 402 µm for the coarse and fine limestone, respectively). This *in vitro* solubility finding is in line with the observed Ca digestibility in the present study, i.e. the Ca digestibility was higher for the fine limestone in the jejunum, and this difference disappeared in the ileum for the low Ca/P ratios but retained for the high Ca/P ratios. Mechanism behind this interactive effect might be that limestone provided only approximately 50% of Ca in the lowest and close to 90% of the Ca in the highest Ca/P ratio diet. In addition, Ca absorption in the proximal small intestine (jejunum) was not adequate for the low Ca/P ratios, and a substantial amount of Ca was absorbed in the distal small intestine (ileum) for the low Ca/P ratios, irrespective of limestone particle size. The superiority of fine limestone over coarse limestone, therefore, disappeared in the ileum for the low Ca/P ratios while it remained for the high Ca/P ratios. In conclusion, coarse limestone has a limited effect on the apparent

distal ileal Ca digestibility at a low Ca/P ratio but may decrease apparent Ca digestibility at a higher Ca/P ratio.

In contrast to Ca digestibility, apparent distal ileal P digestibility was slightly improved by coarse limestone. As mentioned before, the fine limestone released more Ca and improved the apparent Ca digestibility in the upper GIT (jejunum). The rapid Ca release probably promotes insoluble Ca-P complexation in the foregut as phytate is primarily hydrolysed in the upper GIT, specifically in the crop, proventriculus and gizzard [24]. The relatively rapid Ca release and Ca-P complexation, therefore, may decrease P digestion. In an *in vitro* study [25], compared to the limestone with larger particle sizes (137-1306 μm), fine limestone (28 μm) had a higher Ca solubility and a greater depression on phytase efficacy. In addition, expression of the P transporters and sodium-dependent phosphate transporter type IIb gradually decreases along the small intestine [26-28]. Therefore, a rapid Ca release in the upper gut may also decrease P absorption.

The interaction between Ca, P, phytate and phytase in the gut is complex. Complexation of Ca-P is widely acknowledged to be responsible for Ca depressing P digestion. However, P solubility was not affected by the dietary treatments in the present study. Therefore, P solubility or Ca-P complexation did not seem to be a major factor in the Ca and P interaction. It should be noted that the freeze-dried digesta was used in the present study to conduct the P solubility test, while it is unknown if freeze-drying affects the mineral solubility. In an *in vitro* model [29], limestone addition improved P solubility in the gastric phase, while it reduced P solubility in the small intestine phase. The mechanism behind Ca stimulating P solubilization is not clear yet, but it implies that the interaction between Ca, phytase, phytate, inorganic P, and phytate-P in the GIT is complicated, and the Ca-P complexation maybe only part of the reason for Ca depressing P digestion.

The effects of dietary Ca/P ratio on growth performance, digesta pH, Ca and P digestion as well as bone development has been extensively studied [30-32]. These reports supported our findings that the high dietary Ca/P ratio decreased the ileal Ca and P digestibility. The optimal dietary Ca/P ratio was 1.00-1.25 to maximize Ca and P digestion while maintaining growth performance and CTS. By reducing the Ca/P ratio from 1.75 to 1.00, the distal ileal Ca and P apparent digestibility could be improved from 36.6 to 53.7% and 48.0 to 58.3%, respectively. Reducing

dietary Ca/P ratio to below 1.00 impaired CTS, although the apparent Ca and P digestibility could be further improved.

In conclusion, apparent distal ileal Ca digestibility of coarse limestone was equal to fine limestone at a low Ca/P ratio, but it was lower for coarse than fine limestone at a high dietary Ca/P ratio. Coarse limestone slightly improved the apparent distal ileal P digestibility compared to fine limestone. In diets with a total P content of 5.5 g/kg, a reduction of Ca/P ratio improved the apparent Ca and P digestibility, with the optimal Ca/P ratio being 1.00-1.25 to optimize the Ca and P digestion while maintaining growth performance and CTS.

Acknowledgement

This research was conducted by Wageningen Livestock Research, within the framework of the public private partnership "Feed4Foodure" and partially funded by the Ministry of Economic Affairs (Policy Support Research project number BO-31.03-005-00). Y.X. Hu received a scholarship form the Chinese Scholarship Council (CSC) to study at Wageningen University & Research.

Author contributions

M.M. van Krimpen, P. Bikker, M. Duijster and Y.X. Hu designed and conducted the research; Y.X. Hu analysed the samples and data. Y.X. Hu, M.M. van Krimpen, P. Bikker, J. van Baal and W.H. Hendriks interpreted the data and wrote the manuscript. All authors have read and approved the final manuscript. M. Duijster is an employee of De Heus, all other authors declare no conflict of interest.

References

1. Shao Y, Sun G, Cao S *et al.* (2019) Bone phosphorus retention and bone development of broilers at different ages. *Poult Sci* **98**, 2114-2121.

2. Misiura MM, Filipe JAN, Walk CL *et al.* (2018) Do not neglect calcium: a systematic review and meta-analysis (meta-regression) of its digestibility and utilisation in growing and finishing pigs. *Br J Nutr* **119**, 1207-1219.

3. Rodehutscord M (2016) Chapter 10 Interactions between minerals and phytate degradation in poultry–challenges for phosphorus digestibility assays. In *Phytate Destruction-Consequences for Precision Animal Nutrition*, pp. 167-177 [Walk CL, Kühn I, Stein HH, Kidd MT and Rodehutscord M, editors]. Wageningen, the Netherlands: Wageningen Academic Publishers.

4. Van Krimpen M, Van Diepen JTM, Van Wikselaar P *et al.* (2013) Effects of available phosphorus (aP), calcium/aP ratio, and growth rate on P deposition, P digestibility, performance and leg quality in broilers. Wageningen UR Livestock Research, Report 670, Wageningen, the Netherlands. p.12.

5. Anwar MN, Ravindran V, Morel PC *et al.* (2016) Effect of limestone particle size and calcium to non-phytate phosphorus ratio on true ileal calcium digestibility of limestone for broiler chickens. *Br Poult Sci* **57**, 707-713.

6. Anwar MN, Ravindran V, Morel PC *et al.* (2017) Effect of calcium source and particle size on the true ileal digestibility and total tract retention of calcium in broiler chickens. *Anim Feed Sci Technol* **224**, 39-45.

7. Kim SW, Li W, Angel R *et al.* (2018) Effects of limestone particle size and dietary Ca concentration on apparent P and Ca digestibility in the presence or absence of phytase. *Poult Sci* **97**, 4306-4314.

8. CVB (2016) *Voedernormen Varkens en Voederwaarden Voedermiddelen voor Pluimvee (Feeding Standards, Feeding Advices and Nutritional Values of Feeding Ingredients of Poultry)*. Wageningen, the Netherlands: Federatie Nederlandse Diervoederketen.

9. Rodehutscord M (2013) Determination of phosphorus availability in poultry. *Worlds Poult Sci J* **69**, 687-698.

10. ASAE (2008) Method of determining and expressing fineness of feed materials by sieving. In: *American Society of Agricultural and Biological Engineers Standards (Standard No. S319.4)*. MI: St. Joseph.

11. ISO (1999) 6496: *Animal Feeding Stuffs - Determination of Moisture and Other Volatile Matter Content*. Geneva: International Organization for Standardization.

12. ISO (2005) 5983-1: *Animal Feeding Stuffs - Determination of Nitrogen Content and Calculation of Crude Protein Content - Part 1: Kjeldahl Method*. Geneva: International Organization for Standardization.

13. ISO (2002) 5984: *Animal feeding stuffs - Determination of Crude Ash*. Geneva: International Organization for Standardization.

14. ISO (1998) 6491: *Animal feeding stuffs - Determination of Phosphorus Content - Spectrometric method*. Geneva*:* International Organization for Standardization.

15. ISO (2000) 6869: *Animal feeding stuffs - Determination of the Contents of Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Sodium and Zinc - Method using Atomic Absorption Spectrometry*. *Geneva:* International Organization for Standardization.

16. Myers WD, Ludden PA, Nayigihugu V *et al.* (2004) A procedure for the preparation and quantitative analysis of samples for titanium dioxide. *J Anim Sci* **82**, 179-183.

17. Guz BC, Molenaar R, de Jong IC *et al.* (2019) Effects of dietary organic minerals, fish oil, and hydrolyzed collagen on growth performance and tibia characteristics of broiler chickens. *Poult Sci* **98**, 6552-6563.

18. de Vries S & Gerrits WJJ (2018) The use of tracers or markers in digestion studies. In *Feed Evaluation Science*, pp. 275–294 [Moughan PJ and Hendriks WH, editors]. Wageningen: Wageningen Academic Publishers.

19. ROSS. 2014. Ross 308 Broiler: Performance Objective. Accessed Mar. 2018. http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-308-Broiler-PO-2014-EN.pdf.

20. Díaz-Alonso JA, Gómez-Rosales S, Angeles MdL *et al.* (2019) Effects of the level and relationship of calcium and available phosphorus on the growth and tibia mineralization of broiler starter chickens. *J Appl Poult Res* **28**, 339-349.

21. David LS, Abdollahi MR, Ravindran G *et al.* (2019) Studies on the measurement of ileal calcium digestibility of calcium sources in broiler chickens. *Poult Sci* **98**, 5582-5589.

22. Manangi MK, Maharjan P & Coon CN (2018) Calcium particle size effects on plasma, excreta, and urinary Ca and P changes in broiler breeder hens. *Poult Sci* **97**, 2798-2806.

23. Kim SW, Li W, Angel R *et al.* (2019) Modification of a limestone solubility method and potential to correlate with *in vivo* limestone calcium digestibility. *Poult Sci* **98**, 6837-6848.

24. Dersjant-Li Y, Awati A, Schulze H *et al.* (2015) Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. *J Sci Food Agric* **95**, 878-896.

25. Manangi MK & Coon CN (2007) The effect of calcium carbonate particle size and solubility on the utilization of phosphorus from phytase for broilers. *Int J Poult Sci* **6**, 85-90.

26. Liu SB, Hu YX, Liao XD *et al.* (2016) Kinetics of phosphorus absorption in ligated small intestinal segments of broilers. *J Anim Sci* **94**, 3312-3320.

27. Hu Y, Liao X, Wen Q *et al.* (2018) Phosphorus absorption and gene expression levels of related transporters in the small intestine of broilers. *Br J Nutr* **119**, 1346-1354.

28. Shao Y, Wen Q, Zhang S *et al.* (2019) Dietary supplemental vitamin D_3 enhances phosphorus absorption and utilisation by regulating gene expression of related phosphate transporters in the small intestine of broilers. *Br J Nutr* **121**, 9-21.

29. Walk CL, Bedford MR & McElroy AP (2012) Influence of diet, phytase, and incubation time on calcium and phosphorus solubility in the gastric and small intestinal phase of an *in vitro* digestion assay. *J Anim Sci* **90**, 3120-3125.

30. Akter M, Graham H & Iji PA (2016) Response of broiler chickens to different levels of calcium, non-phytate phosphorus and phytase. *Br Poult Sci* **57**, 799-809.

31. Gautier AE, Walk CL & Dilger RN (2017) Influence of dietary calcium concentrations and the calcium-to-non-phytate phosphorus ratio on growth performance, bone characteristics, and digestibility in broilers. *Poult Sci* **96**, 2795-2803.

32. Li W, Angel R, Kim SW *et al.* (2018) Impacts of age and calcium on phytase efficacy in broiler chickens. *Anim Feed Sci Technol* **238**, 9-17.

Supplementary Table 1. Composition and nutrient content (g/kg, as-fed basis, d 30-39) of finisher diets with fine or coarse limestone and incremental Ca/P ratios [1]

Particle size limestone	Fine						Coarse					
Ca/P ratio	0.50	0.75	1.00	1.25	1.50	1.75	0.50	0.75	1.00	1.25	1.50	1.75
Ingredients												
Corn	363	357	351	345	339	333	363	357	351	345	339	333
Wheat	350	350	350	350	350	350	350	350	350	350	350	350
Soybean meal, extracted	238	238	239	239	240	240	238	238	239	239	240	240
Soybean oil	26	28	30	32	34	37	26	28	30	32	34	37
Monosodium phosphate	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1	6.1
Monocalcium phosphate	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Limestone (fine)	3.5	6.8	10.2	13.5	16.8	20.0	0	0	0	0	0	0
Limestone (coarse)	0	0	0	0	0	0	3.5	6.8	10.2	13.5	16.8	20.0
Salt	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
L-Val (98%)	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Met (99%)	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
L-Lys (79%)	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8
Thr (98%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Titanium dioxide (TiO_2)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Premix [2]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Calculated nutrients												
Dry matter	875	876	876	877	878	878	875	876	876	877	878	878
ME, kcal/kg	3059	3059	3059	3059	3059	3059	3059	3059	3059	3059	3059	3059
Crude protein	188	188	188	187	187	187	188	188	188	187	187	187
Lys	10	10	10	10	10	10	10	10	10	10	10	10
Met	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Met+Cys	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2
Thr	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6
Ca	2.5	3.8	5.1	6.3	7.6	8.8	2.5	3.8	5.1	6.3	7.6	8.8
Total P (P)	5.1	5.1	5.1	5.1	5.0	5.0	5.1	5.1	5.1	5.1	5.0	5.0
Available P (aP)	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9
Ca/P	0.50	0.75	1.00	1.25	1.50	1.75	0.50	0.75	1.00	1.25	1.50	1.75
Analysed nutrients												
Dry matter	877	882	884	884	884	884	883	885	885	885	884	883
Crude protein	184	182	182	183	183	182	182	181	185	184	185	181
Crude fat	57.8	58.8	57.3	59.3	59.2	58.4	58.9	59.2	59.8	56.1	55.6	56.2
Ca	2.8	4.1	5.4	6.7	7.7	9.0	3.0	3.7	5.3	6.5	7.2	8.2
P	5.3	5.5	5.8	5.7	5.6	5.7	5.7	5.7	5.7	5.6	5.8	5.3
Ca/P	0.53	0.75	0.93	1.18	1.38	1.58	0.53	0.65	0.93	1.16	1.24	1.55

[1] The Ca content and particle size distribution of the coarse and fine limestone are shown in Table 1.
[2] Provided per kg of diet: 12,000 IE retinol, 2,400 IE cholecalciferol, 50 mg dl-a-tocopherol, 1.5 mg menadione, 2.0 mg thiamine, 7.5 mg riboflavin, 3.5 mg pyridoxine, 20 mg cyanocobalamins, 35 mg niacin, 12 mg D-pantothenic acid, 460 mg choline chloride, 1.0 mg folic acid, 0.2 mg biotin, 80 mg iron, 12 mg copper, 85 mg manganese, 60 mg zinc, 0.4 mg cobalt, 0.8 mg iodine, 0.1 mg selenium, 125 mg antioxidant mixture.

Supplementary Table 2. Effect of Ca/P ratio and particle size of limestone on growth performance in the finisher and overall broilers [1,2,3,4]

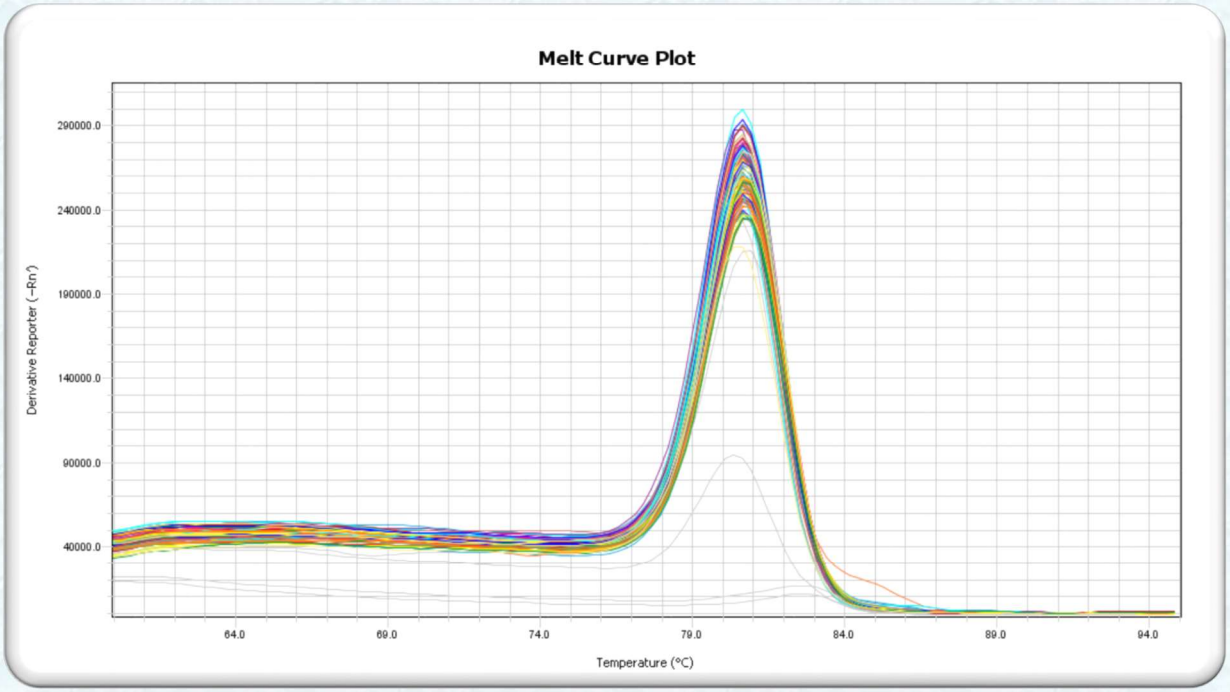
Particle size	Ca/P ratio	BW, g				Finisher period (d 29-39)			Overall period (d 14-39)		
		d 14	d 20	d 29	d 39	BWG g	FI g	FCR g/g	BWG g	FI g	FCR g/g
Fine	0.50	431	893	1702	2756	1054	1918	1.83	2325	3630	1.56
	0.75	436	918	1866	3034	1168	2113	1.83	2597	4035	1.56
	1.00	425	907	1822	3035	1213	2091	1.73	2609	3888	1.49
	1.25	430	915	1874	3043	1169	2137	1.84	2613	4018	1.54
	1.50	431	927	1921	3007	1086	2082	1.97	2576	3957	1.55
	1.75	428	885	1811	2897	1086	2001	1.87	2469	3804	1.54
Coarse	0.50	428	872	1665	2705	1040	1907	1.86	2277	3620	1.59
	0.75	431	909	1813	2957	1145	2029	1.78	2526	3885	1.54
	1.00	432	922	1831	2987	1156	2030	1.78	2555	3868	1.52
	1.25	430	919	1844	2951	1107	2034	1.87	2521	3872	1.54
	1.50	439	926	1823	3006	1182	2060	1.75	2566	3853	1.50
	1.75	435	928	1906	3093	1187	2167	1.85	2659	4055	1.53
Pooled SEM		5.07	10.7	40.6	68.8	57.7	51.7	0.09	67.6	120	0.05
Particle size											
Fine			907	1833	2958	1128	2055	1.84	2532	3889	1.54
Coarse			910	1814	2950	1136	2038	1.82	2517	3859	1.54
Pooled SEM			4.4	16.6	28.1	23.6	21.1	0.04	27.6	49.1	0.02
Ca/P ratio											
0.50			883	1684	2731	1047	1913	1.84	2301	3625	1.58
0.75			914	1840	2996	1156	2071	1.81	2562	3960	1.55
1.00			915	1827	3011	1182	2058	1.75	2582	3878	1.51
1.25			917	1859	2997	1138	2085	1.86	2567	3945	1.54
1.50			927	1872	3007	1139	2070	1.85	2571	3905	1.53
1.75			906	1859	2995	1137	2084	1.86	2564	3930	1.54
Pooled SEM			7.5	28.7	48.6	40.8	36.5	0.07	47.8	85.0	0.04
P-value		0.819									
Particle size			0.423	0.431	0.768	0.839	0.528	0.601	0.746	0.676	0.897
Ca/P ratio			0.004	<0.001	0.001	0.303	0.011	0.845	0.001	0.070	0.807
Particle size × Ca/P ratio			0.076	0.290	0.321	0.570	0.135	0.755	0.337	0.571	0.970
Linear (Ca/P ratio)			0.015	<0.001	0.002	0.333	0.006	0.530	0.002	0.051	0.465
Quadratic (Ca/P ratio)			0.002	0.007	0.003	0.086	0.032	0.486	0.003	0.084	0.315

[1] Dietary P content was fixed at 5.5 and 5.0 g/kg diet on d 14-29 and 30-39, respectively.
[2] Data are presented as treatment means, 6 replicate pens per treatment (n=6).
[3] The Ca content and particle distribution of the coarse and fine limestone are shown in Table 1.
[4] Each treatment had an equal number of 6 pens with 16 birds per pen before d 20, and an unequal number of 4 pens with 4 birds per pen and 2 pens with 16 birds per pen after d 21.
BW, body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.

Supplementary Table 3. Effect of Ca/P ratio and particle size of limestone on apparent Ca and P digestibility (%) in crop, gizzard, duodenum and ceca in broilers [1,2,3]

Particle size	Ca/P ratio	P disappearance/digestibility				Ca disappearance/digestibility			
		Crop	Prov.+Gizzard	Duodenum	Ceca	Crop	Prov.+Gizzard	Duodenum	Ceca
Fine	0.50	6.07	53.2	-73.2	-264	-7.1	36.1	-84.9	-111
	0.75	1.13	51.1	-66.3	-284	-5.7	33.3	-75.6	-184
	1.00	3.50	52.4	-90.9	-348	-1.2	40.8	-74.4	-247
	1.25	2.50	43.8	-74.9	-528	-1.8	-21.7	-131	-698
	1.50	1.21	44.4	-39.1	-654	-15.2	-81.4	-195	-1057
	1.75	-2.00	46.5	-93.4	-811	-23.6	-13.7	-77.3	-1334
Coarse	0.50	6.57	56.6	-76.0	-191	-9.9	-42.1	-149	-56.9
	0.75	9.02	56.9	-43.9	-259	-7.2	-73.5	-442	-112
	1.00	3.80	50.1	-51.6	-309	-4.1	-88.2	-284	-193
	1.25	0.30	51.2	-91.9	-484	-17.2	-64.1	-175	-496
	1.50	-1.39	43.7	-91.8	-508	-44.3	-191	-190	-652
	1.75	0.93	43.2	-84.1	-699	-35.4	-392	-109	-958

[1] Dietary P content was fixed at 5.5 g/kg.
[2] Data are presented as treatment means, 4 replicate pens per treatment (n=4).
[3] The Ca content and particle distribution of the coarse and fine limestone are shown in Table 1.
Prov.+gizzard, proventriculus plus gizzard.


Melt Curve Plot
290000.0
240000.0
190000.0
140000.0
90000.0
40000.0
Derivative Reporter (-Rn')
64.0
69.0
74.0
79.0
84.0
89.0
94.0
Temperature (°C)

CHAPTER 5

Mucosal expression of Ca and P transporters and claudins in the small intestine of broilers is altered by dietary Ca/P in a limestone particle size dependent manner

Y.X. Hu[*†], J. van Baal[†], W.H. Hendriks[†], M. Duijster[‡], M.M. van Krimpen[*], P. Bikker[*]

[*] Wageningen University & Research, Wageningen Livestock Research, 6700 AH Wageningen, the Netherlands

[†] Wageningen University & Research, Animal Nutrition Group, 6700 AH Wageningen, the Netherlands

[‡] R&D department, De Heus Animal Nutrition B.V, 6710 BJ Ede, the Netherlands

Submitted to Plos One

Abstract

As high Ca intake or fine limestone reduces precaecal P absorption independently of P solubility in broilers, this study determined whether incremental dietary Ca/P and limestone particle size affect gene expression of P transporters in the small intestine. A total of 1152 one-day-old Ross 308 male broiler chickens received diets low (0.50), medium (1.00) or high (1.75) in Ca/P containing either fine (160 µm) or coarse (1062 µm) limestone, in a 3×2 factorial arrangement. Expression of genes related to Ca and P absorption were determined using real-time quantitative PCR (RT-qPCR) in duodenal and jejunal mucosa. Results indicated that incremental dietary Ca/P decreased duodenal CaSR, CaBP-D28k, PMCA1 and NaPi-IIb, but not TRPC1 mRNA. This effect was greater with fine limestone when Ca/P increased from low to medium, but greater with coarse limestone when increased from medium to high. A similar inhibitory effect was observed for jejunal CaBP-D28k expression where incremental dietary Ca/P and fine limestone decreased CaSR mRNA, while dietary Ca/P decreased TRPC1 mRNA in the presence of coarse but not fine limestone. It also decreased jejunal NaPi-IIb mRNA irrespective of limestone particle size. Dietary treatments did not affect jejunal PMCA1 mRNA expression or that of PiT-1, PiT2 and XPR1 in both intestinal segments. Dietary Ca increment reduced mucosal CLDN-2 mRNA in both segments, and ZO-1 mRNA in jejunal mucosa only for coarse limestone. In conclusion, dietary Ca/P reduced expression of duodenal and jejunal mucosa Ca and P transporters in a limestone particle size dependent manner. Its inhibitory action on CLDN-2 expression was independent of limestone particle size. Calcium induced mRNA expression of P transporters maybe important in reducing intestinal P absorption in broilers.

Key words: calcium, phosphorus, digestive tract, transporters and claudins, broilers

Introduction

Calcium (Ca) and phosphorus (P) are two macro minerals involved in multiple biological processes, including cell signalling, synaptic transmission, muscle contraction, bone mineralization and many other biochemical reactions in both humans and animals [1]. It is generally accepted that free Ca ions reduce intestinal P absorption by the formation of insoluble Ca-P and Ca-phytate complexes [2], although the presence of such complexes has still not been demonstrated in digesta of animals. Experimental data showed that reduction of dietary Ca/P improves intestinal P absorption but may in the case of excessive reduction compromise growth performance of broilers. In our recent broiler study [3], aimed to clarify the impact of dietary Ca/P combined with limestone particle size on intestinal Ca and P digestibility, we demonstrated that incremental dietary Ca/P linearly reduced apparent P absorption in the bird's gastrointestinal tract (GIT). Strikingly, P solubility was not affected by dietary Ca level or limestone coarseness in digesta collected from the crop, jejunum or ileum. These findings indicate that luminal Ca-P or Ca-phytate complexation are not the only mechanism for the reduction in intestinal P absorption in response to high dietary Ca/P. Uptake of Ca and P by enterocytes occurs through transcellular and paracellular pathways, which are orchestrated predominantly by the cross-talk between parathyroid hormone (PTH), fibroblast growth factor 23 (FGF-23) and vitamin-D_3 [4, 5]. Together with our previous observation that fine limestone is better digestible than coarse limestone [3], we hypothesised that intake of high dietary Ca/P in broilers reduces intestinal P absorption by reducing the local gene expression of P transporters and that this inhibitory effect is greater for fine compared to coarse limestone.

It is presumed that the molecular mechanisms for Ca and P absorption in chickens are rather similar to those of mammals due to the high homology of candidate genes involved [6]. The transcellular route is an overall active, saturated process, which requires the entry of ions through protein carriers in the apical plasma membrane, intracellular diffusion and extrusion via transporters in the basolateral membrane [7]. Regarding intestinal absorption and renal reabsorption of P, apical P uptake is considered to be mediated by members of the sodium-coupled P cotransporter family, predominantly NaPi-IIb/SLC34A2. A possible role of inorganic phosphate transporter 1 (PiT-1/SLC20A1) and/or inorganic phosphate transporter 2 (PiT-2/SLC20A2) has also been postulated, but its relevance for

whole body P homeostasis remains elusive [8, 9]. In respect to basolateral P extrusion, some evidence put forward xenotropic and polytropic retrovirus receptor 1 (XPR1) as a novel candidate for basolateral P exportation in animals [10, 11]. As for Ca, the ion first enters the intestinal epithelial cell across the apical membranes passively through TRPV5 or TRPV6 [12]. This is followed by binding of Ca to calbindin proteins (in birds exclusively calbindin D28k (CaBP-D28k)) and transferred to the basolateral membrane. Basolateral exit is then driven by the plasma membrane Ca-ATPase 1 (PMCA1) and/or sodium-Ca exchanger (NCX1) [13] against the electrochemical gradient and thus by active transport. Recently, transient receptor potential canonical 1 (TRPC1) is put forward as a novel candidate apical Ca transporter as its expression level increases upon maturity in layers [14].

Compared to transcellular transport, the mechanisms involved in the paracellular routes for Ca and P are far less understood. Increasing evidence indicates that certain members of the claudin (CLDN) family control the paracellular permeability of the tight junctions in epithelial cells by forming a selective seal (i.e. barrier) or pore (i.e. gate) for ions in tight junctions (TJ) [15]. Indeed, in mice CLDN-2, -12, and -15 are associated with intestinal Ca uptake [16], while CLDN-1 and -5 are linked to sealing functions that could diminish Ca transport [17, 18]. Unfortunately, the literature is devoid of information demonstrating the importance on the expression pattern of TJ proteins in the broiler's GIT in response to dietary intake levels of Ca and/or P.

The objective of the present study was to investigate the influence of incremental dietary Ca/P and limestone particle size on the mRNA expression levels of transporters and TJ proteins related to Ca and P absorption in two segments of the small intestinal tract, i.e. duodenum and jejunum of broilers in order to obtain a better understanding of the consequences of such diets on the broiler's Ca and P homeostasis.

Experimental methods

The experiment was conducted in the broiler research accommodation of De Heus (Eerde, the Netherlands). All procedures complied with the Dutch law on animal experiments and the study was approved by the Ethical Committee of Wageningen University & Research, Wageningen, the Netherlands (no. 2016.D-0065.004).

Animals, experimental design and tissue collection

This research is a successive study to that of Hu *et al*. [3] concerning the interactive *in vivo* effects of limestone particle size and dietary Ca/P on intestinal Ca and P digestibility coefficient, growth performance and tibia breaking strength in broilers. Details regarding the experimental design, feed composition, animal husbandry, feeding regime, and sample collection can be found in our previous article [3]. Briefly, 1152 one-day-old Ross 308 broiler chickens had *ad libitum* access to a standard commercial starter feed from day 0-13. From day 14 onwards, birds received one of twelve diets in a 6×2 factorial arrangement containing one of six Ca/P (0.50, 0.75, 1.00, 1.25, 1.50 and 1.75) feeds either with fine or coarse limestone (geometric mean diameter 160 or 1062 μm). Each treatment was replicated six times with 16 birds per replicate pen. Fine and coarse limestone were separated from the same batch of product (Sibelco, Maastricht, the Netherlands) via sieving through a 500 μm screen. Dietary Ca ranged from 2.7 to 9.6 g/kg and P content was fixed at 5.5 g/kg (3.2 g/kg digestible P). On day 20 and 21, six birds per pen were randomly selected from four of the six replicate pens and euthanized by electrocution. After cleansed in water, the mucosa was scraped from the middle of the duodenum, jejunum, ileum and colon, immediately frozen in liquid nitrogen and stored at -80 °C until further analysis.

Since, on average, over 85% of the precaecal digestible Ca and P was absorbed upstream of the ileum [3], we selected duodenum and jejunum mucosa for analysis of mRNA abundance of genes related to trans- and paracellular Ca and P absorption. To this end, three broilers per pen were randomly taken from the treatment groups with a low (0.50), medium (1.00) and high (1.75) Ca/P for both fine and coarse limestone. Broilers from the other treatment groups were not analysed for this purpose. Dietary Ca content met, was below or greater than the minimal Ca requirement for chickens for the applied medium, low and high Ca/P, respectively.

Real-time quantitative PCR

Deep-frozen intestinal mucosa samples were ground in liquid nitrogen and total RNA was isolated with TRIzol (ThermoFisher Scientific, Waltham, MA) 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 integrity of RNA were determined with the NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific) and 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies), respectively. Five hundred ng RNA was reverse transcribed with a Superscript III kit (ThermoFisher Scientific) and mRNA levels were assessed by real-time 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 conditions: 95 °C for 15 s and 60 °C for 30 s for 40 cycles. This was followed by a melting curve analysis ramping from 60 to 95 °C with a rate of 0.1 °C/s in order to confirm PCR specificity. The used primer sequences were designed with Primer Express Software (Life Technologies, Bleiswijk, the Netherlands), and where possible recommended primer sets that span an intron were selected and presented in **Table 1**. Absolute quantitative mRNA measurement was performed by establishing a linear calibration curve using 10-fold serial dilutions of cDNA template for corresponding genes. Since the Normfinder [19] algorithm demonstrated that the combination of two reference genes, importin 8 (IPO8) and eukaryotic elongation factor 2 (EEF2), was most stable among other candidates (beta-actin (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 60S acidic ribosomal protein P0 (RPLP0)), expression levels were normalized to the geometric mean of IPO8 and EEF2.

Table 1. Primers used for real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Gene	Accession no.	Sense 5'-3'	Antisense 5'-3'
Ca transporters			
CaSR	XM_416491.6	GCCAATCTGCTGGGACTCTT	CTGATGCTCGTCATTGGGGA
CaBP-D28k	NM_205513.1	CGAGATCTGGCACCACTACG	ACCTGAGCAAGCTCAACGAT
PMCA1	NM_001168002.3	ACTCTGATGGCAGTTTCCGA	GTCACGGTCCCTAGGTCTGA
TRPC1	NM_001004409.2	GAATACGATGGGACCAGCCC	AGCTGATTGCGTGACTCTTCT
TRPV6	XM_004938143.3	GACCAGAGCAAAGAGGGACC	CCGCCTCTGCATGAGGTATT
P transporters			
NaPi-IIa	XM_015293846.2	GAAGCCAGGTGCCTCTGATG	AGAGGATGGCGTTGTCCTTG
NaPi-IIb	NM_204474.2	TGGCTTTGTCCCTGCTTGTT	CCAGCCAGCCAAGTAAAAGG
PiT-1	XM_015297502.2	TGAAGCTTCCCATCTCGGGT	AGGACAACACGATTTTTAGCAGC
PiT-2	NM_001305398.1	GCTGGGAGCAAAAGTAGGAGA	AAACAGCAGAACCAACCATCG
XPR1	XM_422258.6	AACCTGGAGACAACACGAGG	CGTTGGTCACCACTTCCTCT

Table 1 (continued). Primers used for real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Gene		Accession no.	Sense 5'-3'
Tight junction proteins and VDR			
CLDN-2	NM_001277622.1	CAACTGGAAGATCAGCTCCT	TGTAGATGTCGCACTGAGTG
CLDN-12	XM_025148431.1	CTCTTATTCCTCCTCGCATG	GTCAAAGCTAAAGACAGGCT
CLDN-16	XM_426702.4	GGGATCCAAACATGTGATGA	AGAGAAATCCAAATCCTGCC
ZO-1	XM_015278981.2	CCGCAGTCGTTCACGATCT	GGAGAATGTCTGGAATGGTCTGA
VDR	NM_205098.1	GGCTCAGGTTTTGCAGATTTG	CAGCATCGCCTTTCCCATT
Reference genes			
ACTB	NM_205518.1	GCCCTGGCACCTAGCACAAT	GCGGTGGACAATGGAGGGT
EEF2	NM_205368.1	CAGTTGGCTTTGGTTCTGGC	AAAGTATCTGTCTCCCCACAGC
GAPDH	NM_204305.1	ATCCCTGAGCTGAATGGGAAG	AGCAGCCTTCACTACCCTCT
IPO8	XM_015287054.2	ACCTCCGAGCTAGATCCTGT	GGCTCTTCTTCGCCAACTCT
RPLP0	NM_204987.2	TTGGGCATCACCACAAAGATT	CCCACTTTGTCTCCGGTCTTAA

ACTB, beta actin; CaBP-D28k, calbindin D28k; CaSR, Ca sensing receptor; CLDN, claudin; EEF2, eukaryotic elongation factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPO8, importin 8; NaPi-IIa, sodium dependent phosphate transporter IIa; NaPi-IIb, sodium dependent phosphate transporter IIb; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; PMCA1, plasma membrane Ca-ATPase 1; RPLP0, 60S acidic ribosomal protein P0; TRPC1, transient receptor potential canonical 1; TRPV6, transient receptor potential cation channel subfamily V member 6; VDR, vitamin-D_3 receptor; XPR1, xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1.

Statistical analysis

The experimental unit for data analysis was pen. Data were subjected to a two-way ANOVA using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). Limestone particle size, Ca/P and their interaction were used as main effects and pen as random effect. Distribution and variance homogeneity of Studentized residuals were visually checked via graphics plotted using ODS GRAPHICS function. The LSMEANS procedure with a PDIFF option was used to estimate the difference between means. Probability was considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.1$.

Results

Duodenum

The RT-qPCR analysis revealed that the transcript level of the extracellular Ca-sensing receptor (CaSR) was reduced in the duodenal mucosa of broilers fed with incremental dietary Ca/P from low (0.5) to high (1.75) in combination with fine

limestone, but was reduced only when dietary Ca/P was increased from low to medium (1.00) in the presence of coarse limestone ($P_{interaction}$=0.047, **Table 2**). In addition, incremental dietary Ca/P also downregulated mRNA expression of Ca-ATPase PMCA1 and CaBP-D28k (P<0.001) in this segment. However, the latter reduction was found with fine limestone when dietary Ca/P was increased from low to medium, but with coarse limestone from medium to high ($P_{interaction}$=0.016 and 0.037, respectively). Moreover, expression of TRPC1 was not affected by either dietary Ca or limestone particle size. Similarly, an interaction effect between Ca/P and limestone particle size was observed for the Na-P cotransporter NaPi-IIb ($P_{interaction}$=0.035), with a reduction in its expression level when dietary Ca/P was increased from low to medium together with fine limestone but with coarse limestone only from medium to high. Expression of the other Na-P cotransporters PiT-1 and PiT-2, and the putative basolateral P channel XPR1 were not affected by the dietary treatments. As for TJ proteins, CLDN-2 was downregulated with high dietary Ca intake (P<0.001) irrespective of limestone size (no interaction effect). Expression of zonula occludens-1 (ZO-1) and CLDN-12, however, remained unaffected by dietary treatments. The same was true for the transcript level of vitamin D_3 receptor (VDR). We did not detect expression of NaPi-IIa, TRPV6 or CLDN-16 in the broiler's duodenal mucosa.

Table 2. Least square mean of mRNA expression levels of Ca and P transporters and claudins in the duodenal mucosa in broilers as affected by dietary Ca/P and limestone particle sizes [1,2,3,4]

Limestone	Ca/P	CaSR	TRPC1	CaBP-D28k	PMCA1	NaPi-IIb	PiT-1	PiT-2	XPR1	ZO-1	CLDN-2	CLDN-12	VDR
	0.50	0.18^{a}	1.29	1319^{a}	478^{a}	2652^{a}	13.0	1.47	12.8	1.89	108	1.77	2.59
Fine	1.00	0.12^{b}	1.30	921^{cd}	373^{b}	2054^{bc}	11.3	1.46	13.1	1.98	93	1.88	2.54
	1.75	0.07^{c}	1.27	998^{bcd}	371^{b}	2173^{abc}	11.3	1.20	13.2	1.93	77	1.88	3.04
	0.50	0.23^{a}	1.29	1248^{ab}	410^{b}	2519^{ab}	11.8	1.24	12.8	2.01	107	1.83	2.64
Coarse	1.00	0.11^{bc}	1.24	1144^{abc}	412^{b}	2633^{a}	12.6	1.43	12.7	1.88	93	1.79	3.00
	1.75	0.11^{bc}	1.26	754^{d}	311^{c}	1875^{c}	12.9	1.44	12.9	1.84	76	1.85	2.76
Pooled SEM		0.020	0.084	125.4	27.7	245.9	1.73	0.252	0.86	0.094	6.0	0.095	0.263
Ca/P													
0.50		0.21	1.29	1283	444	2586	12.4	1.36	12.8	1.95	108^{a}	1.80	2.62
1.00		0.12	1.27	1032	393	2344	12.0	1.44	12.9	1.93	93^{b}	1.84	2.77
1.75		0.09	1.27	876	341	2024	12.1	1.32	13.1	1.88	76^{c}	1.86	2.90
Pooled SEM		0.014	0.059	88.7	19.6	173.9	1.22	0.141	0.61	0.066	4.2	0.067	0.186
Limestone													
Fine		0.13	1.27	1079	408	2293	11.9	1.38	13.0	1.93	93	1.84	2.72
Coarse		0.15	1.26	1049	378	2343	12.5	1.37	12.8	1.91	92	1.82	2.80
Pooled SEM		0.012	0.049	72.4	16.0	142.0	1.00	0.115	0.50	0.054	3.5	0.055	0.152
P-value													
Ca/P		<0.001	0.913	<0.001	<0.001	0.009	0.926	0.671	0.907	0.593	<0.001	0.651	0.335
Limestone		0.030	0.629	0.673	0.068	0.727	0.561	0.962	0.609	0.707	0.853	0.652	0.651
Ca/P × limestone		0.047	0.914	0.037	0.016	0.035	0.463	0.265	0.926	0.170	0.976	0.417	0.151

[1] Dietary P content was fixed at 5.5 g/kg diet.
[2] Data are presented as treatment means, 4 pens per treatment and 3 birds per pen (n=12).
[3] Fine and coarse limestone were separated from the same product via sieving (Sibelco, the Netherlands; geometric mean diameter 160 vs. 1062 μm).
[4] Determined using absolute quantification and normalized by the geometric mean of EEF2 and IPO8.
[a-d] Values lacking a common superscript within a column differ ($P \leq 0.05$).
CaBP-D28k, calbindin D28k; CaSR, Ca sensing receptor; CLDN, claudin; EEF2, eukaryotic elongation factor 2; IPO8, importin 8; NaPi-IIa, sodium dependent phosphate transporter IIa; NaPi-IIb, sodium dependent phosphate transporter IIb; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; PMCA1, plasma membrane Ca-ATPase 1; TRPC1, transient receptor potential canonical 1; TRPV6, transient receptor potential cation channel subfamily V member 6; VDR, vitamin-D_3 receptor; XPR1, xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1. Of note, NaPi-IIa, TRPV6 and CLDN-16 mRNA were not detectable.

Jejunum

Jejunal mucosa displayed lower mRNA abundances of all measured genes compared to duodenal mucosa except for PiT-1 and CLDN-12 (**Table 3**). Expression of CaBP-D28k, NaPi-IIb and XPR1 in the jejunum were approximately 50% lower. Jejunal mucosal expression of CaSR was reduced with high dietary Ca/P ($P<0.001$) and with fine limestone intake ($P=0.002$). An interaction effect was observed for CaBP-D28k mRNA levels due to the fact that the inhibitory effect was greatest with fine limestone at an increase of Ca/P from low to medium but with coarse limestone from medium to high ($P_{interaction}=0.004$). Contrary to findings in the duodenum, PMCA1 mRNA levels remained unaffected by the dietary treatments in the jejunum. Besides, dietary Ca increment reduced jejunal expression of TRPC1 but only for diets supplied with coarse limestone ($P_{interaction}=0.05$). Incremental dietary Ca/P also reduced CLDN-2 gene expression levels ($P<0.015$) independent of limestone size. Compared to the low Ca/P group, high dietary Ca/P downregulated ZO-1 mRNA levels only in the presence of coarse limestone ($P_{interaction}=0.047$). Neither CLDN-12 nor VDR mRNA levels were influenced by the dietary treatments. Regarding P absorption, NaPi-IIb expression was downregulated by incremental dietary Ca/P ($P=0.004$) regardless of the addition of the different limestone sizes. Similarly to duodenal mucosa, jejunal mucosal transcript levels of PiT-1, PiT-2 and XPR1 were not affected in response to the dietary treatments ($P>0.05$; Table 3) and the levels of NaPi-IIa, TRPV6 and CLDN-16 were below the limit of detection (data not shown).

Table 3. Least square mean of mRNA expression levels of Ca and P transporters and claudins in the jejunal mucosa as affected by dietary Ca/P and limestone particle sizes in broilers [1,2,3,4]

Limestone	Ca/P	CaSR	TRPC1	CaBP-D28k	PMCA1	NaPi-IIb	PiT-1	PiT-2	XPR1	ZO-1	CLDN-2	CLDN-12	VDR
	0.50	0.94	1.47^{b}	668^{a}	356	1276	16.0	1.26	8.31	2.42^{abc}	70.1	1.85	1.71
Fine	1.00	0.70	1.61^{ab}	418^{c}	287	971	15.1	1.01	8.01	2.54^{ab}	51.2	2.03	1.71
	1.75	0.43	1.41^{b}	530^{b}	330	952	16.0	0.92	8.56	2.35^{b}	45.5	1.85	2.10
	0.50	1.14	1.69^{a}	664^{a}	327	1282	16.2	1.10	8.40	2.64^{a}	65.4	2.02	2.17
Coarse	1.00	0.95	1.44^{b}	561^{ab}	320	1296	16.0	1.15	8.58	2.41^{bc}	56.1	1.81	1.94
	1.75	0.65	1.43^{b}	401^{c}	295	846	14.7	1.12	8.37	2.27^{c}	45.6	1.83	2.00
Pooled SEM		0.115	0.109	53.8	36.1	151.8	2.81	0.137	0.617	0.100	6.28	0.111	0.229
Ca/P													
0.50		1.04^{a}	1.58	666	342	1279^{a}	16.1	1.18	8.35	2.53	67.8^{a}	1.94	1.94
1.00		0.83^{b}	1.53	489	304	1133^{a}	15.5	1.08	8.30	2.47	53.6^{b}	1.92	1.82
1.75		0.54^{c}	1.42	466	312	899^{b}	15.3	1.02	8.46	2.31	45.6^{b}	1.84	2.05
Pooled SEM		0.081	0.077	38.1	25.5	107.4	1.99	0.097	0.436	0.071	4.44	0.078	0.163
Limestone													
Fine		0.69	1.50	539	324	1066	15.7	1.06	8.30	2.43	55.7	1.91	1.84
Coarse		0.92	1.52	542	314	1141	15.6	1.12	8.45	2.44	55.6	1.89	2.04
Pooled SEM		0.066	0.063	31.1	20.8	87.6	1.63	0.079	0.356	0.058	3.63	0.064	0.133
P-value													
Ca/P		<0.001	0.125	<0.001	0.308	0.004	0.925	0.222	0.927	0.015	<0.001	0.451	0.420
Limestone		0.002	0.738	0.915	0.626	0.398	0.972	0.510	0.704	0.891	0.973	0.604	0.135
Ca/P × limestone		0.949	0.050	0.004	0.351	0.125	0.867	0.153	0.677	0.047	0.553	0.066	0.271

[1] Dietary P content was fixed at 5.5 g/kg diet.
[2] Data are presented as treatment means, 4 pens per treatment and 3 birds per pen (n=12).
[3] Fine and coarse limestone were separated from the same product via sieving (Sibelco, the Netherlands; geometric mean diameter 160 vs. 1062 µm).
[4] Determined using absolute quantification normalized by EEF2 and IPO8.
[a-c] Values lacking a common superscript within a column differ ($P \leq 0.05$).
CaBP-D28k, calbindin D28k; CaSR, Ca sensing receptor; CLDN, claudin; EEF2, eukaryotic elongation factor 2; IPO8, importin 8; NaPi-IIa, sodium dependent phosphate transporter IIa; NaPi-IIb, sodium dependent phosphate transporter IIb; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; PMCA1, plasma membrane Ca-ATPase 1; TRPC1, transient receptor potential canonical 1; TRPV6, transient receptor potential cation channel subfamily V member 6; VDR, vitamin-D_3 receptor; XPR1, xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1. Of note, TRPV6, NaPi-IIa and CLDN-16 mRNA were not detectable.

Discussion

The data in the present study demonstrates that high dietary Ca/P reduces duodenal mRNA level of NaPi-IIb in broilers to a greater extent in the presence of fine limestone than coarse limestone. The inhibitory effect on the jejunal NaPi-IIb mRNA level, however, appears to be independent of limestone size. Similarly, in the duodenum, incremental dietary Ca/P downregulated the expression of the Ca absorption-related genes CaBP-D28k and PMCA1 in a limestone size dependent manner, but this dependency was not seen for the latter gene in the jejunum. Irrespective of limestone size, the expression of CLDN-2 in both intestinal segments was linearly downregulated with incremental dietary Ca/P.

An intriguing observation is that our RT-qPCR procedure did not detect any substantial gene expression of TRPV6 in the duodenal and jejunal mucosa, while ample studies have demonstrated that TRPV6 is responsible for the highly selective apical uptake of Ca by absorptive epithelial cells in mammals. The expression of TRPV6 in the chicken's intestine is controversial; the absence of TRPV6 mRNA in the chicken small intestine is also reported by others [6, 20, 21], while the presence is supported by the evidence of an immunoreactive protein in the small intestine of chickens [20, 22]. Gloux *et al*. [14] postulated that TRPC1 may be a new candidate Ca transporter which also allows apical Ca entry in the gut of chickens. Indeed, we observed a downregulation of TRPC1 mRNA expression in response to dietary Ca increment combined with coarse limestone in jejunal but not duodenal mucosa. However, expression of TRPC1 was not regulatable when the diet contained fine limestone. Further studies are thus required to establish the exact transport system for Ca by the intestinal epithelium of chickens.

Our observation that high dietary Ca/P downregulates expression of NaPi-IIb, CaBP-D28k and PMCA1 in the duodenum and jejunum in broilers is in agreement with studies in mammals. We previously reported that in these birds, incremental dietary Ca/P led to a linear increase in serum Ca concentration, accompanied by a numerical decrease in serum 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$). The VDR is expressed in the small intestine and unaffected by the dietary treatments. The observed lowering of CaBP-D28k, PMCA1 and NaPi-IIb mRNA levels may be ascribed to the decrement of $1,25(OH)_2D_3$ since these genes contain a responsive element on their promoter sequence [23].

Since NaPi-IIb is considered as the rate-limiting step in the process of P absorption across the epithelial cells [24], its downregulation with high Ca/P indicates a lower capacity of P absorption by the intestinal segment. This notion is in line with our previous observation [3] that high dietary Ca/P lowered serum P levels in these birds. In another recently performed broiler study from our group (Chapter 6), dietary Ca increment also reduced serum P accompanied with a downregulation of duodenal expression of NaPi-IIb, PiT-2 and XPR1, which is in line with results obtained in the present study.

We also observed that the limestone particle size treatment modulated the effect of dietary Ca/P on the duodenal expression of NaPi-IIb, CaBP-D28k and PMCA1, i.e. mRNA levels of these genes were in the combination with fine limestone only higher with low dietary Ca/P, while for coarse limestone with low and medium Ca/P. An explanation is that fine limestone is more soluble [25], providing a greater Ca availability for absorption in the duodenum [3]. Combined with a medium Ca/P level, this may lead to an earlier overload of the Ca transcellular pathway compared to the combination with coarse limestone and that the epithelial cells will prevent excessive cellular uptake long term by reducing expression of these three genes to lower the active Ca and P transport capacity in the duodenum. Noteworthy is that we previously demonstrated that serum Ca, P and $1,25(OH)_2D_3$ were independent of limestone particle size, which is probably because the broilers were under steady-state conditions thus representing intestinal Ca and P absorption from the total tract rather than proximal part of the gut. Furthermore, an acute release of Ca from fine limestone could activate extracellular CaSR, thereby, directly downregulating expression of Ca transporters independent of serum circulation of PTH or $1,25(OH)_2D_3$ [26].

Incremental dietary Ca/P might also reduce paracellular Ca permeation. No information is available about the intestinal Ca absorption via the paracellular route in broilers. In mammals, Ca permeation was considered to be stable and independent of $1,25(OH)_2D_3$ [27, 28]. However, in Caco-2 cells, it has been demonstrated that CLDN-2 formed pores selective for Ca ions, increasing Ca permeation across the monolayer and its expression level was upregulated after $1,25(OH)_2D_3$ stimulation [29]. In the present study, we found a drastic reduction of mRNA expression of CLDN-2 in the broilers evoked by high dietary Ca/P intake in both duodenum and jejunum, which could also be regulated by $1,25(OH)_2D_3$. This

is in agreement with the numerical drop of serum $1,25(OH)_2D_3$ levels in these birds [3] and that the CLDN-2 gene has a responsive element to vitamin D_3 in its promoter. In support of this notion is the reduced CLDN-2 expression by a high dietary Ca/P in these segments (Table 2 and 3) and the serum $1,25(OH)_2D_3$ levels were not affected by limestone particle size [3]. The lower mRNA expression of CLDN-2 probably attributes to a decreased capacity of intestinal paracellular Ca permeation. Unfortunately, CLDN that may facilitate paracellular P permeation in animals have not been identified yet and awaits, therefore, further studies.

Our previous study demonstrated that over 85% of precaecal digestible Ca and P is absorbed proximal to the ileum [3]. Here, we report that the duodenal mucosa displayed a higher expression level of NaPi-IIb, CaBP-D28k and PMCA1 than jejunum. Considering that active apical entry of P [24] and intracellular diffusion of Ca [30] are regarded as the rate-limiting processes for active translocation of Ca and P across the intestinal epithelial cell, a gradual reduction of NaPi-IIb and CaBP-D28k may indicate a lower contribution of active Ca and P absorption along the small intestine. Indeed, by using *in situ* ligated intestinal loops infused with solutions varying in P concentration, Liu *et al*. [31] confirmed that P absorption is a carrier-mediated process in the duodenum while a passive unsaturated process in the jejunum and ileum. It should be noted that an abundant expression of Ca and P transporters does not necessarily mean substantial absorption of Ca and P in the duodenum, as our previous broiler study [3] showed that digesta mean retention time was rather short in this segment (approximately 1 min).

In conclusion, dietary Ca increment reduces expression of Ca transporters in a limestone particle size dependent manner in both duodenal and jejunal mucosa. Expression of Ca permeable claudin (CLDN-2) is also downregulated with dietary Ca increment irrespective of limestone particle size. Calcium induced mRNA expression of P transporters maybe important in reducing intestinal P absorption in broilers.

Acknowledgements

This research was conducted by Wageningen Livestock Research within the framework of the public private partnership "Feed4Foodure" and partially funded by the Ministry of Economic Affairs (Policy Support Research project number BO-31.03-005-00). The authors acknowledge the Chinese Scholarship Council (CSC) for the scholarship of Y.X. Hu to study at Wageningen University & Research.

Author contributions

M.M. van Krimpen, M. Duijster and Y.X. Hu designed and conducted the study; Y.X. Hu conducted sample and data analysis; Y.X. Hu, J. van Baal, P. Bikker, M.M. van Krimpen, W.H. Hendriks and M. Duijster critically discussed and interpreted data, and wrote the paper. All authors, except the late M.M. van Krimpen, have read and approved the final manuscript. M. Duijster is an employee of De Heus, all other authors declare no conflict of interest.

References

1. Weaver CM & Peacock M (2019) Calcium. *Adv Nutr* **10**, 546-548.

2. Dersjant-Li Y, Awati A, Schulze H *et al.* (2015) Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. *J Sci Food Agric* **95**, 878-896.

3. Hu YX, Bikker P, Duijster M *et al.* (2020) Coarse limestone does not alleviate the negative effect of a low Ca/P ratio diet on characteristics of tibia strength and growth performance in broilers. *Poult Sci* **99**, 4978–4989.

4. Christakos S, Dhawan P, Verstuyf A *et al.* (2016) Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. *Physiol Rev* **96**, 365-408.

5. Khundmiri SJ, Murray RD & Lederer E (2016) PTH and vitamin D. *Compr Physiol* **6**, 561-601.

6. Proszkowiec-Weglarz M & Angel R (2013) Calcium and phosphorus metabolism in broilers: effect of homeostatic mechanism on calcium and phosphorus digestibility. *J Appl Poult Res* **22**, 609-627.

7. Kellett GL (2011) Alternative perspective on intestinal calcium absorption: proposed complementary actions of $Ca_v1.3$ and TRPV6. *Nutr Rev* **69**, 347-370.

8. Hernando N, Gagnon KB & Lederer ED (2020) Phosphate transport in epithelial and nonepithelial tissue. *Physiol Rev* **101**, 1–35.

9. Hu Y, Liao X, Wen Q *et al.* (2018) Phosphorus absorption and gene expression levels of related transporters in the small intestine of broilers. *Br J Nutr* **119**, 1346-1354.

10. Legati A, Giovannini D, Nicolas G *et al.* (2015) Mutations in XPR1 cause primary familial brain calcification associated with altered phosphate export. *Nat Genet* **47**, 579-581.

11. Michigami T, Kawai M, Yamazaki M *et al.* (2018) Phosphate as a signaling molecule and its sensing mechanism. *Physiol Rev* **98**, 2317-2348.

12. McGoldrick LL, Singh AK, Saotome K *et al.* (2018) Opening of the human epithelial calcium channel TRPV6. *Nature* **553**, 233-237.

13. Khanal RC & Nemere I (2008) Regulation of intestinal calcium transport. *Annu Rev Nutr* **28**, 179-196.

14. Gloux A, Le Roy N, Brionne A *et al.* (2019) Candidate genes of the transcellular and paracellular calcium absorption pathways in the small intestine of laying hens. *Poult Sci* **98**, 6005-6018.

15. Tsukita S, Tanaka H & Tamura A (2019) The claudins: from tight junctions to biological systems. *Trends Biochem Sci* **44**, 141-152.

16. Fujita H, Chiba H, Yokozaki H *et al.* (2006) Differential expression and subcellular localization of claudin-7, -8, -12, -13, and -15 along the mouse intestine. *J Histochem Cytochem* **54**, 933-944.

17. Inai T, Kobayashi J & Shibata Y (1999) Claudin-1 contributes to the epithelial barrier function in MDCK cells. *Eur J Cell Biol* **78**, 849-855.

18. Amasheh S, Schmidt T, Mahn M *et al.* (2005) Contribution of claudin-5 to barrier properties in tight junctions of epithelial cells. *Cell Tissue Res* **321**, 89-96.

19. Andersen CL, Jensen JL & Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* **64**, 5245-5250.

20. Yang JH, Hou JF, Farquharson C *et al.* (2011) Localisation and expression of TRPV6 in all intestinal segments and kidney of laying hens. *Br Poult Sci* **52**, 507-516.

21. Proszkowiec-Weglarz M, Schreier LL, Miska KB *et al.* (2018) Effect of early neonatal development and delayed feeding post-hatch on jejunal and ileal calcium and phosphorus transporter genes expression in broiler chickens. *Poult Sci* **98**, 1861–1871

22. Huber K, Zeller E & Rodehutscord M (2015) Modulation of small intestinal phosphate transporter by dietary supplements of mineral phosphorus and phytase in broilers. *Poult Sci* **94**, 1009-1017.

23. Belkacemi L, Züegel U, Steinmeyer A *et al.* (2005) Calbindin-D28k (CaBP28k) identification and regulation by 1,25-dihydroxyvitamin D_3 in human choriocarcinoma cell line JEG-3. *Mol Cell Endocrinol* **236**, 31-41.

24. Biber J, Hernando N & Forster I (2013) Phosphate transporters and their function. *Annu Rev Physiol* **75**, 535-550.

25. Kim SW, Li W, Angel R *et al.* (2019) Modification of a limestone solubility method and potential to correlate with *in vivo* limestone calcium digestibility. *Poult Sci* **98**, 6837-6848.

26. Lee JJ, Liu X, O'Neill D *et al.* (2019) Activation of the calcium-sensing receptor attenuates TRPV6-dependent intestinal calcium absorption. *JCI insight* **4**, e128013.

27. Bronner F (2009) Recent developments in intestinal calcium absorption. *Nutr Rev* **67**, 109-113.

28. Song Y, Kato S & Fleet JC (2003) Vitamin D receptor (VDR) knockout mice reveal VDR-independent regulation of intestinal calcium absorption and $ECaC_2$ and calbindin D_{9k} mRNA. *J Nutr* **133**, 374-380.

29. Fujita H, Sugimoto K, Inatomi S *et al.* (2008) Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca^{2+} absorption between enterocytes. *Mol Biol Cell* **19**, 1912-1921.

30. Kiela PR & Ghishan FK (2018) Molecular mechanisms of intestinal transport of calcium, phosphate, and magnesium. In *Physiology of the Gastrointestinal Tract (Sixth Edition)*, pp. 1405-1449 [Said HM, Ghishan FK, Kaunitz JD, Merchant JL and Wood JD, editors]. London: Academic Press Elsevier.

31. Liu SB, Hu YX, Liao XD *et al.* (2016) Kinetics of phosphorus absorption in ligated small intestinal segments of broilers. *J Anim Sci* **94**, 3312-3320.

CHAPTER 6

Low-Ca diets increase duodenal mRNA expression of Ca and P transporters and claudins but compromise growth performance irrespective of microbial phytase inclusion in broilers

Y.X. Hu[*†], J. van Harn[*], W.H. Hendriks[†], J. van Baal[†], M.A. Dijkslag[‡], M.M. van Krimpen[*], P. Bikker[*]

[*] Wageningen University & Research, Wageningen Livestock Research, 6700 AH Wageningen, the Netherlands

[†] Wageningen University & Research, Animal Nutrition Group, 6700 AH Wageningen, the Netherlands

[‡] ForFarmers, Kwinkweerd 12, 7241 CW Lochem, the Netherlands

Submitted to Poultry Science

Abstract

The hypothesis that dietary inclusion of microbial phytase improves apparent calcium (Ca) digestibility thereby allowing a lower dietary Ca inclusion without compromising growth performance was tested. One-day-old male Ross 308 broilers (25 birds/pen, nine pens/treatment) were assigned to one of eight experimental diets containing one of four dietary Ca to retainable P (rP) ratios (1.3, 1.8, 2.3 and 2.8) with (1000 FTU/kg) or without microbial phytase inclusion. On day 21-23, eight birds per pen were dissected to collect digesta from different gut segments for analysis of apparent Ca and P digestibility. Mid duodenal mucosa was collected for expression of Ca and P transporters by RT-qPCR. Dietary phytase inclusion in low Ca/rP diets increased apparent Ca digestibility in the distal ileum ($P_{interaction}$=0.023) but not the proximal or distal jejunum. Broilers receiving the lowest Ca/rP displayed the lowest body weight gain and highest feed conversion ratio (P<0.001) irrespective of dietary phytase inclusion. Incremental dietary Ca/rP linearly reduced apparent P digestibility to a greater extent in the absence of phytase in the distal jejunum and ileum ($P_{interaction}$=0.021 and 0.001, respectively). Incremental dietary Ca/rP linearly reduced serum P more in phytase-free diets ($P_{interaction}$<0.001), and lowered duodenal expression of P transporters NaPi-IIb, PiT-2 and XPR1 (P=0.052, 0.071 and 0.028, respectively). Incremental dietary Ca/rP linearly increased (P<0.001) serum Ca irrespective of phytase inclusion, accompanied by a lower (P<0.001) duodenal expression of Ca transporters CaSR, CaBP-D28k and PMCA1 and Ca-pore forming claudins CLDN-2 and CLDN-12. Dietary phytase inclusion increased (P=0.026) NaPi-IIb but reduced (P=0.029) CLDN-2 expression. Incremental Ca/rP reduced apparent Ca and P digestibility, increased serum Ca, lowered serum P and inhibited mRNA levels of Ca and P-related transporters, indicating that these transporters and CLDN contribute to the observed effect of Ca level and phytase on Ca and P absorption in broilers. Despite the improvement in apparent Ca digestibility, inclusion of dietary phytase did not restore the compromised growth performance of broilers fed a Ca-deficient diet, leading to rejection of the hypothesis.

Key words: Ca and P digestibility, intestinal gene expression, paracellular pathway, transcellular pathway, broilers

Introduction

Phosphorus (P) is the 3rd most expensive ingredient in farm animal diets and plays an important role in many biological processes such as muscle contraction, energy production, cell signalling and bone formation [1]. Inositol phosphate bound P (IP-P) is the major form of P storage (60-80%) in most cereal grains and oil seeds [2]. This IP-P is poorly digestible in non-ruminant animals such as pigs and broilers. It is generally accepted that reduction of dietary calcium (Ca) content improves IP degradation and IP-P digestion [3]. However, over-reduction of dietary Ca content may compromise growth performance and tibia breaking strength [4] because a minimum supply of dietary Ca is required to suffice post-absorption P utilisation and bone development. Improving dietary Ca digestibility seems to be a promising approach to reduce dietary Ca inclusion without compromising growth performance or bone development in broilers.

Dietary inclusion of microbial phytase is widely practised to improve IP-P absorption in animal diets [5]. A number of studies reported that microbial phytase also improves dietary Ca digestibility although the results are inconsistent. The mechanism involved may be that dietary phytase liberates Ca ions by degradation of its chelator IP. Ravindran *et al*. [6] showed that microbial phytase inclusion (500 and 2000 FTU/kg) enhanced the true ileal Ca digestibility in broilers fed canola-based diets, and also in corn-soybean meal based diets at a dosage of 2000 but not 500 FTU phytase/kg diet. These findings are largely in line with Majeed *et al*. [7] who demonstrated that microbial phytase inclusion (1000 FTU/kg) significantly increased apparent ileal Ca digestibility from 44 to 68% in diets supplemented with coarse limestone, while it reduced Ca digestibility from 70 to 61% in diets with fine limestone. These findings indicate that dietary phytase may improve Ca digestibility depending on dose of microbial phytase and source of Ca, probably because they affect Ca-IP complexation in the gastrointestinal tract (GIT) of broilers. It seems possible that inclusion of microbial phytase improves Ca digestibility and allows a reduction of dietary Ca inclusion without compromising growth performance. Moreover, a reduction in dietary Ca may abate the process of Ca-IP complexation, which in turn, may enhance microbial phytase efficacy in the GIT. The interaction between Ca and microbial phytase on Ca and P digestibility is currently not fully clarified with studies reporting a high dietary Ca content hampering or either enhancing microbial phytase efficacy.

The binding capacity of IP towards Ca decreases drastically at a pH <5 [8], indicating that phytase efficacy would be hampered more in the distal than proximal segments of the GIT. However, Ca and P absorption along the intestinal tract in broilers is not fully elucidated. Moreover, a gradual reduction in expression of Ca and P transporters along the small intestine of broilers was observed [9], which may suggest a lower absorption capacity of Ca and P in the distal compared to the proximal part of the small intestine. The candidate genes involved in intestinal Ca and P absorption in broilers are presumed to be rather similar to those in mammals, due to their relative high degree in protein sequence homology, implicating functional similarity [10]. Transcellular intestinal absorption of Ca or P starts with the apical uptake into the enterocytes via distinct transporters, diffusion across the cytoplasm and basolateral exit by other transporters [11]. The paracellular mechanism is a passive transport process that occurs across the majority of the intestine and is a linear function of luminal Ca and P concentration and is likely mediated by claudins (CLDN), integral structures of the tight junction complex [12]. Recently, it has been demonstrated that Ca absorbed through the paracellular shunt is also significantly regulated [13]. More insight into the modulation of intestinal Ca and P transporters and CLDN through dietary intervention is an important element in poultry nutrition in order to better understand and improve intestinal Ca and P absorption, reduce feed costs and alleviate P pollution in the environment.

We hypothesized that microbial phytase in the diet would increase intestinal Ca absorption in broilers, allowing lower dietary Ca inclusion without affecting the growth performance. Therefore, we investigated the interactive effect of dietary Ca content and microbial phytase inclusion on the growth performance, apparent Ca and P digestibility, serum Ca and P content as well as duodenal mRNA expression of genes related to Ca and P absorption in broilers.

Materials and methods

The experiment was approved by the ethical committee of Wageningen University & Research (2016.D-0065.022) and conducted in the facilities of ForFarmers, Bathmen, the Netherlands. All procedures agreed with Dutch laws on animal experiments.

Experimental design and diets

A total of 1800 one-day-old Ross 308 male broilers were equally divided over 72 pens, receiving one of eight experimental diets containing one of four Ca to retainable P ratios (Ca/rP) in the presence (1000 FTU/kg) or absence of microbial phytase in a 4×2 completely randomized block design, with nine replicate pens per treatment and 25 birds per pen. Pens were blocked by location within the barn and allotted to one of eight experimental treatments. The intended rP content was fixed at 80% of CVB [(14)] recommendation for all treatment groups (i.e. 3.2, 2.5 and 2.2 g/kg, excluding the contribution of microbial phytase, in the starter, grower and finisher period, respectively). The intended dietary Ca/rP of 1.3, 1.8, 2.3 and 2.8 was realised by inclusion of various amounts of limestone.

The animal experiment lasted for 36 d and included three feeding phases. Starter, grower and finisher diets were supplied from d 0-10, 10-29 and 29-36, respectively. In each of the three feeding phases, a basal diet, which met or exceeded the minimum requirement of all nutrients for broilers [(14)] except for Ca and rP, was prepared and then divided into eight equal portions. Subsequently the eight experimental diets were made by adding various amounts of limestone (Faunacal®, Wülfrath, Germany; mean particle size 90 µm as reported by the manufacturer), phytase (Axtra Phy, Danisco Animal Nutrition, Marlborough, United Kingdom) and diamol (Damolin, Kønsborgvej, Denmark) according to the experimental design. Titanium dioxide (TiO_2) was added to the basal grower diets at 5 g/kg as an indigestible marker. All diets were pelleted (starter diets 2.5 mm, grower and finisher diets 3.2 mm). Diet samples were taken with an automatic sampling device during production. Dietary composition is reported in **Table 1**. Intended and analysed Ca, P and phytase activity are shown in **Table 2**.

Table 1. Ingredients and composition of the diets, g/kg as fed unless otherwise specified

Item	Starter	Grower	Finisher
Ingredients			
Wheat	320.0	318.0	350.0
Maize	257.0	260.0	253.0
Soybean meal (48% crude protein)	307.0	278.0	238.0
Rapeseed meal	24.8	39.8	59.1
Sunflower seed meal	5.3	10.0	10.9
Soybean oil	27.6	36.0	38.7
Palm oil	5.0	10.0	10.0
Oat hulls	10.0	10.0	10.0
Lauric fatty acids	5.0	5.0	5.0
Premix vitamins [1]	2.0	1.5	1.25
Premix trace minerals [2]	1.5	1.5	1.25
Premix xylanase (6.25%) [3]	1.0	1.0	1.0
DL-Methionine	2.8	2.3	2.1
L-Lysine (HCl)	2.4	1.7	1.8
L-Threonine	1.0	0.5	0.5
Valine	0.7	0.1	0.1
Sodium bicarbonate	2.3	3.0	2.7
Sodium chloride	1.8	1.0	1.0
Monocalcium phosphate	9.3	5.5	4.4
Experimental mixture [4]	13.5	10.1	9.2
Titanium dioxide	0.0	5.0	0.0
Nutrients, calculated			
AMEn, kcal/kg	2900	3000	3025
Crude protein	219	211	200
Crude fibre	33	34	36
Crude ash	57	50	46
Crude fat	63	77	80
Starch	357	360	372
Nutrients, analysed			
Dry matter	890	882	881
Crude ash	55	51	43
Crude fibre	32	33	34
Crude protein	216	207	199
Starch	367	355	369
Crude fat	65	75	81

[1] Vitamin premix (Trouw Nutrition, Putten, the Netherlands) contained: (IU/kg) vitamin A 5,000,000; vitamin D_3 2,222,000; vitamin E 13,333; (mg/kg) vitamin K_3 1,000; vitamin B_1 667; vitamin B_2 2,667; vitamin B_3 26,667; vitamin B_5 7246; vitamin B_6 2,000; vitamin B_7 89; vitamin B_{11} 667; vitamin B_{12} 10.
[2] Trace mineral premix (Trouw Nutrition, Putten, the Netherlands) contained: (g/kg) $FeSO_4 \cdot H_2O$ 23.3; $Ca(IO_3)_2$ 1; $CuSO_4 \cdot 5H_2O$ 8; MnO 46.7; $ZnSO_4 \cdot H_2O$ 53.3; (mg/kg) Na_2SeO_3 133.
[3] Xylanase 6.25% (Trouw Nutrition, Putten, the Netherlands).
[4] Composition shown in Table 2.

Table 2. Inclusion of limestone, phytase and diamol, and calculated and analysed content of calcium (Ca), phosphorus (P) and phytase activity in the starter, grower and finisher diets, g/kg as fed unless otherwise specified

Phytase, FTU/kg	0				1000			
Ca/rP	1.3	1.8	2.3	2.8	1.3	1.8	2.3	2.8
Starter period (d 0-10)								
Limestone [1]	0.5	4.9	9.2	13.5	0.5	4.9	9.2	13.5
Phytase [2]	0	0	0	0	0.2	0.2	0.2	0.2
Diamol [3]	13.0	8.6	4.3	0	13.0	8.6	4.3	0
Ca calculated	4.2	5.8	7.4	9.0	4.2	5.8	7.4	9.0
P calculated	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Retainable P calculated [4]	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Ca analysed	4.5	6.0	6.8	9.0	4.5	6.5	8.0	9.0
P analysed	5.7	5.7	5.6	5.6	5.7	5.6	5.7	5.6
Phytase analysed, FTU/kg	119	34	404	246	1395	1550	1362	1511
Grower period (d 10-29)								
Limestone [1]	0.06	3.4	6.8	10.1	0.06	3.4	6.8	10.1
Phytase [2]	0	0	0	0	0.2	0.2	0.2	0.2
Diamol [3]	10.1	6.7	3.3	0	10.1	6.7	3.3	0
Ca calculated	3.2	4.5	5.7	6.9	3.2	4.5	5.7	6.9
P calculated	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Retainable P calculated [4]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Ca analysed	3.5	4.7	6.0	7.1	3.5	4.7	5.9	6.7
P analysed	4.7	4.8	4.9	4.9	5.1	4.9	5.2	5.0
Phytase analysed, FTU/kg	237	135	55	293	1386	1060	1205	860
Finisher period (d 29-36)								
Limestone [1]	0.1	3.1	6.2	9.2	0.1	3.1	6.2	9.2
Phytase [2]	0	0	0	0	0.2	0.2	0.2	0.2
Diamol [3]	9.1	6.1	3.0	0	9.1	6.1	3.0	0
Ca calculated	2.9	4.0	5.2	6.3	2.9	4.0	5.2	6.3
P calculated	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8
Retainable P calculated [4]	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
Ca analysed	3.2	4.1	5.5	6.1	3.2	4.2	5.2	6.1
P analysed	4.8	4.7	4.8	4.8	4.8	4.9	4.7	4.8
Phytase analysed, FTU/kg	252	315	281	157	966	1208	673	842

[1] Faunacal®, Wülfrath, Germany, mean particle size 90 µm as reported by the manufacture.
[2] Axtra Phy, Marlborough, United Kingdom.
[3] Damolin, Kønsborgvej, Denmark.
[4] Calculated retainable P content does not include contribution of microbial phytase.

Animal husbandry and management

The birds were housed in 2 m^2 pens with wooden shavings (0.9-1.0 kg/m^2) as bedding material. During the first 3 days continuous light (24L:0D) was provided, thereafter, a dark/light schedule of 18 h light and 6 h dark (18L:6D, 20 lux light intensity) was used throughout the experimental period except for the week of dissection (d 17-22) during which continuous lighting was used (24L:0D) to ensure a constant feed intake and steady-state passage of digesta in the GIT. The birds were weighed on d 10, at dissection (d 21-23), d 29 and d 36. On d 21-23 eight birds per pen were randomly selected and sacrificed for collection of digesta, mucosa scrapings and tibia (see below). The remaining 17 birds per pen were kept until d 36 to determine the growth performance in the overall period (d 0-36) until reaching a commonly used commercial slaughter weight. Broilers were dissected per block with 3 blocks per day on d 21-23. From d 18-23, a cardboard was placed in the pens to prevent birds from consuming excreta or bedding material since this might influence the observed Ca and P digestibility. During the entire period, birds had free access to feed and water. Ventilation and temperature in the barn were computer controlled and were appropriate for the age of birds.

Sample collection and chemical analysis

On d 21-23, eight birds per pen were weighed and electrocuted. The abdominal cavity was opened, the GIT carefully taken out and laid out on the dissection table, before the proximal and distal half of the jejunum as well as the distal half of the ileum were enclosed with plastic forceps and quantitatively emptied by flushing with deionized water. The collected digesta were stored at -20 °C until determination of Ca, P and Ti. After electrocution blood was collected from the carotid artery of three of the eight dissected birds per pen, and centrifuged at 3,000×g for 10 min at 4 °C to harvest serum. The serum was stored at -80 °C pending analysis of Ca and P. After cleansing with tap water, mucosa was scraped from the mid duodenum (approximately 5 cm) of one bird, randomly selected out of the eight dissected birds per pen, snap-frozen in liquid nitrogen and stored at -80 °C prior to analysis of gene expression. The right tibia from three birds, randomly selected out of the eight dissected birds per pen was separated and stored at -20 °C prior to breaking test.

Fresh diets and lyophilised digesta were ground to pass a 1-mm sieve (Retsch GmbH, Germany) prior to subsequent analyses. Diets were analysed for dry matter [15], crude ash [16], crude fibre [17], crude protein (N [18]x6.25), starch [19], and crude fat [20] before commencement of the trial. The Ca, P and Ti content in the diets and lyophilised digesta were determined using ICP-OES [21] (ThermoFisher, MA) after destruction of the samples with a mixture of 37% HCl (6 ml), 65% HNO_3 (2 ml) and 48% HF (2 ml) in a microwave (CEM, NC) [22]. Serum Ca and P concentrations were determined with a C701 Photometric measuring unit (Roche Diagnostics Limited, Rotkreuz, Switzerland). The characteristics of tibia strength were determined by a breaking test using an Instron Texture Analyzer (type 3366, MA). Tibia diameter, maximum compressive load, bone deflection at maximum compressive load and stiffness (slope) were determined as characteristics of tibia strength, as described by Guz *et al*. [23].

Expression of genes in the duodenal mucosa was determined using real-time quantitative PCR (RT-qPCR) following the standard protocol in our lab. Briefly, scrapings of duodenal mucosa were ground in liquid nitrogen and subsampled (50-100 mg). In the subsample, total RNA was isolated using TRIzol (ThermoFisher Scientific) and then 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. The RNA integrity number values ranged from 9.5 to 10. A total of 500 ng RNA was reverse transcribed with Superscript III kit (ThermoFisher Scientific) and mRNA levels were assessed by 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 s and 60 °C for 30 s for 40 cycles. Absolute quantitative mRNA measurement was performed by establishing a linear calibration curve using 10-fold serial dilutions of cDNA template for corresponding genes. Expression levels of gene of interest were normalized to the geometric mean expression level of importin 8 (IPO8), eukaryotic elongation factor 2 (EEF2), beta actin (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 60S ribosomal protein P0 (RPLP0). The used primer sequences were designed with Primer Express Software (Life Technologies, Bleiswijk, the Netherlands), and where possible recommended primer sets that span an intron were selected and presented in **Table 3**.

Table 3. Gene-specific primers used for the analysis of mRNA levels using real-time quantitative PCR (RT-qPCR)

Gene	Accession no.	Sense 5'-3'	Antisense 5'-3'
Ca transporters			
CaSR	XM_416491.6	GCCAATCTGCTGGGACTCTT	CTGATGCTCGTCATTGGGGA
CaBP-D28k	NM_205513.1	CGAGATCTGGCACCACTACG	ACCTGAGCAAGCTCAACGAT
PMCA1	NM_001168002.3	ACTCTGATGGCAGTTTCCGA	GTCACGGTCCCTAGGTCTGA
TRPC1	NM_001004409.2	GAATACGATGGGACCAGCCC	AGCTGATTGCGTGACTCTTCT
TRPV6	XM_004938143.3	GACCAGAGCAAAGAGGGACC	CCGCCTCTGCATGAGGTATT
P transporters			
NaPi-IIa	XM_015293846.2	GAAGCCAGGTGCCTCTGATG	AGAGGATGGCGTTGTCCTTG
NaPi-IIb	NM_204474.2	TGGCTTTGTCCCTGCTTGTT	CCAGCCAGCCAAGTAAAAGG
PiT-1	XM_015297502.2	TGAAGCTTCCCATCTCGGGT	AGGACAACACGATTTTTAGCAGC
PiT-2	NM_001305398.1	GCTGGGAGCAAAAGTAGGAGA	AAACAGCAGAACCAACCATCG
XPR1	XM_422258.6	AACCTGGAGACAACACGAGG	CGTTGGTCACCACTTCCTCT
Tight junction proteins and VDR			
CLDN-2	NM_001277622.1	CAACTGGAAGATCAGCTCCT	TGTAGATGTCGCACTGAGTG
CLDN-12	XM_025148431.1	CTCTTATTCCTCCTCGCATG	GTCAAAGCTAAAGACAGGCT
CLDN-16	XM_426702.4	GGGATCCAAACATGTGATGA	AGAGAAATCCAAATCCTGCC
ZO-1	XM_015278981.2	CCGCAGTCGTTCACGATCT	GGAGAATGTCTGGAATGGTCTGA
VDR	NM_205098.1	GGCTCAGGTTTTGCAGATTTG	CAGCATCGCCTTTCCCATT
Reference genes			
ACTB	NM_205518.1	GCCCTGGCACCTAGCACAAT	GCGGTGGACAATGGAGGGT
EEF2	NM_205368.1	CAGTTGGCTTTGGTTCTGGC	AAAGTATCTGTCTCCCCACAGC
GAPDH	NM_204305.1	ATCCCTGAGCTGAATGGGAAG	AGCAGCCTTCACTACCCTCT
IPO8	XM_015287054.2	ACCTCCGAGCTAGATCCTGT	GGCTCTTCTTCGCCAACTCT
RPLP0	NM_204987.2	TTGGGCATCACCACAAAGATT	CCCACTTTGTCTCCGGTCTTAA

ACTB, beta actin; CaBP-D28k, calbindin D28k; CaSR, Ca sensing receptor; CLDN, claudin; EEF2, eukaryotic elongation factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPO8, importin 8; NaPi-IIa, sodium dependent phosphate transporter IIa; NaPi-IIb, sodium dependent phosphate transporter IIb; PMCA1, plasma membrane Ca-ATPase 1; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; RPLP0, 60S acidic ribosomal protein P0; TRPC1, transient receptor potential canonical 1; TRPV6, transient receptor potential cation channel subfamily V member 6; VDR, vitamin-D_3 receptor; XPR1, xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1.

Calculations and statistical analysis

The apparent Ca and P digestibility coefficient was calculated according to de Vries and Gerrits [24]:

$$\text{Apparent digestibility, \%} = (1-(X_{digesta}/X_{diet})\times(Ti_{diet}/Ti_{digesta}))\times 100$$

where $X_{digesta}$ and X_{diet} are the Ca or P content in the freeze-dried digesta and diet (g/kg), respectively, and Ti_{diet} and $Ti_{digesta}$ the Ti content in diet and freeze-dried digesta (g/kg), respectively.

Pen was the experimental unit for data analysis. All data were subjected to a two-way ANOVA using the MIXED procedure of SAS (version 9.4, SAS Institute, Cary, NC) with dietary Ca/rP, phytase and their interaction as fixed effects and block and pen as random effects. The LSMEANS procedure with a PDIFF option was used to separate the means. Distribution and variance homogeneity of the Studentized residual were visually checked by the graphics plotted using the ODS GRAPHICS procedure. A CONTRAST procedure was used to estimate the linear and quadratic effect of dietary Ca/rP irrespective of phytase inclusion. Probability was considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

Results

Growth performance

The mean initial body weight (BW) of the newly hatched broilers (d 0) across dietary treatments was approximately 47 g (**Table 4**). Intake of microbial phytase (1000 FTU/kg) increased body weight gain (BWG) and feed intake (FI) and lowered feed conversion ratio (FCR) in the first 3 weeks of life (P=0.002, 0.029 and 0.001, respectively). Furthermore, dietary Ca content quadratically affected BWG, FI and FCR (P<0.001, 0.004 and <0.001, respectively), with the maximum values of BWG and FI being observed in broilers fed a dietary Ca/rP of 1.8. This impact of dietary Ca/rP and phytase on growth performance persisted until the end of the trial (d 36). No significant Ca/rP × phytase interactions for BWG, FI and FCR were observed during the entire experiment (d 0-36).

Table 4. Least square means of initial body weight on d 0 (BW_d0), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of broilers from d 0 to dissection (d 21-23) and d 36 as affected by dietary Ca to retainable P ratio (Ca/rP) and microbial phytase supplementation [1,2]

Ca/rP	Phytase	BW_d0	D 0 to dissection (d 21-23)			D 0-36		
	FTU/kg	g	BWG, g	FI, g	FCR, g/g	BWG, g	FI, g	FCR, g/g
1.3	0	47.2	1132	1488	1.32	2483	3790	1.53
1.8	0	46.9	1190	1518	1.28	2661	3897	1.46
2.3	0	46.8	1178	1497	1.27	2656	3868	1.46
2.8	0	46.9	1125	1451	1.29	2609	3803	1.46
1.3	1000	47.1	1138	1476	1.30	2511	3781	1.51
1.8	1000	46.7	1233	1552	1.26	2734	3968	1.45
2.3	1000	47.0	1204	1526	1.27	2667	3888	1.46
2.8	1000	46.9	1204	1518	1.26	2699	3901	1.45
Pooled SEM		0.35	23.8	26.5	0.010	39.9	52.2	0.011
Ca/rP								
1.3		47.2	1135^{c}	1482^{b}	1.31^{a}	2497^{b}	3785^{c}	1.52^{a}
1.8		46.8	1211^{a}	1535^{a}	1.27^{b}	2697^{a}	3932^{a}	1.46^{b}
2.3		46.9	1191^{ab}	1511^{ab}	1.27^{b}	2661^{a}	3878^{ab}	1.46^{b}
2.8		46.9	1165^{bc}	1484^{b}	1.28^{b}	2654^{a}	3852^{bc}	1.46^{b}
Pooled SEM		0.24	16.9	18.7	0.007	27.8	37.7	0.008
Phytase								
0		47.0	1156	1488	1.29	2602	3839	1.48
1000		46.9	1195	1518	1.27	2653	3884	1.47
Pooled SEM		0.17	11.9	13.2	0.005	19.6	37.5	0.006
P-value								
Ca/rP		0.491	<.001	0.019	<0.001	<0.001	0.003	<0.001
Linear (Ca/rP)		-	0.197	0.800	<0.001	<0.001	0.340	<0.001
Quadratic (Ca/rP)		-	<0.001	0.004	<0.001	<0.001	0.001	<0.001
Phytase		0.845	0.002	0.029	0.001	0.010	0.073	0.057
Ca/rP × Phytase		0.878	0.186	0.235	0.286	0.367	0.393	0.585

[1] Calculated rP content was 3.2, 2.5 and 2.2 g/kg in the starter, grower and finisher diets, respectively, excluding the contribution of microbial phytase.
[2] Data are presented as treatment means, 9 replicate pens per treatment (n=9).
[a-c] Values within a column without common superscript differ significantly ($P \leq 0.05$).

Apparent digestibility in different GIT segments and serum concentration of Ca and P

Overall, apparent Ca digestibility gradually increased from the proximal jejunum to distal ileum (**Table 5**). Incremental dietary Ca/rP linearly reduced ($P<0.001$) apparent Ca digestibility in all three intestinal segments. Phytase inclusion did not affect apparent Ca digestibility in the jejunum, whereas in the distal ileum, we

observed a Ca/rP × phytase interaction ($P_{interaction}$=0.023). In particular, addition of phytase enhanced apparent Ca digestibility only in the 1.3 and 1.8 Ca/rP diets compared to the non-phytase-treated broilers. Despite its inhibitory action on apparent Ca digestibility, incremental dietary Ca/rP, but not phytase inclusion (P=0.296), linearly and quadratically elevated (P≤0.001) serum Ca concentration.

Table 5. Least square means of calcium (Ca) and phosphorus (P) content in the serum and apparent Ca and P digestibility in different gastrointestinal tract segments on d 21-23 in broilers, in response to dietary Ca to retainable P ratio (Ca/rP) and microbial phytase supplementation [1,2]

Ca/rP	Phytase	Serum, mM		Apparent Ca digestibility, %			Apparent P digestibility, %		
	FTU/kg	Ca	P	Proximal jejunum	Distal jejunum	Distal ileum	Proximal jejunum	Distal jejunum	Distal ileum
1.3	0	2.43	2.31^{a}	46.1	56.1	67.3^{bc}	61.2	73.1^{b}	78.8^{b}
1.8	0	2.64	2.14^{c}	43.6	54.2	63.2^{c}	49.2	62.0^{c}	65.3^{e}
2.3	0	2.68	1.97^{d}	37.1	47.1	54.2^{d}	43.9	54.6^{d}	56.2^{f}
2.8	0	2.74	1.72^{e}	27.4	40.4	48.9^{e}	32.3	46.2^{e}	49.3^{g}
1.3	1000	2.43	2.34^{a}	55.8	63.4	75.6^{a}	76.0	84.3^{a}	89.4^{a}
1.8	1000	2.62	2.25^{ab}	51.5	59.0	69.6^{b}	72.6	82.3^{a}	83.7^{a}
2.3	1000	2.64	2.17^{bc}	33.8	45.7	54.8^{d}	59.8	72.9^{b}	75.2^{c}
2.8	1000	2.71	2.17^{bc}	27.4	38.2	46.9^{e}	57.3	68.5^{b}	70.3^{d}
Pooled SEM		0.042	0.061	4.66	3.30	2.62	4.14	2.58	1.75
Ca/rP									
1.3		2.43^{c}	2.32	51.0^{a}	59.7^{a}	71.4	68.7^{a}	78.7	84.2
1.8		2.63^{b}	2.20	47.6^{b}	56.6^{a}	66.4	60.9^{b}	72.2	74.5
2.3		2.66^{b}	2.07	35.5^{c}	46.4^{b}	54.5	51.9^{c}	63.8	65.8
2.8		2.73^{a}	1.94	27.4^{d}	39.3^{c}	47.9	44.9^{d}	57.4	59.9
Pooled SEM		0.030	0.043	3.29	2.33	1.85	2.93	1.82	1.24
Phytase									
0		2.62	2.03	38.6	49.4	58.4	46.7	59.0	62.5
1000		2.60	2.23	42.1	51.6	61.7	66.5	77.1	79.7
Pooled SEM		0.021	0.030	2.33	1.65	1.31	2.07	1.30	0.87
P-value									
Ca/rP		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Linear (Ca/rP)		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Quadratic (Ca/rP)		0.001	0.959	0.317	0.237	0.540	0.866	0.966	0.038
Phytase		0.296	<0.001	0.133	0.195	0.014	<0.001	<0.001	<0.001
Ca/rP × Phytase		0.952	<0.001	0.159	0.124	0.023	0.207	0.021	0.001

[1] Calculated rP content was 3.2, 2.5 and 2.2 g/kg in the starter, grower and finisher diets, respectively, excluding the contribution of microbial phytase.
[2] Data are presented as treatment means, 9 replicate pens per treatment (n=9).
[a-g] Values within a column without common superscript differ significantly (P≤0.05).

A substantial increase in apparent P digestibility from the proximal to distal jejunum, with only a slight subsequent increase in the distal ileum (Table 5) was observed. Incremental dietary Ca/rP linearly reduced (P<0.001) apparent P digestibility in all three intestinal segments. In the distal jejunum and distal ileum, a Ca/rP × phytase interaction on apparent P digestibility ($P_{interaction}$=0.021 and 0.001, respectively) was observed. Increasing dietary Ca/rP from 1.3 to 2.8 reduced distal ileal P digestibility, which was more pronounced in broilers fed phytase free compared to phytase supplemented diets (29.5 vs. 19.1% units reduction). Incremental dietary Ca/rP linearly decreased serum P concentration, but this reduction was much smaller with phytase-supplemented diets than with phytase-free diets ($P_{interaction}$<0.001).

Duodenal mRNA expression of transporters and CLDN

Analysis of the broiler's duodenal mucosa on d 21-23 by RT-qPCR revealed that incremental dietary Ca/rP linearly reduced the expression levels of extracellular Ca sensing receptor (CaSR), calbindin-D28k (CaBP-D28k) and plasma membrane Ca-ATPase 1 (PMCA1) by 70, 51 and 40%, respectively (P<0.001, **Table 6**). Phytase had no impact on these Ca homeostasis-related genes. Transcript levels of apical Ca channel (TRPV6) were beyond the limit of detection and that of transient receptor potential canonical 1 (TRPC1), a nonspecific cation channel, were affected neither by dietary Ca/rP nor phytase treatment.

Duodenal expression of the apical sodium dependent phosphate transporters, NaPi-IIb, PiT-2 and the candidate basolateral P extrusion channel, xenotropic and polytropic retrovirus receptor 1 (XPR1), were either significantly reduced or showed a trend with incremental dietary Ca/rP in a linear fashion (14, 8 and 23% reduction; P=0.052, 0.071 and 0.028, respectively). Moreover, phytase increased NaPi-IIb mRNA by 20% (P=0.026), but did not alter the levels of PiT-2 and XPR1 mRNA (P=0.398 and 0.912, respectively). Expression of PiT-1 was affected by an interaction between Ca/rP and phytase ($P_{interaction}$=0.035), with the expression being 57% higher with phytase in the lowest Ca/rP diet compared to the non-phytase group.

On the one hand, incremental dietary Ca/rP linearly decreased duodenal zonula occludens-1 (ZO-1), CLND-2 and -12 mRNA irrespective of phytase inclusion (13, 40 and 13% reduction, P=0.005, <0.001 and 0.010, respectively) while phytase inclusion, on the other hand, reduced CLDN-2 expression by 15% (P=0.029). Neither dietary Ca/rP nor phytase affected the expression of vitamin D_3 receptor (VDR) mRNA. Expression of CLDN-16 in the broiler's duodenal mucosa was beyond the limit of detection.

Table 6. Least square means of mRNA expression level of calcium (Ca) and phosphorus (P) transporters, vitamin D_3 receptor (VDR) and claudins (CLDN) on day 21-23 in the broiler's duodenal mucosa in response to dietary Ca to retainable P ratio (Ca/rP) and microbial phytase intake [1,2,3]

Ca/rP	Phytase	Ca transporters				P transporters				VDR and CLDN			
	FTU/kg	CaSR	TRPC1	CaBP-D28k	PMCA1	NaPi-IIb	PiT-1	PiT-2	XPR1	VDR	ZO-1	CLDN-2	CLDN-12
1.3	0	0.36	0.39	455	101	3.30	2.18^{c}	392	5.32	2.75	1.44	23.0	0.23
1.8	0	0.25	0.38	349	80.5	3.53	3.60^{a}	382	5.32	2.76	1.53	18.3	0.23
2.3	0	0.16	0.39	291	71.0	2.76	2.45^{bc}	366	5.03	3.05	1.34	18.4	0.22
2.8	0	0.12	0.37	231	62.3	2.71	2.87^{abc}	353	4.76	2.83	1.25	15.8	0.20
1.3	1000	0.39	0.41	455	98.2	3.83	3.43^{ab}	406	5.41	2.93	1.50	23.3	0.23
1.8	1000	0.17	0.49	346	90.7	4.30	2.68^{abc}	466	5.44	2.67	1.58	16.2	0.25
2.3	1000	0.14	0.41	200	59.6	3.28	3.29^{ab}	317	4.57	2.72	1.29	14.4	0.19
2.8	1000	0.10	0.38	213	56.8	3.40	3.39^{ab}	379	4.90	2.89	1.30	11.9	0.20
Pooled SEM		0.046	0.045	53.9	10.75	0.549	0.540	44.2	0.433	0.446	0.124	2.17	0.020
Ca/rP													
1.3		0.37^{a}	0.40	455^{a}	99.4^{a}	3.57	2.81	399	5.37	2.84	1.47^{ab}	23.2^{a}	0.23^{ab}
1.8		0.21^{b}	0.44	347^{b}	85.6^{a}	3.91	3.14	424	5.38	2.71	1.55^{a}	17.2^{b}	0.24^{a}
2.3		0.15^{bc}	0.40	246^{c}	65.3^{b}	3.02	2.87	342	4.80	2.88	1.32^{bc}	16.4^{b}	0.21^{bc}
2.8		0.11^{c}	0.37	222^{c}	59.5^{b}	3.05	3.13	366	4.83	2.86	1.28^{c}	13.8^{c}	0.20^{c}
Pooled SEM		0.033	0.032	38.1	7.60	0.388	0.382	31.3	0.307	0.315	0.088	1.54	0.014
Phytase													
0		0.22	0.38	332	78.6	3.07	2.77	373	5.11	2.85	1.39	18.9	0.22
1000		0.20	0.42	303	76.3	3.70	3.20	392	5.08	2.80	1.42	16.4	0.22
Pooled SEM		0.023	0.022	26.9	5.38	0.274	0.270	22.1	0.217	0.223	0.062	1.09	0.010

Table 6 (continued). Least square means of mRNA expression level of calcium (Ca) and phosphorus (P) transporters, vitamin D_3 receptor (VDR) and claudins (CLDN) on day 21-23 in the broiler's duodenal mucosa in response to dietary Ca to retainable P ratio (Ca/rP) and microbial phytase intake [1,2,3]

Ca/rP	Phytase	Ca transporters				P transporters				VDR and CLDN			
	FTU/kg	CaSR	TRPC1	CaBP-D28k	PMCA1	NaPi-IIb	PiT-1	PiT-2	XPR1	VDR	ZO-1	CLDN-2	CLDN-12
P-value													
Ca/rP		<0.001	0.104	<0.001	<0.001	0.071	0.746	0.053	0.097	0.952	0.008	<0.001	0.026
Linear (Ca/rP)		<0.001	0.275	<0.001	<0.001	0.052	0.562	0.071	0.028	0.826	0.005	<0.001	0.010
Quadratic (Ca/rP)		0.010	0.163	0.113	0.457	0.573	0.903	0.982	0.961	0.829	0.338	0.131	0.523
Phytase		0.412	0.271	0.287	0.671	0.026	0.119	0.398	0.912	0.841	0.662	0.029	0.863
Ca/rP × Phytase		0.454	0.385	0.571	0.542	0.986	0.035	0.218	0.727	0.873	0.927	0.460	0.519

[1] Calculated rP content was 3.2, 2.5 and 2.2 g/kg in the starter, grower and finisher diets, respectively, excluding the contribution of microbial phytase.
[2] Data are presented as treatment means, 9 replicate pens per treatment (n=9).
[3] Determined using absolute quantification normalized by geometric mean expression level of ACTB, EEF2, GAPDH, IPO8 and RPLP0.
[a-c] Values within a column without common superscript differ significantly ($P \leq 0.05$).
ACTB, beta actin; CaBP-D28k, calbindin D28k; CaSR, Ca sensing receptor; CLDN, claudin; EEF2, eukaryotic elongation factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPO8, importin 8; NaPi-IIb, sodium dependent phosphate transporter type IIb; PMCA1, plasma membrane Ca-ATPase 1; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; RPLP0, 60S ribosomal protein P0; TRPC1, transient receptor potential canonical 1; VDR, vitamin-D_3 receptor; XPR1, xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1. Of note, mRNA expression of transient receptor potential cation channel subfamily V member 6 (TRPV6), sodium dependent phosphate transporter type IIa (NaPi-IIa) and CLDN-16 were beyond limit of detection.

Characteristics of tibia strength

Incremental dietary Ca/rP linearly and quadratically enhanced the stiffness of tibia in broilers, with a steeper slope in phytase-included than phytase-free diets (**Table 7**), as evidenced by the interaction effect ($P_{interaction}$=0.001). Furthermore, the maximum compressive load was linearly and quadratically increased (P<0.001) with incremental Ca/rP or phytase inclusion, and tibia diameter tended to be quadratically increased with incremental Ca/rP reaching the maximal tibia diameter at Ca/rP of 1.8 (P=0.075).

Table 7. Effects of dietary Ca to retainable P ratio (Ca/rP) and microbial phytase intake on the least square means of diameter and characteristics of breaking strength of tibia collected from broilers on day 21-23 [1,2]

Ca/rP	Phytase FTU/kg	Diameter mm	Stiffness N/mm	Maximum compressive load N	Deflection at maximum compressive load, mm
1.3	0	6.0	57.0[d]	116	4.4
1.8	0	6.1	68.9[c]	139	4.3
2.3	0	6.2	77.1[b]	160	4.4
2.8	0	6.0	73.5[bc]	158	4.4
1.3	1000	5.9	57.1[d]	122	4.5
1.8	1000	6.2	81.0[b]	165	4.3
2.3	1000	6.1	88.7[a]	178	4.4
2.8	1000	6.0	95.0[a]	181	4.0
Pooled SEM		0.13	3.81	7.4	0.16
Ca/rP					
1.3		5.9	57.1	119[c]	4.4
1.8		6.2	75.0	152[b]	4.3
2.3		6.1	82.9	169[a]	4.4
2.8		6.0	84.2	169[a]	4.2
Pooled SEM		0.09	2.71	5.2	0.11
Phytase					
0		6.1	69.1	143	4.4
1000		6.1	80.5	161	4.3
Pooled SEM		0.07	1.91	3.7	0.08
P-value					
Ca/rP		0.075	<0.001	<0.001	0.156
Linear (Ca/rP)		0.392	<0.001	<0.001	0.066
Quadratic (Ca/rP)		0.016	<0.001	<0.001	0.599
Phytase		0.706	<0.001	<0.001	0.627
Ca/rP × Phytase		0.591	0.001	0.242	0.133

[1] Calculated rP content was 3.2, 2.5 and 2.2 g/kg in the starter, grower and finisher diets, respectively, excluding the contribution of microbial phytase.
[2] Data are presented as treatment means, 9 replicate pens per treatment (n=9).
[a-d] Values within a column without common superscript differ significantly (P≤0.05).

Discussion

Previous study indicated that the reduction of dietary Ca content improves apparent P digestibility, but a too low dietary Ca content compromises growth performance and tibia breaking strength in broilers [4]. In the present study, we hypothesized that microbial phytase supplementation improves dietary Ca digestibility, allowing a lower dietary Ca inclusion without compromising growth performance. This hypothesis, however, is not supported by our experimental results. We found that broilers fed with microbial phytase indeed displayed improved apparent distal ileal Ca digestibility, particularly in the lowest Ca/rP diet (Table 5). However, the additional absorbed Ca (0.32 g/kg diet) in the presence of phytase at the lowest Ca/rP diet was insufficient to alleviate the negative impact of a low Ca intake on growth performance, as indicated by a lower BWG and FI and higher FCR when birds received the lowest Ca/rP diet regardless of microbial phytase inclusion (Table 4). Furthermore, although mRNA expression of Ca absorption-related genes, including CaBP-D28k, PMCA1, CLDN-2 and -12 was highest in the duodenal mucosa for birds fed the lowest Ca/rP diet, this was independent of microbial phytase inclusion (Table 6). Therefore, our results did not support a reduction in dietary Ca inclusion in the presence of microbial phytase without compromising growth performance in broilers, which led us to reject the hypothesis.

Dietary Ca and microbial phytase interaction

Reducing dietary Ca content has been intensively studied since Ca can bind to IP which hampers the digestion of IP to lower IP esters, i.e. IP6 to IP1 [25]. It is demonstrated that microbial phytase inclusion enhances intestinal IP degradation [26], thus it may also enhance dietary Ca digestibility. This assumption is supported by Li *et al*. [27] who reported that microbial phytase inclusion (1000 FTU/kg) significantly improved standardized ileal Ca digestibility (38 vs. 49%) in broilers, which largely supports our results (Table 5). Interestingly, dietary phytase inclusion increased apparent distal ileal Ca digestibility to a greater extent in the lowest Ca/rP diet, while phytase improved BWG and FI to a numerically greater extent in the higher Ca/rP diets (Table 4). This finding suggests that release of IP-Ca might not have been a major element for dietary phytase to augment the

broiler's growth performance. Amerah *et al*. [28] reported that microbial phytase inclusion improved BWG, FI and FCR in broilers fed a Ca-sufficient, but not a Ca-deficient diet, which accompanied with an increased ileal digestibility of P, gross energy and amino acids. They concluded that the positive impact of phytase on growth performance was attributed to a higher digestibility of other nutrients, such as amino acids, energy and P rather than Ca. Thus, despite of a positive impact on Ca digestibility, dietary phytase inclusion would not restore the compromised growth performance of broilers when a Ca-deficient diet was offered.

Numerous studies have been published (e.g. Bello *et al*. [29], Krieg *et al*. [30] and Ajuwon *et al*. [31]) demonstrating that microbial phytase inclusion improves IP degradation and P absorption in broilers. We also observed an improved apparent P digestibility in diets supplemented with microbial phytase. Noteworthy is the rather high distal ileal apparent P digestibility (i.e. 78.8%) without microbial phytase inclusion in the lowest Ca/rP diet (Table 5), indicating that broilers had a large potential to degrade IP and absorb IP-P upon low-Ca intake. Since the intrinsic phytase activity in the basal diet was low (Table 2), the high ileal P digestibility observed in the absence of exogenous phytase inclusion is probably attributed to the activity of endogenous epithelial and/or microbial phosphatases in the GIT of broilers. This idea is supported by Rodehutscord and Rosenfelder [25], who in their literature survey found that approximately 70% of dietary IP6 is degraded in the distal ileum of broilers fed a low-Ca and low-P content diets devoid of phytase. Furthermore, we also observed a steep linear inhibitory effect on apparent ileal P digestibility with incremental dietary Ca/rP in the absence of phytase, indicating that the catalytic activity of endogenous epithelial and/or microbial phosphatases is significantly inhibited by dietary Ca. This is in line with the findings of Sommerfeld *et al*. [32], who showed a strong inhibition of ileal IP degradation with dietary inclusion of mineral Ca and P in broilers fed phytase-free diets. To further clarify the source of endogenous phosphatase, these researchers conducted a similar study by feeding gamma-irradiated diets to gnotobiotic broilers [3]. Since their data revealed a similar reduction of ileal IP degradation upon mineral Ca and P supplementation, it can be concluded that broilers have a great potential to absorb P from IP due to a high endogenous epithelial phosphatase activity, which can be inhibited by high levels of dietary Ca.

Dietary phytase inclusion alleviated the negative impact of dietary Ca/rP increment on apparent P digestibility in the distal jejunum and ileum (Table 5), which is largely in agreement with Sommerfeld *et al*. [32], who reported that the negative impact of dietary supplementation of Ca and P on ileal IP degradation in broilers could be fully recovered by the inclusion of microbial phytase. As mentioned above, in the absence of microbial phytase, a high dietary Ca intake inhibited endogenous epithelial phosphatases activity and reduced distal ileal P digestibility. In contrast, in the presence of microbial phytase, the negative impact of a high dietary Ca intake on P digestibility was much smaller. It appears that the efficacy of the exogenous microbial phytase is less affected by dietary Ca inclusion compared to the endogenous epithelial phosphatases. An explanation for this difference is likely pH related, that exogenous microbial phytase is primarily active in the proventriculus and gizzard [8], while endogenous epithelial phosphatases are mostly active in the small intestine. The luminal pH in the small intestine is favorable to Ca-IP complexation, impeding the efficacy of endogenous epithelial phosphatases, while the low pH in the proventriculus and gizzard (approximately at pH 3), is unfavorable to Ca-IP complexation, making the efficacy of exogenous microbial phytase in the latter segments less sensitive to dietary Ca intake.

Expression of Ca and P related transporters and tight junctions

We observed a downregulation of CaBP-D28k and PMCA1, CLDN-2 and -12 mRNA with incremental Ca/rP (Table 6) in the duodenal mucosa. These results are in line with the reduced apparent Ca digestibility at a higher Ca intake in the posterior small intestinal segments (Table 5), suggesting that both intestinal Ca transporters and CLDN are regulatable and contribute to whole body Ca homeostasis in broilers. The higher serum Ca level obtained with high Ca intake (Table 5) would activate the extracellular CaSR on the parathyroid gland, supressing parathyroid hormone (PTH) expression and secretion [33]. A reduced PTH circulation would reduce serum circulation of 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), thereby, downregulating expression of Ca transporters and CLDN in the GIT since the promoter of CaBP-D28k, PMCA1, CLDN-2 and -12 contains a VDR responsive element [10; 34]. Another possible mechanism for the downregulation of Ca transporters might be that a high dietary Ca/rP increased luminal Ca content, which might activate extracellular CaSR, a transmembrane G protein-coupled receptor expressed in the GIT, thereby,

directly reducing CaBP-D28k and PMCA1 expression, independent of circular $1,25(OH)_2D_3$ [35; 36].

Incremental Ca intake also led to the downregulation of duodenal P transporters NaPi-IIb, PiT-2 and XPR1 (Table 6), which is consistent with the lower apparent P digestibility in the jejunal and ileal part under these conditions (Table 5). Furthermore, the downregulation of P transporters was accompanied by a lower concentration of serum P, and dietary phytase inclusion increased serum P while upregulating duodenal expression of NaPi-IIb. Our observations are in line with the work of Huber *et al*. [37], who demonstrated a positive correlation between plasma P and jejunal NaPi-IIb mRNA expression in laying hens. Expression of NaPi-IIb in the intestine of broilers might be under control of systemic hormones and regulators such as $1,25(OH)_2D_3$ and fibroblast growth factor 23 (FGF23). It is conceivable that high Ca intake decreased serum $1,25(OH)_2D_3$ [4], via the parathyroid-kidney axis, which in turn reduced intestinal NaPi-IIb mRNA expression. Furthermore, dietary phytase inclusion enhanced serum P concentration (Table 5), which might have stimulated FGF23 secretion from the bone [38]. A higher serum FGF23 circulation has been shown to be coupled with a greater NaPi-IIb protein expression in the duodenal mucosa of layers [39]. Taken together, expression of P transporters is consistent with intestinal P absorption in broilers.

Site of Ca and P absorption

It is generally accepted that Ca and P are primarily absorbed in the small intestine of chickens [40]. In this study, we observed that an average of 95 and 85% of the precaecal digestible P and Ca were absorbed proximal to the ileum, respectively indicating a limited contribution of the ileum to Ca and P absorption. Our previous study in broilers [4] demonstrated that approximately 85% of the distal ileal digestible P and Ca were absorbed anterior to the ileum, which is in line with results of the present study. Rodehutscord *et al*. [41] compared apparent P digestibility in the proximal, medial and distal ileum in broilers receiving diets with various P content. They found that P absorption was trivial in the ileum particularly in broilers fed diets with a P content above 5 g/kg. Their results are in line with the present study (P content 4.8 g/kg), and shows that the ileum plays a minor role in P

absorption in broilers. Ileal Ca absorption has been less investigated but may not be substantial according to the present and our previous study [4].

In conclusion, dietary phytase inclusion improves distal ileal apparent Ca digestibility but cannot recover the compromised growth performance in broilers fed a Ca-deficient diet. Incremental dietary Ca/rP reduces apparent Ca and P digestibility paralleled with a reduced duodenal expression of Ca and P transporters and CLDN, indicating that these transporters and CLDN contribute to the observed effect of Ca level and phytase on Ca and P absorption in chicken.

Acknowledgement

This research was conducted by Wageningen Livestock Research, within the framework of the public private partnership "Feed4Foodure" and partially funded by the Ministry of Economic Affairs (Policy Support Research project number BO-31.03-005-00). Mr L. Kruijt assisted with RT-qPCR analysis, and Y.X. Hu received a scholarship form the Chinese Scholarship Council (CSC) to study at Wageningen University & Research.

Author contributions

P. Bikker, J. van Harn, M.M. van Krimpen, M.A. Dijkslag and Y.X. Hu designed and conducted the research; Y.X. Hu analysed the samples and data; J. van Baal designed and validated the RT-qPCR primers. Y.X. Hu, P. Bikker, J. van Baal, W.H. Hendriks and J van Harn interpreted the data and wrote the manuscript. All authors with the exception of the late M.M. van Krimpen have read and approved the final manuscript. M.A. Dijkslag is an employee of ForFarmers, all other authors declare to have no conflict of interest.

References

1. Chang AR & Anderson C (2017) Dietary phosphorus intake and the kidney. *Annu Rev Nutr* **37**, 321-346.

2. Rodehutscord M, Ruckert C, Maurer HP *et al.* (2016) Variation in chemical composition and physical characteristics of cereal grains from different genotypes. *Arch Anim Nutr* **70**, 87-107.

3. Sommerfeld V, Van Kessel AG, Classen HL *et al.* (2019) Phytate degradation in gnotobiotic broiler chickens and effects of dietary supplements of phosphorus, calcium, and phytase. *Poult Sci* **98**, 5562-5570.

4. Hu YX, Bikker P, Duijster M *et al.* (2020) Coarse limestone does not alleviate the negative effect of a low Ca/P ratio diet on characteristics of tibia strength and growth performance in broilers. *Poult Sci* **99**, 4978–4989.

5. Lei XG, Weaver JD, Mullaney E *et al.* (2013) Phytase, a new life for an "old" enzyme. *Annu Rev Anim Biosci* **1**, 283-309.

6. Ravindran V, David LS, Abdollahi MR *et al.* (2020) True ileal calcium digestibility in soybean meal and canola meal, and true ileal phosphorous digestibility in maize-soybean meal and maize-canola meal diets, without and with microbial phytase, for broiler growers and finishers. *Br Poult Sci* **62**, 293-303.

7. Majeed S, Qudsieh R, Edens FW *et al.* (2020) Limestone particle size, calcium and phosphorus levels, and phytase effects on live performance and nutrients digestibility of broilers. *Poult Sci* **99**, 1502–1514.

8. Dersjant-Li Y, Awati A, Schulze H *et al.* (2015) Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. *J Sci Food Agric* **95**, 878-896.

9. Li P, Wang R, Jiao H *et al.* (2018) Effects of dietary phosphorus level on the expression of calcium and phosphorus transporters in laying hens. *Front Physiol* **9**, 627.

10. Proszkowiec-Weglarz M & Angel R (2013) Calcium and phosphorus metabolism in broilers: effect of homeostatic mechanism on calcium and phosphorus digestibility. *J Appl Poult Res* **22**, 609-627.

11. Kiela PR & Ghishan FK (2018) Molecular mechanisms of intestinal transport of calcium, phosphate, and magnesium. In *Physiology of the Gastrointestinal Tract (Sixth Edition)*, pp. 1405-1449 [Said HM, Ghishan FK, Kaunitz JD, Merchant JL and Wood JD, editors]. Cambridge: Academic Press.

12. Tsukita S, Tanaka H & Tamura A (2019) The claudins: from tight junctions to biological systems. *Trends Biochem Sci* **44**, 141-152.

13. Hashimoto N, Matsui I, Ishizuka S *et al.* (2020) Lithocholic acid increases intestinal phosphate and calcium absorption in a vitamin D receptor dependent but transcellular pathway independent manner. *Kidney Int* **97**, 1164–1180.

14. CVB (2018) *Voedernormen Varkens en Voederwaarden Voedermiddelen voor Pluimvee (Feeding Standards, Feeding Advices and Nutritional Values of Feeding Ingredients of Poultry)*. Wageningen, the Netherlands: Federatie Nederlandse Diervoederketen.

15. ISO (1999) 6496: *Animal Feeding Stuffs - Determination of Moisture and Other Volatile Matter Content*. Geneva: International Organization for Standardization.

16. ISO (2002) 5984: *Animal feeding stuffs - Determination of Crude Ash*. Geneva: International Organization for Standardization.

17. ISO (2002) 6865: *Animal feeding stuffs - Determination of Crude Fibre Content — Method with Intermediate Filtratione Ash.* Geneva: International Organization for Standardization.

18. ISO (2005) 5983-1: *Animal Feeding Stuffs - Determination of Nitrogen Content and Calculation of Crude Protein Content - Part 1: Kjeldahl Method*. Geneva: International Organization for Standardization.

19. ISO (2004) 15914: *Animal Feeding Stuffs - Enzymatic Determination of Total Starch Content*. Geneva: International Organization for Standardization.

20. ISO (1999) 6492: *Animal Feeding Stuffs - Determination of Fat Content*. Geneva: International Organization for Standardization.

21. ISO (2009) 27085: *Animal Feeding Stuffs - Determination of Calcium, Sodium, Phosphorus, Magnesium, Potassium, Iron, Zinc, Copper, Manganese, Cobalt, Molybdenum, Arsenic, Lead and Cadmium by ICP-AES*. Geneva: International Organization for Standardization.

22. Wang J, Nakazato T, Sakanishi K *et al.* (2004) Microwave digestion with HNO_3/H_2O_2 mixture at high temperatures for determination of trace elements in coal by ICP-OES and ICP-MS. *Anal Chim Acta* **514**, 115-124.

23. Guz BC, Molenaar R, de Jong IC *et al.* (2019) Effects of dietary organic minerals, fish oil, and hydrolyzed collagen on growth performance and tibia characteristics of broiler chickens. *Poult Sci* **98**, 6552-6563.

24. de Vries S & Gerrits WJJ (2018) The use of tracers or markers in digestion studies. In *Feed Evaluation Science*, pp. 275–294 [Moughan PJ and Hendriks WH, editors]. Wageningen, the Netherlands: Wageningen Academic Publishers.

25. Rodehutscord M & Rosenfelder P (2016) Chapter 1 Update on phytate degradation pattern in the gastrointestinal tract of pigs and broiler chickens. In *Phytate Destruction-Consequences for Precision Animal Nutrition*, pp. 15-28 [Walk CL, Kühn I, Stein HH, Kidd MT and Rodehutscord M, editors]. Wageningen, the Netherlands: Wageningen Academic Publishers.

26. Walk CL & Rama Rao SV (2020) Dietary phytate has a greater anti-nutrient effect on feed conversion ratio compared to body weight gain and greater doses of phytase are required to alleviate this effect as evidenced by prediction equations on growth performance, bone ash and phytate degradation in broilers. *Poult Sci* **99**, 246-255.

27. Li W, Angel R, Plumstead PW *et al.* (2020) Effects of limestone particle size, phytate, calcium source, and phytase on standardized ileal calcium and phosphorus digestibility in broilers. *Poult Sci* **100**, 900–909.

28. Amerah AM, Plumstead PW, Barnard LP *et al.* (2014) Effect of calcium level and phytase addition on ileal phytate degradation and amino acid digestibility of broilers fed corn-based diets. *Poult Sci* **93**, 906-915.

29. Bello A, Dersjant-Li Y & Korver DR (2019) The efficacy of 2 phytases on inositol phosphate degradation in different segments of the gastrointestinal tract, calcium and phosphorus digestibility, and bone quality of broilers. *Poult Sci* **98**, 5789-5800.

30. Krieg J, Siegert W, Berghaus D *et al.* (2020) Phytase supplementation effects on amino acid digestibility depend on the protein source in the diet but are not related to InsP6 degradation in broiler chickens. *Poult Sci* **99**, 3251-3265.

31. Ajuwon KM, Sommerfeld V, Paul V *et al.* (2020) Phytase dosing affects phytate degradation and Muc2 transporter gene expression in broiler starters. *Poult Sci* **99**, 981-991.

32. Sommerfeld V, Schollenberger M, Kühn I *et al.* (2018) Interactive effects of phosphorus, calcium, and phytase supplements on products of phytate degradation in the digestive tract of broiler chickens. *Poult Sci* **97**, 1177-1188.

33. Musgrove J & Wolf M (2020) Regulation and effects of FGF23 in chronic kidney disease. *Annu Rev Physiol* **82**, 365-390.

34. Fujita H, Sugimoto K, Inatomi S *et al.* (2008) Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca^{2+} absorption between enterocytes. *Mol Biol Cell* **19**, 1912-1921.

35. Lee JJ, Liu X, O'Neill D *et al.* (2019) Activation of the calcium-sensing receptor attenuates TRPV6-dependent intestinal calcium absorption. *JCI insight* **4**, e128013.

36. Chanpaisaeng K, Teerapornpuntakit J & Wongdee K *et al.* (2021) Emerging roles of calcium-sensing receptor in the local regulation of intestinal transport of ions and calcium. *Am J Physiol Cell Physiol* **320**, C270-C278.

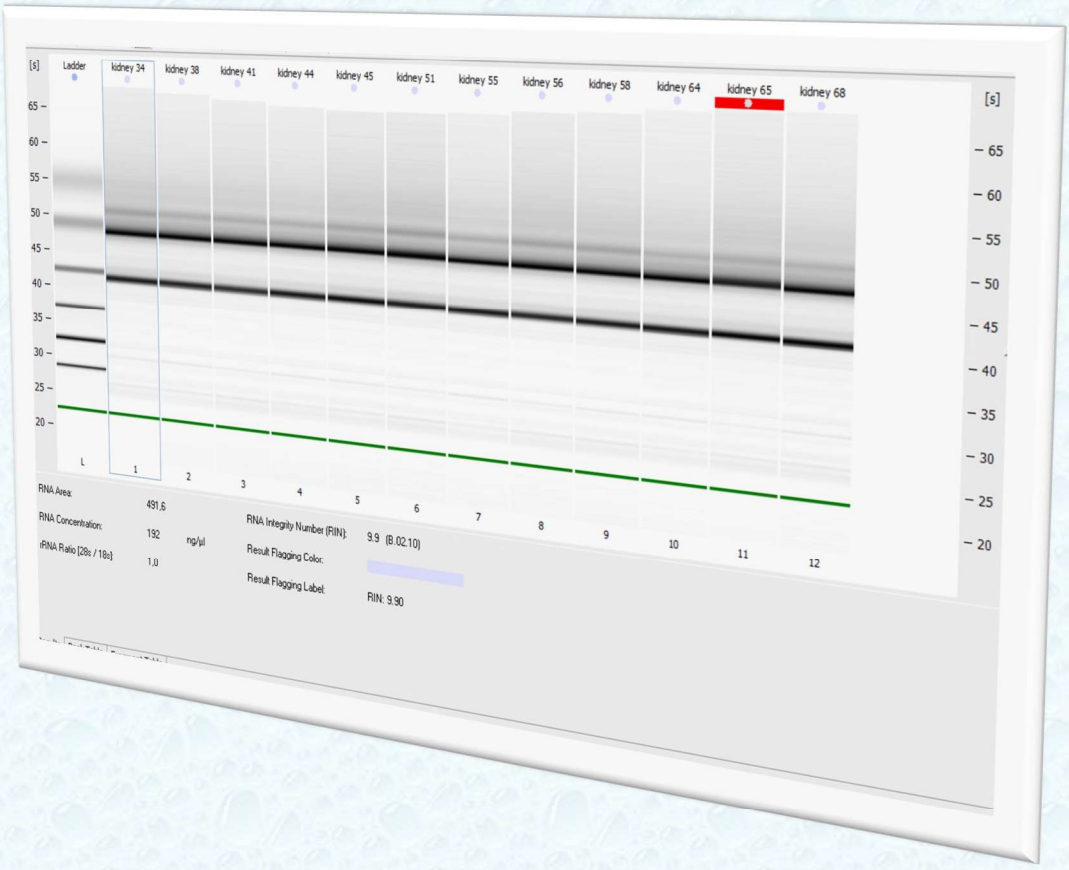
37. Huber K, Hempel R & Rodehutscord M (2006) Adaptation of epithelial sodium-dependent phosphate transport in jejunum and kidney of hens to variations in dietary phosphorus intake. *Poult Sci* **85**, 1980-1986.

38. Ren ZZ, Butz DE, Wahhab AN *et al*. (2017) Additive effects of fibroblast growth factor 23 neutralization and dietary phytase on chick calcium and phosphorus metabolism. *Poult Sci* **96**, 1167-1173.

39. Ren ZZ, Yan JK, Pan C *et al*. (2020) Supplemental nicotinamide dose-dependently regulates body phosphorus excretion via altering type II sodium-phosphate co-transporter expressions in laying hens. *J Nutr* **150**, 2070-2076.

40. Veum T (2010) Phosphorus and calcium nutrition and metabolism. In *Phosphorus and Calcium Utilization and Requirements in Farm Animals*, pp. 94-111 [Vitti DMSS and Kebreab E, editors]. London: CAB International.

41. Rodehutscord M, Dieckmann A, Witzig M *et al.* (2012) A note on sampling digesta from the ileum of broilers in phosphorus digestibility studies. *Poult Sci* **91**, 965-971.


[s]
Ladder
kidney 34
kidney 38
kidney 41
kidney 44
kidney 45
kidney 51
kidney 55
kidney 56
kidney 58
kidney 64
kidney 65
kidney 68
[s]
65
60
55
50
45
40
35
30
25
20
L
1
2
3
4
5
6
7
8
9
10
11
12
RNA Area:
491.6
RNA Concentration:
192
ng/µl
rRNA Ratio [28s / 18s]:
1.0
RNA Integrity Number (RIN):
9.9 (B.02.10)
Result Flagging Color:
Result Flagging Label:
RIN: 9.90

CHAPTER 7

Renal expression of Ca and P transporters: contrasting responses to dietary Ca and microbial phytase in growing chickens and pigs

Y.X. Hu[*†], P. Bikker[*] W.H. Hendriks[†], M.M. van Krimpen[*], J. van Baal[†]

[*] Wageningen University & Research, Wageningen Livestock Research, 6700 AH Wageningen, the Netherlands

[†] Wageningen University & Research, Animal Nutrition Group, 6700 AH Wageningen, the Netherlands

To be submitted

Abstract

In previous studies incremental dietary Ca intake was shown to downregulate intestinal expression of Ca transporters CaSR, CaBP-D28k and PMCA1, and cation-permeable CLDN-2 and -12 in broilers. In contrast, dietary Ca reduced intestinal expression of Ca transporters TRPV5, TRPV6 and CaBP-D9k, and upregulated CLDN-2, indicating a shift from transcellular to paracellular Ca absorption pathways with increasing Ca intake in pigs. These observations indicate that the regulation of intestinal absorption and urinary excretion of Ca may not be the same in pigs and poultry. Here, we hypothesized that renal expression of Ca- and P-related transporters and pore-forming CLDN respond differently to dietary Ca and P intake in broilers compared to growing pigs. In experiment (Exp) 1 we varied dietary Ca content (2.0 vs. 9.6 g/kg) and phytase (0 vs. 500 FTU/kg) in growing pigs, in Exp 2 dietary Ca/P (0.50, 1.00 and 1.75) and limestone particle size (fine vs. coarse) and in Exp 3 dietary Ca/retainable P (rP) (1.3, 1.8, 2.3 and 2.8) and phytase (0 vs. 1000 FTU/kg) in broilers. Results of RT-qPCR demonstrate that in Exp 1 incremental dietary Ca intake reduces the expression of epithelial Ca transporters TRPV5, TRPV6, CaBP-D28k and NCX1, and Ca permeable CLDN-12 and -16 in pig kidney. In contrast, in Exp 3 high Ca intake increased renal expression of TRPV6, CaBP-D28k and cation-selective CLDN-2 in broilers. Moreover, while dietary phytase inclusion enhanced the reducing effect of dietary Ca on renal TRPV5, CaBP-D28k and NCX1 expression in pigs ($P_{interaction}<0.05$), it reduced the positive impact of dietary Ca on renal TRPV6 and CaBP-D28k expression in broilers ($P_{interaction}<0.05$). In pig kidney, incremental dietary Ca upregulated PiT-1 ($P<0.001$) while phytase downregulated XPR1 ($P=0.035$), without effect on other P transporters. In the broiler kidney, however, incremental dietary Ca intake downregulated NaPi-IIa, PiT-1, PiT-2 and XPR1 ($P<0.01$), while phytase downregulated NaPi-IIa ($P<0.001$) but upregulated PiT-2 and XPR1 ($P<0.05$). In Exp 2, limestone particle size had no effect on the above-mentioned transporter genes, except that coarse limestone numerically ($P=0.053$) decreased CaBP-D28k expression in broiler kidneys. Incremental dietary Ca and microbial phytase inclusion downregulate renal Ca transporters and CLDN in growing pigs, but upregulate these genes in broilers, confirming the hypothesis and showing important differences in the regulation of Ca and P homeostasis between birds and mammals.

Key words: calcium and phosphorus, transporters and claudins, kidney, pigs, broilers

Introduction

Calcium (Ca) and phosphorus (P) are two essential macro minerals for vertebrates and play an important role in many biological processes such as muscle contraction, cell differentiation and bone formation [1]. Calcium and P homeostasis is controlled through the absorption of these minerals in the gastrointestinal tract (GIT), their storage and release in bone and excretion via urine by the kidney. Of these three organs, the kidney is generally considered as the central regulator of whole body Ca and P homeostasis [2].

The transporters involved in renal Ca and P reabsorption in broilers [3] and pigs [4] are presumed to be rather similar to those in rodents, due to their relative high degree in protein sequence homology, implicating functional similarity. Studies in rodents indicate that after glomerular filtration, bulk Ca is reabsorbed in the proximal tubules and thick ascending limb via paracellular diffusion, and to a lesser extent but tightly controlled in the distal convoluted and connecting tubules via an active ATP-consuming transcellular route [5]. Active transcellular Ca reabsorption begins with Ca diffusion into the renal epithelium predominantly via transient receptor potential channel V member 5 (TRPV5) and 6 (TRPV6) [6]. Intracellular Ca binds to calbindin-D28k (CaBP-D28k) and is exported via basolateral sodium-Ca exchanger (NCX1) or plasma membrane Ca-ATPase 1 (PMCA1) [7]. The reabsorption of P occurs mainly in the proximal tubules via both transcellular and paracellular pathways [2]. Transcellular uptake of P is facilitated by sodium dependent phosphate transporter type IIa (NaPi-IIa) [8], and putative xenotropic and polytropic retrovirus receptor 1 (XPR1) [9].

Paracellular ion reabsorption occurs via channels formed by claudins (CLDN, for review see Otani and Furuse [10]) whereby CLDN-2, -12 and -16 have been demonstrated to be permeable for Ca [11]. CLDN-4 is associated with chloride reabsorption in the kidney of mice [12], and CLDN-7 putatively forms channels for magnesium in Caco-2 [13] or for sodium in porcine kidney cell lines (LLC-PK1) [14]. CLDN-10 may facilitate both cation and anion absorption depending on the spliced variants [15]. Noteworthy, CLDN forming paracellular pores for P have not been identified, yet.

Improving dietary P absorption and retention has been intensively studied in a number of animal species. In some studies and reports dealing with Ca and P

absorption and excretion, it is (inherently) assumed that regulatory mechanism of Ca and P metabolism are largely identical between pigs and poultry. For instance, based on earlier findings in chicken kidney, Colston and Feldman [16] postulated the function and metabolism of 1,25-dihydroxycalciferol ($1,25(OH)_2D_3$, the bioactive form of vitamin D_3) in pig kidney and tested their hypothesis in the porcine kidney cell lines (LLC-PK1). Symeou *et al*. [17] directly used broiler data to predict efficacy of mucosal phosphatases to degrade dietary inositol phosphate in the GIT of pigs when the relevant pig data are absent, in their *in silico* model that simulates P intake, absorption and retention in pigs. However, this simplification ignores the fact that striking differences have been observed in Ca and P absorption and metabolism between pigs and broilers. An example is that in the study of Jendza *et al.* [18], which investigated the efficacy and equivalency of microbial phytase inclusion in pigs and broilers, dietary phytase inclusion of 500 FTU/kg enhanced the apparently digested P content by 0.6 vs. 0.9 g/kg in broilers and pigs, respectively. Interestingly, they also found that based on bone ash content, 500 FTU/kg phytase was equivalent to 1.2 vs. 1.0 g of inorganic P from monosodium phosphate in broilers and pigs, respectively. Their results indicate that although phytase inclusion (500 FTU/kg) enhances intestinal P absorption to a lesser extent in broilers compared to pigs, it increases bone ash to a greater extent in broilers. This indicates a difference in urinary Ca and P excretion in the kidney between pigs and broilers.

The potential difference in Ca and P absorption and excretion between pigs and broilers was also observed in our previous studies. In Chapter 5 and 6 it was found that incremental dietary Ca intake downregulated expression of both Ca-related transporters and CLDN in the duodenum and jejunum of broilers. By contrast, in the jejunum of pigs, a higher Ca intake reduced expression of Ca transporters while it increased Ca permeable CLDN (Chapter 3). The differences in modulating intestinal expression of Ca transporters and CLDN presumably are related to a difference in Ca and P post-absorptive metabolism and excretion via urine between pigs and broilers. This assumption is supported by the difference in kidney morphology between pigs and poultry, where 70-90% [19] of the avian nephron is reptilian-type and without a loop of Henle (for comparison of different nephron types see Dantzler [20]). We hypothesized that the response of the renal expression of Ca- and P-related transporters and CLDN to dietary Ca and microbial

phytase inclusion differs between growing pigs and broilers. As such, the impact of dietary Ca content, limestone particle size and microbial phytase inclusion on mRNA expression of Ca- and P-related transporters and CLDN in kidneys of growing pigs and broilers was compared.

Materials and methods

Kidneys from a study in pigs (Experiment (Exp) 1) and two studies in broilers (Exp 2 and 3) that were approved by the Ethical Committee of Wageningen University & Research and conducted in accordance with the Dutch law on animal experiments were used. Experiment 1 was performed in the facilities of the Swine Research Centre of Trouw Nutrition (Sint Anthonis, the Netherlands; approval no. 2016.D-0065.006), while Exp 2 and 3 were conducted in the broiler research facility of De Heus (Eerde, the Netherlands; approval no. 2016.D-0065.004) and ForFarmer (Bathmen, the Netherlands; approval no. 2016.D-0065.022), respectively.

Experiment 1

The experimental design, dietary composition, animal husbandry, sample collection, as well as results regarding faecal and urinary Ca and P excretion are described in detail in Chapter 2. Briefly, sixty individually housed male growing pigs (Hypor Libra×Maxter, 30.4±1.3 kg) received one of six diets containing either low, medium or high Ca content (2.0, 5.8, and 9.6 g/kg, respectively) in the presence or absence of microbial phytase (0 vs. 500 FTU/kg) in a 3×2 factorial arrangement. The study was conducted over time in two runs with five replicate pigs per treatment per run. Dietary P content in the basal diet was fixed at marginal deficiency for all treatment groups (4.7 g/kg, digestible P 1.7 g/kg excluding the contribution of microbial phytase). Limestone (Sibelco, Maastricht, the Netherlands; particle size less than 250 µm as reported by the manufacture) was added to the basal diet to realise the intended Ca content of 2.0, 5.8 and 9.6 g/kg, respectively. The animal trial lasted for 21 days. At dissection, the renal cortex of the left kidney was sliced off with a scalpel, collected in cryovials, snap-frozen in liquid nitrogen, and stored at -80 °C until analysis. For analysis of gene expression

in the kidney, all pigs from the treatments with the lowest and highest dietary Ca content, with and without phytase were selected.

Experiment 2

Experiment 2 is described in detail in Chapter 4. Briefly, 1152 0-d-old male Ross 308 broilers were equally divided over 72 pens and allocated to one of twelve diets containing one of six Ca levels from either fine or coarse limestone in a 6×2 factorial arrangement, with six replicate pens per treatment and 16 birds per replicate pen. The total P content in the basal diet was sufficient (5.5 g/kg, retainable P (rP) 3.2 g/kg) to meet the requirements of broilers. Fine or coarse limestone was added to the basal diet to realise the intended Ca/P of 0.50, 0.75, 1.00, 1.25, 1.50 and 1.75, respectively. The coarse and fine limestone were derived from the same limestone product (Sibelco, Maastricht, the Netherlands) via sieving. The analysed geometric mean diameter was 160 vs. 1062 µm for the fine and coarse limestone, respectively. Broilers were given a common commercial starter diet (apparent metabolizable energy 12.5 MJ/kg, crude protein 212 g/kg, Ca 8.6 g/kg, P 5.5 g/kg) from d 0-13. Thereafter dietary treatments were applied for six days (d 14-20). At dissection (d 20), the left kidney were sampled (3 birds per pen) from four of the six replicate pens, snap-frozen in liquid nitrogen and stored at -80 °C. For gene expression analysis, samples from the lowest, medium and highest Ca/P (0.50, 1.00 and 1.75) with fine and coarse limestone were selected.

Experiment 3

As described in Chapter 6, in Exp 3, 1800 male 0-d-old Ross 308 broilers were equally divided among 72 pens and allocated to one of eight experimental diets containing either one of four Ca levels in the presence (1000 FTU/kg) or absence of microbial phytase in a 4×2 factorial arrangement, with nine replicate pens per treatment and 25 birds per replicate pen. The animal trial lasted for 23 d including a starter and grower period from d 0-9 and 10-23, respectively. Dietary rP was fixed at 80% of CVB [21] recommendation in the basal diet (3.2 and 2.5 g/kg, excluding the contribution of microbial phytase in the starter and grower diet,

respectively). Limestone (Faunacal®, Wülfrath, Germany; mean particle size 90 µm as reported by the manufacture) was added to the basal diet to realise Ca/rP of 1.3, 1.8, 2.3 and 2.8. At dissection, one of the kidneys of one bird per pen was sampled, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis.

Gene expression

Determination of mRNA expression of Ca- and P-related transporters and CLDN was performed following the standard protocol in our laboratory. Briefly, deep-frozen kidney tissue was ground in liquid nitrogen, subsampled (50-100 mg) and used to isolate the total RNA using TRIzol (ThermoFisher Scientific). 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. Reverse transcription of RNA (500 ng) was achieved with a Superscript III kit (ThermoFisher Scientific) and mRNA levels were assessed by real-time 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 conditions: 95 °C for 15 s and 60 °C for 30 s for 40 cycles. This was followed by a melting curve analysis ramping from 60 to 95 °C with a rate of 0.1 °C/s in order to confirm PCR specificity. Absolute quantitative mRNA levels in the three experiments were calculated by establishing a linear calibration curve using 10-fold serial dilutions of cDNA template for corresponding genes. Expression levels of genes of interest in Exp 1 were normalized to the geometric mean of importin 8 (IPO8), eukaryotic elongation factor 2 (EEF2) and beta actin (ACTB) [22]. Expression levels in Exp 2 and 3 were normalized by the geometric mean of IPO8, EEF2, ACTB, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 60S acidic ribosomal protein P0 (RPLP0) [23]. The used primer sequences were designed with Primer Express Software (Life Technologies, Bleiswijk, the Netherlands), and where possible recommended primer sets that span an intron were selected (**Table 1**).

Table 1. Gene-specific primers used for the analysis of mRNA levels using real-time quantitative PCR (RT-qPCR)

Genes	Primers used in pig study (5'-3')	Primers used in broiler studies (5'-3')
Ca transporters		
CaSR	F TTGCTCATGCCCTGCAAGAT	F GCCAATCTGCTGGGACTCTT
	R AAGTGCCGTAGGTGCTTCAG	R CTGATGCTCGTCATTGGGGA
CaBP-D9k	F TATGCAGCCAAAGAAGGGGAT	-
	R CTAGGGTTCTCGGACCTTTCAG	-
CaBP-D28k	F GGCTTCATTTCGACGCTGAC	F CGAGATCTGGCACCACTACG
	R TCGGGTGATAACTCCAATCCAG	R ACCTGAGCAAGCTCAACGAT
NCX1	F GTTTGTGGCGCTTGGAACTT	-
	R TGCCCGTGACGTTACCTATG	-
PMCA1	-	F ACTCTGATGGCAGTTTCCGA
	-	R GTCACGGTCCCTAGGTCTGA
TRPM7	F AGCACCATCTTGGACTCTTGC	-
	R CCCAGGACCACAGATTTCACG	-
TRPV5	F GCTGCAACAGAAGAGGATTCG	-
	R CCGAAAGTCACAGGTTCGGT	-
TRPV6	F AGCAGAAGCGGATCTGGG	F GACCAGAGCAAAGAGGGACC
	R GGGCTCCTTTCTGGTGGACA	R CCGCCTCTGCATGAGGTATT
P transporters		
NaPi-IIa	F GACAGGACTGACTTCCGGC	F GAAGCCAGGTGCCTCTGATG
	R CACGGATGTTGAAGGAGGCT	R AGAGGATGGCGTTGTCCTTG
NaPi-IIb	F CTCTGTAGCTGCCGGGTCCTAA	F TGGCTTTGTCCCTGCTTGTT
	R GGTCAGAGTCGACGAGAACAC	R CCAGCCAGCCAAGTAAAAGG
NaPi-IIc	F GCATCCTGTTGTGGTACGTG	-
	R AGAAGCCGAGCAACAGGTAG	-
PiT-1	F GATGTTTGGTTCTGCTGTGTG	F TGAAGCTTCCCATCTCGGGT
	R AGACCACTTGACACCCTCCTG	R AGGACAACACGATTTTTAGCAGC
PiT-2	F TCACGAACCAAGCCACGAA	F GCTGGGAGCAAAAGTAGGAGA
	R GATCTCACCCCCATCACACC	R AAACAGCAGAACCAACCATCG
XPR1	F GGTGGCCTCTTGCCAAATGA	F AACCTGGAGACAACACGAGG
	R GCACTGGATAAAGCGAAGCC	R CGTTGGTCACCACTTCCTCT
Tight junction proteins		
CLDN-2	F CTGCCCACTGCAAGGAAATC	F CAACTGGAAGATCAGCTCCT
	R ACTCACTCTTGGCTTTGGGT	R TGTAGATGTCGCACTGAGTG
CLDN-4	F ATGGGTGCCTCGCTCTACAT	-
	R GAGTAGGGCTTGTCGGTACG	-
CLDN-7	F GCAGGTCTTTGTGCGTTGAT	-
	R AAGATGGCAGGGCCAAACTC	-
CLDN-10	F GACCGGGTGTTCCCTGTATG	-
	R GGCTCCTGCCCATCCAATAA	-
CLDN-12	F ATGACGTCCGTTCTGCTCTT	F CTCTTATTCCTCCTCGCATG
	R TACGTATGCATGCTGGGAGG	R GTCAAAGCTAAAGACAGGCT
CLDN-16	F TTTTTGGCTGGTGCTGTCCT	F GGGATCCAAACATGTGATGA
	R CATACGACTTGGCCATCGAAAC	R AGAGAAATCCAAATCCTGCC
ZO-1	F GTTGGACAACCAGACGTGGA	F CCGCAGTCGTTCACGATCT
	R ACTAACTTCATGCTGGGCCG	R GGAGAATGTCTGGAATGGTCTGA

Table 1 (continued). Gene-specific primers used for the analysis of mRNA levels using real-time quantitative PCR (RT-qPCR)

Genes	Primers used in pig study (5'-3')	Primers used in broiler studies (5'-3')
Vitamin D_3 related proteins		
CYP24	F GGGCGGAGGATTTGAGGAAT	-
	R ATCAACACGGTTCCTTTGGGT	-
VDR	F CTCTCCAGACACGATGGAGC	F GGCTCAGGTTTTGCAGATTTG
	R TTGGCAAAGCCGATGACCTT	R CAGCATCGCCTTTCCCATT
Reference genes		
ACTB	F CGTGAGAAGATGACCCAGATCA	F GCCCTGGCACCTAGCACAAT
	R TCTCCGGAGTCCATCACGAT	R GCGGTGGACAATGGAGGGT
EEF2	F AGTCCACTCTGACGGACTCA	F CAGTTGGCTTTGGTTCTGGC
	R AGAGGGAAATGGCCGTTGAT	R AAAGTATCTGTCTCCCCACAGC
GAPDH	-	F ATCCCTGAGCTGAATGGGAAG
	-	R AGCAGCCTTCACTACCCTCT
IPO8	F TGCCATGGTATTTCTCCTCAAA	F ACCTCCGAGCTAGATCCTGT
	R GCAGAAGAGGCATCATGTCTGT	R GGCTCTTCTTCGCCAACTCT
RPLP0	-	F TTGGGCATCACCACAAAGATT
	-	R CCCACTTTGTCTCCGGTCTTAA

- Gene not determined.
ACTB, beta actin; CaBP-D9k, calbindin D8k; CaBP-D28k, calbindin D28k; CaSR, Ca sensing receptor; CLDN, claudin; CYP24, vitamin D_3 24 hydroxylase; EEF2, eukaryotic elongation factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPO8, importin 8; NaPi-IIa, sodium dependent phosphate transporter IIa; NaPi-IIb, sodium dependent phosphate transporter IIb; NaPi-IIc, sodium dependent phosphate transporter IIc;NCX1, sodium-Ca exchanger; PMCA1, plasma membrane Ca-ATPase 1; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; RPLP0, 60S acidic ribosomal protein P0; TRPC1, transient receptor potential canonical 1; TRPM7, transient receptor potential cation channel subfamily M member 7; TRVP5, transient receptor potential cation channel subfamily V member 5 TRPV6, transient receptor potential cation channel subfamily V member 6; VDR, vitamin-D_3 receptor; XPR1, xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1.

Statistical analysis

Pen was the experimental unit in all three studies. Data were submitted to a 2-way ANOVA using the MIXED procedure of SAS (version 9.4, SAS Institute, Cary, NC). In Exp 1 and 3, dietary Ca content (or Ca/rP in Exp 3), phytase and their interaction were used as fixed effects and block as random effect. In Exp 2, dietary Ca/P, limestone particle size and their interaction were used as fixed effect, block and pen as random effect. Distribution and variance homogeneity of the Studentized residuals were visually checked via graphics plotted by the ODS GRAPHICS procedure. The LSMEANS procedure with a PDIFF option was used to separate means. In Exp 3, linear and quadratic effect of dietary Ca/rP were determined using the CONTRAST procedure. Probability was considered significant at 0.05 and a trend between 0.05 and 0.1.

Results

Dietary effects in pig kidney

Quantification of gene expression levels in the kidney cortex of pigs by RT-qPCR revealed that extracellular Ca sensing receptor (CaSR) mRNA expression was increased more (62 vs. 21%, $P_{interaction}$=0.039, **Table 2**) with high (9.6 g/kg) dietary Ca content in the absence and presence of phytase compared to low (2.0 g/kg), respectively. Overall, mRNA expression of Ca-related transporters was reduced with higher Ca content, with a greater impact in the presence compared to absence of phytase. In particular, a high dietary Ca content reduced TRPV5 mRNA expression only in the presence of phytase (69% reduction, $P_{interaction}$=0.026). Similar to TRPV5, a high Ca intake (9.6 g/kg) reduced renal CaBP-D28k mRNA expression by 77 vs. 36% ($P_{\text{interaction}}$=0.025), and NCX1 mRNA expression by 57 vs. 21% ($P_{\text{interaction}}$=0.024) in presence and absence of phytase, respectively. Irrespective of phytase inclusion ($P_{interaction}$=0.791), renal TRPV6 mRNA expression was 40% lower (P<0.001) at a high Ca intake (Table 2). In contrast, expression of CaBP-D9k was increased with a high dietary Ca content in the phytase-free but not phytase-supplemented diets ($P_{interaction}$=0.002). In general, expression of TRPV5 mRNA in the pig renal cortex was approximately 6 times greater than that of TRPV6, and CaBP-D28k mRNA expression approximately 100 times greater than CaBP-D9k mRNA expression. Expression of TRPM7 remained unaffected by dietary treatment.

Expression of P transporters in the kidney of pigs was not affected by dietary Ca content or microbial phytase inclusion, apart from PiT-1, which showed a 47% greater expression level in response to dietary Ca intake (P<0.001) and for XPR1, which expression level was 7% lower with phytase inclusion (P=0.035).

Except for ZO-1 and CLDN-4, renal expression of all measured tight junction proteins was affected by dietary Ca content and/or phytase inclusion without significant interactions in pigs. In particular, expression of the Ca-related CLDN, including CLDN-12 (P=0.013) and -16 (P<0.001), was reduced by 9 and 38% with dietary Ca intake, respectively. Expression of Ca pore-forming CLDN-2 was 7% greater with phytase inclusion (P=0.032). Phytase inclusion also reduced renal expression of CLDN-7 and -10 by 17 and 10%, respectively (P=0.005). Furthermore, dietary Ca content and phytase inclusion increased vitamin D_3 receptor (VDR) and 24-hydroxylase (CYP24) mRNA expression in the kidney cortex of pigs.

Table 2. Least square means of mRNA expression level of various calcium (Ca) and phosphorus (P) related transporters, tight junction proteins, vitamin D_3 24 hydroxylase and its receptor in the kidney cortex of growing pigs as affected by dietary Ca content and microbial phytase supplementation [1,2,3]

Ca	Phytase	Ca transporters							P transporters					
g/kg	FTU/kg	CaSR	CaBP-D9k	CaBP-D28k	NCX1	TRPM7	TRPV5	TRPV6	NaPi-IIa	NaPi-IIb	NaPi-IIc	PiT-1	PiT-2	XPR1
2.0	0	0.13^{c}	0.06^{b}	20.1^{a}	0.43^{a}	1.37	0.29^{a}	0.06	4.00	0.002	0.13	1.79	10.8	0.46
	500	0.14^{bc}	0.04^{b}	22.2^{a}	0.49^{a}	1.31	0.36^{a}	0.05	3.93	0.002	0.14	1.84	11.0	0.44
9.6	0	0.21^{a}	0.34^{a}	12.9^{b}	0.34^{b}	1.35	0.29^{a}	0.03	4.12	0.002	0.15	2.93	10.2	0.50
	500	0.17^{b}	0.04^{b}	5.0^{c}	0.21^{c}	1.30	0.11^{b}	0.03	4.12	0.001	0.12	2.41	9.7	0.45
Pooled SEM		0.016	0.056	2.95	0.052	0.059	0.075	0.007	0.159	0.001	0.015	0.251	2.01	0.022
Ca														
2.0		0.14	0.05	21.1	0.46	1.34	0.32	0.05	3.97	0.002	0.14	1.82	10.9	0.45
9.6		0.19	0.19	8.9	0.27	1.33	0.20	0.03	4.12	0.002	0.13	2.67	9.9	0.47
Pooled SEM		0.012	0.041	2.08	0.038	0.043	0.053	0.005	0.112	0.001	0.011	0.178	1.43	0.016
Phytase														
0		0.17	0.20	16.5	0.39	1.36	0.29	0.04	4.06	0.002	0.14	2.36	10.5	0.48
500		0.16	0.04	13.6	0.35	1.31	0.24	0.04	4.03	0.002	0.13	2.13	10.4	0.45
Pooled SEM		0.012	0.041	2.08	0.038	0.043	0.053	0.005	0.112	0.001	0.011	0.178	1.43	0.016
P-value														
Ca		<0.001	0.003	<0.001	<0.001	0.569	0.028	<0.001	0.182	0.405	0.860	<0.001	0.429	0.204
Phytase		0.334	0.001	0.177	0.400	0.224	0.288	0.422	0.777	0.467	0.457	0.201	0.921	0.035
Ca × phytase		0.039	0.002	0.025	0.024	0.945	0.026	0.791	0.722	0.619	0.195	0.122	0.779	0.502

Table 2 (continued). Least square means of mRNA expression level of various calcium (Ca) and phosphorus (P) related transporters, tight junction proteins, vitamin D_3 24 hydroxylase and its receptor in the kidney cortex of growing pigs as affected by dietary Ca content and microbial phytase supplementation [1,2,3]

Ca	Phytase	Tight junction proteins, CYP24 and VDR								
g/kg	FTU/kg	ZO-1	CLDN-2	CLDN-4	CLDN-7	CLDN-10	CLDN-12	CLDN-16	CYP24	VDR
2.0	0	0.278	0.172	0.13	0.023	3.36	0.35	0.18	0.56	0.51
	500	0.286	0.176	0.13	0.020	3.11	0.35	0.19	0.86	0.69
9.6	0	0.283	0.164	0.14	0.025	3.44	0.34	0.11	2.32	0.74
	500	0.272	0.185	0.11	0.020	2.98	0.31	0.12	3.00	0.88
Pooled SEM		0.011	0.007	0.014	0.001	0.161	0.017	0.009	0.301	0.086
Ca										
2.0		0.282	0.174	0.13	0.022	3.24	0.35	0.18	0.71	0.60
9.6		0.277	0.174	0.12	0.023	3.21	0.32	0.11	2.66	0.81
Pooled SEM		0.008	0.006	0.010	0.001	0.114	0.012	0.006	0.219	0.063
Phytase										
0		0.280	0.168	0.13	0.024	3.40	0.35	0.14	1.44	0.62
500		0.279	0.180	0.12	0.020	3.05	0.33	0.15	1.93	0.78
Pooled SEM		0.008	0.006	0.010	0.001	0.114	0.012	0.006	0.219	0.063
P-value										
Ca		0.471	0.982	0.532	0.446	0.809	0.013	<0.001	<0.001	0.002
Phytase		0.857	0.032	0.182	0.005	0.005	0.135	0.171	0.034	0.017
Ca × phytase		0.254	0.127	0.098	0.541	0.369	0.382	0.478	0.386	0.754

[1] Dietary P content was fixed at 4.7 g/kg.
[2] Data are presented as treatment means, 10 replicate pens per treatment (n=10).
[3] Determined using absolute quantification and normalized by the geometric mean expression of ACTB, EEF2 and IPO8.
[a-c] Least square means within a column lacking a common superscript differ significantly ($P \leq 0.05$).
ACTB, beta actin; CaBP-D9k, calbindin-D9k; CaBP-D28k, calbindin-D28k; CaSR, Ca sensing receptor; CLDN, claudin; CYP24, vitamin D_3 24 hydroxylase; EEF2, eukaryotic elongation factor 2; IPO8, importin 8; NaPi-IIa, sodium-dependent phosphate transporter type IIa; NaPi-IIb, sodium-dependent phosphate transporter type IIb; NaPi-IIc, sodium-dependent phosphate transporter type IIc; NCX1, sodium-Ca exchanger; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; TRPV5, transient receptor potential cation channel subfamily V member 6; TRPV6, transient receptor potential cation channel subfamily V member 6; TRPM7, transient receptor potential cation channel subfamily M member 7; VDR, vitamin D_3 receptor; XPR1, xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1.

Dietary effects in chicken kidney

Irrespectively of limestone particle size (no interaction effect), incremental Ca/P enhanced expression of TRPV6 (P=0.008) and CaBP-D28k mRNA (P<0.001) by 34 and 86%, respectively, and tended (P=0.073) to enhance renal expression of CaSR by 10% in the kidney of broilers (**Table 3**). Limestone particle size had no impact (P>0.10) on expression of Ca- and P-related transporters, apart from a renal CaBP-D28k mRNA expression which tended (P=0.053) to increase (16%) with fine limestone. Dietary treatments did not alter transcript levels of renal PMCA1.

Renal NaPi-IIa mRNA expression tended ($P_{interaction}$=0.063, Table 3) to be lower (31%) in diets supplemented with coarse compared to fine limestone at the lowest dietary Ca content only. Irrespective of limestone particle size (no interaction effect), incremental dietary Ca content reduced PiT-1 and PiT-2 mRNA expression by 86 and 30%, respectively (P<0.001 and 0.032). By contrast, XPR1 mRNA expression remained unaffected.

No interaction effect between limestone particle size and inclusion level on any of the measured tight junction proteins was observed in the kidney of broilers. CLDN-2 mRNA expression was enhanced by 25% with incremental Ca intake (P=0.045, Table 3). In addition, expression of ZO-1 mRNA tended (P=0.075) to be lower (9%) in the presence of coarse compared to fine limestone. CLDN-12 and -16 mRNA expression were not affected by dietary treatment. In contrast, VDR mRNA expression was 74% higher (P<0.001) in broilers with a high compared to low Ca intake.

Table 3. Least square means of mRNA expression level of calcium (Ca) and phosphorus (P) related transporters, tight junction proteins and vitamin D_3 receptor in the kidney of broilers as affected by dietary Ca/P and limestone particle size [1,2,3]

Limeston	Ca/P	Ca transporters					P transporters					Tight junction proteins and VDR				
		CaSR	CaBP-D28k	PMCA1	TRPC1	TRPV6	NaPi-IIa	NaPi-IIb	PiT-1	PiT-2	XPR1	ZO-1	CLDN-2	CLDN-12	CLDN-16	VDR
	0.50	1.7	52.9	17.5	0.55	41.9	286	1.5	76.2	1016	7.6	0.65	0.53	1.9	2.0	0.51
Coarse	1.00	2.2	59.0	17.4	0.61	44.3	313	2.2	32.7	813	9.5	0.75	0.68	2.2	2.6	0.60
	1.75	2.1	97.6	18.7	0.65	62.4	319	2.1	15.4	905	8.9	0.79	0.68	2.5	2.6	0.93
	0.50	2.1	61.8	17.9	0.61	48.8	375	2.4	82.6	1231	8.6	0.78	0.58	2.3	2.4	0.57
Fine	1.00	2.3	65.6	18.5	0.69	42.5	284	2.6	15.0	980	9.5	0.82	0.75	2.3	2.7	0.63
	1.75	2.0	116.2	17.9	0.65	59.4	337	1.7	6.7	671	8.1	0.81	0.72	2.2	2.6	0.95
Pooled SEM		0.21	9.92	1.76	0.054	8.26	34.8	0.45	15.63	177.8	1.26	0.066	0.097	0.23	0.29	0.095
Ca/P																
0.50		1.9	57.4^{b}	17.7	0.58	45.3^{b}	331	1.9	79.4^{a}	1124^{a}	8.1	0.71	0.56^{b}	2.1	2.2	0.54^{b}
1.00		2.2	62.3^{b}	18.0	0.65	43.4^{b}	298	2.4	23.8^{b}	896^{ab}	9.5	0.79	0.72^{a}	2.3	2.7	0.61^{b}
1.75		2.0	106.9^{a}	18.3	0.65	60.9^{a}	328	1.9	11.0^{b}	788^{b}	8.5	0.80	0.70^{a}	2.4	2.6	0.94^{a}
Pooled SEM		0.15	7.02	1.24	0.038	5.84	24.6	0.32	11.05	125.8	0.89	0.050	0.068	0.16	0.21	0.067
Limestone																
Coarse		2.0	69.8	17.9	0.60	49.5	306	1.9	41.4	911	8.7	0.73	0.63	2.2	2.4	0.68
Fine		2.1	81.2	18.1	0.65	50.2	332	2.2	34.7	961	8.8	0.80	0.69	2.3	2.6	0.72
Pooled SEM		0.12	5.73	1.02	0.031	4.77	20.1	0.26	9.03	102.7	0.73	0.038	0.056	0.13	0.17	0.055
P-value																
Ca/P		0.073	<0.001	0.873	0.117	0.008	0.356	0.229	<0.001	0.032	0.270	0.146	0.045	0.352	0.103	<0.001
Limestone		0.215	0.053	0.823	0.135	0.885	0.203	0.195	0.463	0.629	0.897	0.075	0.312	0.614	0.346	0.528
Ca/P × limestone		0.261	0.667	0.742	0.612	0.652	0.063	0.136	0.549	0.158	0.614	0.481	0.982	0.108	0.647	0.970

[1] Dietary P was fixed at 5.5 g/kg.
[2] Data are presented as treatment means, 4 pens per treatment and 3 birds per pen (n=12).
[3] Determined using absolute quantification and normalized by the geometric mean expression of ACTB, EEF2, GAPDH, IPO8 and RPLP0.
[a-b] Least square means within a column lacking a common superscript differ significantly ($P \leq 0.05$).
ACTB, beta actin; CaBP-D28k, calbindin-D28k; CaSR, Ca sensing receptor; CLDN, claudin; EEF2, eukaryotic elongation factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPO8, importin 8; NaPi-IIa, sodium dependent phosphate transporter type IIa; NaPi-IIb, sodium dependent phosphate transporter type IIb; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; PMCA1, plasma membrane Ca-ATPase 1; RPLP0, 60S acidic ribosomal protein P0; TRPC1, transient receptor potential canonical 1; TRPV6, transient receptor potential cation channel subfamily V member 6; VDR, vitamin D_3 receptor; XPR1, xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1.

In Exp 3, incremental dietary Ca intake enhanced TRPV6 mRNA expression ($P_{interaction}$=0.002, **Table 4**) by 67 vs. 10%, and increased CaBP-D28k mRNA expression ($P_{interaction}$<0.001) by 123 vs. 36% in phytase-free and phytase-supplemented diets, respectively. However, CaSR and PMCA1 mRNA expression were not significantly affected by dietary treatment in Exp 3 (Table 4).

No interaction effect between dietary Ca and phytase intake on expression of any of the measured P transporters was observed in the kidney of broilers. Incremental Ca intake reduced expression of NaPi-IIa, PiT-1, PiT-2 and XPR1 by 11, 81, 24 and 10%, respectively in the broiler's kidney in Exp 3 (P<0.01, Table 4). In addition, phytase inclusion reduced (P<0.001) NaPi-IIa mRNA expression by 15% and enhanced PiT-2 (P<0.001) and XPR1 (P<0.030) by 4 and 19%, respectively. Renal expression of NaPi-IIb mRNA was not affected by the dietary treatments.

In Exp 3, CLDN-2 mRNA expression was increased linearly with dietary Ca/rP in the kidney of broilers (35% increase with Ca/rP from 1.3 to 2.8, P=0.002). CLDN-12 expression tended (P=0.073) to be lower (4%) with phytase inclusion. ZO-1 and CLDN-16 mRNA expression were not affected by dietary treatments, while VDR mRNA expression was linearly elevated with incremental dietary Ca/rP (71% increase with Ca/rP from 1.3 to 2.8, P<0.001).

Table 4. Least square means of mRNA expression level of calcium (Ca) and phosphorus (P) related transporters, tight junction proteins and vitamin D_3 receptor in the kidney of broilers as affected by dietary Ca to retainable P ratio (Ca/rP) and microbial phytase inclusion [1,2,3]

Phytase	Ca/rP	Ca transporters					P transporters					Tight junction proteins and VDR				
FTU/kg		CaSR	CaBP-D28k	PMCA1	TRPC1	TRPV6	NaPi-IIa	NaPi-IIb	PiT-1	PiT-2	XPR1	ZO-1	CLDN-2	CLDN-12	CLDN-16	VDR
0	1.3	1.75	26.1^{d}	5.66	0.45	35.1^{bc}	272	1.42	18.57	1011	6.28	0.94	0.12	0.42	0.76	0.44
	1.8	1.64	26.6^{cd}	5.60	0.45	33.7^{bc}	233	1.35	9.79	983	6.94	0.93	0.12	0.42	0.76	0.56
	2.3	1.71	38.2^{b}	5.60	0.46	41.2^{b}	261	1.08	3.99	806	6.05	0.98	0.14	0.43	0.81	0.77
	2.8	1.67	58.1^{a}	5.59	0.46	58.5^{a}	268	1.09	3.48	803	5.59	0.98	0.14	0.43	0.75	0.90
1000	1.3	1.80	26.0^{d}	5.89	0.46	33.5^{c}	262	1.40	21.35	1234	6.89	0.97	0.09	0.42	0.81	0.53
	1.8	1.66	25.8^{d}	5.85	0.46	30.5^{c}	209	1.36	6.83	1069	6.84	0.93	0.15	0.39	0.74	0.65
	2.3	1.64	29.6^{bcd}	5.50	0.45	32.0^{c}	201	1.34	5.22	1056	6.25	0.97	0.12	0.39	0.77	0.73
	2.8	1.66	35.5^{bc}	5.80	0.44	36.8^{bc}	207	1.48	3.96	912	6.26	0.91	0.14	0.41	0.78	0.75
Pooled SEM		0.118	4.32	0.237	0.024	3.90	18.8	0.208	2.579	66.1	0.312	0.042	0.014	0.020	0.046	0.086
Ca/rP																
1.3		1.77	26.1	5.77	0.45	34.3	267^{a}	1.41	19.96^{a}	1122^{a}	6.59^{ab}	0.96	0.10^{b}	0.42	0.78	0.48^{b}
1.8		1.65	26.2	5.72	0.46	32.1	221^{b}	1.36	8.31^{b}	1026^{b}	6.89^{a}	0.93	0.13^{a}	0.40	0.75	0.60^{b}
2.3		1.67	33.9	5.55	0.45	36.6	231^{b}	1.21	4.61^{c}	931^{c}	6.15^{bc}	0.97	0.13^{a}	0.41	0.79	0.75^{a}
2.8		1.67	46.8	5.70	0.45	47.7	238^{b}	1.28	3.72^{c}	858^{c}	5.92^{c}	0.95	0.14^{a}	0.42	0.77	0.82^{a}
Pooled SEM		0.084	3.05	0.167	0.017	2.76	13.3	0.147	1.823	46.7	0.220	0.030	0.010	0.014	0.032	0.061
Phytase																
0		1.69	37.3	5.61	0.46	42.1	259	1.23	8.96	901	6.21	0.96	0.13	0.42	0.77	0.67
1000		1.69	29.2	5.76	0.45	33.2	220	1.40	9.34	1068	6.56	0.95	0.12	0.40	0.77	0.66
Pooled SEM		0.059	2.16	0.118	0.012	1.95	9.4	0.104	1.289	33.0	0.156	0.021	0.007	0.010	0.023	0.043

Table 4 (continued). Least square means of mRNA expression level of calcium (Ca) and phosphorus (P) related transporters, tight junction proteins and vitamin D_3 receptor in the kidney of broilers as affected by dietary Ca to retainable P ratio (Ca/rP) and microbial phytase inclusion [1,2,3]

Phytase	Ca/rP	Ca transporters					P transporters					Tight junction proteins and VDR				
FTU/kg		CaSR	CaBP-D28k	PMCA1	TRPC1	TRPV6	NaPi-IIa	NaPi-IIb	PiT-1	PiT-2	XPR1	ZO-1	CLDN-2	CLDN-12	CLDN-16	VDR
P-value																
	Ca/rP	0.456	<0.001	0.575	0.818	<0.001	0.007	0.556	<0.001	<0.001	<0.001	0.524	0.002	0.591	0.609	<0.001
	Linear (Ca/rP)	0.257	<0.001	0.447	0.924	<0.001	0.067	0.265	<0.001	<0.001	<0.001	0.898	0.001	0.978	0.996	<0.001
	Quadratic (Ca/rP)	0.337	0.004	0.413	0.816	0.001	0.007	0.545	<0.001	0.731	0.092	0.958	0.102	0.181	0.822	0.579
	Phytase	0.967	<0.001	0.214	0.988	<0.001	<0.001	0.122	0.767	<0.001	0.030	0.566	0.348	0.073	0.868	0.942
	Ca/rP × phytase	0.901	0.001	0.699	0.647	0.002	0.142	0.446	0.454	0.214	0.257	0.383	0.098	0.708	0.405	0.158

[1] Dietary rP was fixed at 80% of minimal requirement of rP for broilers (3.2 and 2.5 g/kg excluding the contribution of microbial phytase in the starter and grower period, respectively).
[2] Data are presented as treatment means, 9 replicate pens per treatment (n=9).
[3] Determined using absolute quantification and normalized by the geometric mean expression of ACTB, EEF2, GAPDH, IPO8 and RPLP0.
[a-d] Least square means within a column lacking a common superscript differ significantly ($P \leq 0.05$).
ACTB, beta actin; CaBP-D28k, calbindin-D28k; CaSR, Ca sensing receptor; CLDN, claudin; EEF2, eukaryotic elongation factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPO8, importin 8; NaPi-IIa, sodium dependent phosphate transporter type IIa; NaPi-IIb, sodium dependent phosphate transporter type IIb; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; PMCA1, plasma membrane Ca-ATPase 1; RPLP0, 60S acidic ribosomal protein P0; TRPC1, transient receptor potential canonical 1; TRPV6, transient receptor potential cation channel subfamily V member 6; VDR, vitamin D_3 receptor; XPR1, xenotropic and polytropic retrovirus receptor 1; ZO-1, zonula occludens-1.

Discussion

To improve dietary P utilization and reduce P excretion, studies have been performed assuming an identical regulatory mechanism of Ca and P metabolism between pigs and broilers (e.g. Colston and Feldman [16]). In the present study, we demonstrate that the renal expression of Ca- and P-related transporters and CLDN in pigs and chicken respond differently to Ca and microbial phytase intake. Thus, caution should be taken with the extrapolation from mammals to birds and *vice versa*. Indeed, mRNA expression of TRPV5, TRPV6, CaBP-D28k, NCX1 and CLDN-12 were all downregulated with incremental dietary Ca content in the kidney of pigs, as demonstrated in Exp 1 (Table 2). The opposite effect was observed in broilers: incremental dietary Ca content linearly upregulated the renal expression of TRPV6, CaBP-D28k and CLDN-2 in both Exp 2 and 3 (Tables 3 and 4).

Modulation of Ca-related transporters and CLDN in the kidney of pigs

In Chapter 2 we observed that incremental dietary Ca intake substantially increased urinary Ca excretion in pigs, which is in agreement with the downregulated renal expression of Ca-related transporters and CLDN, involved in Ca reabsorption in the kidney, with a higher Ca intake (Table 2). This downregulation likely reflects a reduction in Ca reabsorption in the kidney. In Chapter 3, we further showed that pigs fed a diet with higher Ca content had elevated serum Ca concentration, which might have activated extracellular CaSR in parathyroid gland and triggered a lower secretion of parathyroid hormone (PTH) as observed in Chapter 3. A lower PTH concentration reduced serum $1,25(OH)_2D_3$ concentration (Chapter 3) probably by catalysing $1,25(OH)_2D_3$ 24-hydroxylation, as indicated by the upregulated renal expression of CYP24 mRNA expression at a high Ca intake (Table 2). The reduced $1,25(OH)_2D_3$ concentration, in turn, decreased expression of TRPV5, TRPV6, CaBP-D28k and NCX1, which could be due to the similarity between these four genes in VDR response elements in their promoter region [24]. In addition, the negative impact of incremental Ca intake on renal TRPV5, CaBP-D28k and NCX1 mRNA expression was greater in pigs fed phytase included diets (Table 2), which is in line with the greater reduction of serum $1,25(OH)_2D_3$ with incremental Ca intake in the presence of phytase (Chapter 3).

Besides systemic regulation of $1,25(OH)_2D_3$ and PTH, increasing Ca intake might also downregulate expression of Ca-related transporters via activation of the cell surface CaSR, a G protein-coupled receptor, expressed in kidney tissue. Specifically, a higher Ca intake elevated serum Ca concentration (Chapter 3) and might result in an increase in tubular Ca concentration that stimulated CaSR, which is capable of integrating Ca signals deriving from the tubular fluid and/or the interstitial plasma [25]. CaSR couples to G_q-proteins to activate enzymes of the phospholipase C family, which eventually leads to the stimulation of urinary Ca excretion [26; 27].

Paracellular Ca transport is mediated via channels in the tight junction proteins formed by CLDN [28]. We observed a positive correlation between the expression levels of Ca-related transporters and CLDN in the kidney of pigs, i.e. lower TRPV5, TRPV6, CaBP-D28k and NCX1 mRNA expression was accompanied with lower CLDN-12 and -16 mRNA expression in pigs with a higher Ca intake (Table 2). A study in mice indicates that CLDN-12 forms Ca-selective pores and its expression is upregulated by exposure to $1,25(OH)_2D_3$ [29]. In murine kidney, CLDN-16 is located on the distal convoluted tubule where it interacts with TRPV5 to mediate Ca reabsorption [30; 31]. Thus, downregulated expression of CLDN-12 and -16 presumably reduced paracellular Ca reabsorption in the kidney, which agrees with the greater urinary Ca excretion in pigs fed a higher Ca diet (Chapter 2). Taken together, expression of both Ca-related transporters and CLDN in the kidney cortex of pigs appears to be tightly controlled in responses to changes in dietary Ca and phytase intake, indicating that kidneys in pigs play a pivotal role in whole body Ca homeostasis.

Modulation of Ca-related transporters and CLDN in the kidney of broilers

In contrast to pigs, incremental Ca intake enhanced renal mRNA expression of TRPV6 and CaBP-D28k in broilers (Tables 3 and 4). In Chapter 4 and 6 it was found that broilers with a higher Ca intake had increased serum Ca concentration. Thus, unlike mammals, expression of Ca transporters in the kidney of broilers was positively correlated with serum Ca concentration. It is tempting to speculate that this is due to the fact that chicken CaSR, which shares high homology (84%) with the amino acids sequence of human CaSR, has a shorter cytoplasmic C-terminal

domain compared with its mammalian homologues [32]. Since the C-terminal tail has been shown to be involved in agonist-promoted receptor internalization of many G protein-coupled receptors, it is conceivable that chicken CaSR exhibits different spatial and temporal regulation of intracellular signalling [33, 34]. Furthermore, 70-90% of the chicken nephron is reptilian type and does not contain a loop of Henle [19], which may also play a role in the apparently different regulation of Ca reabsorption in renal tubules of chickens.

Our observations are in line with several *in vivo* studies in chickens. For instance, Bar *et al.* [35] reported that dietary low P or high Ca content increased serum Ca concentration in chickens, which was associated with a higher renal mRNA expression of CaBP-D28k. Similar modulation of renal CaBP-D28k expression was observed in another *in vivo* study from these researchers [36]. The latter study showed that a lower dietary Ca content reduced serum Ca concentration, which was accompanied by an elevated serum $1,25(OH)_2D_3$ level and reduced CaBP-D28k protein expression in the kidney of chickens. A similar effect on these three parameters was obtained with low dietary P content [36]. It is noteworthy that both studies [35; 36] also demonstrated that CaBP-D28k expression in the GIT was reduced upon high Ca intake in these animals. Those findings agree with our results that in broilers, high Ca intake reduced mRNA expression of Ca-related transporters and CLDN in the GIT (Chapter 5 and 6) while it enhanced their mRNA expression in the kidney (Table 3 and 4).

Researchers of the previous studies [35; 36] claimed that CaBP-D28k expression in the kidney of chickens were anti-homeostatic. Thus, they speculated that CaBP-D28k might not be involved in the process of transcellular Ca reabsorption in chickens, but in the sequestering of free intracellular Ca to prevent apoptotic responses [37]. However, we question this interpretation, since CaBP-D28k protein is highly conserved during phylogeny with an overall 79% homology between chickens and rats [38]. Moreover, CaBP-D28k has shown to be dynamically involved in the process of transcellular Ca absorption in the chicken intestine, since its distribution was altered from villus core to the basolateral membrane with Ca absorption [39]. Furthermore, CaBP-D28k mRNA levels positively correlated with that of TRPV6 and CLDN-2 in the broiler kidney (Table 3 and 4). We postulate that CaBP-D28k is actively involved in transcellular Ca reabsorption in chickens.

The upregulation of Ca-related transporters and CLDN in the kidney of broilers in response to higher Ca intake demonstrates that whole body Ca homeostasis in poultry is differently regulated compared to mammals. An earlier study in chickens indicated that vitamin D_3 treatment enhanced CaBP-D28k mRNA expression eightfold more in the duodenal mucosa than kidney [40]. Apparently, CaBP-D28k expression in the GIT of broilers is more sensitive to changes in serum $1,25(OH)_2D_3$ than in the kidney, indicating that the GIT plays a primary role in whole body Ca homeostasis in these species. Indeed, duodenal mRNA expression of CaSR, CaBP-D28k and PMCA1 negatively correlated with serum Ca concentration in broilers (Chapter 5 and 6), presumably to maintain serum Ca concentration within a normal physiological range. Furthermore, it is noteworthy that the chicken GIT may reflux urine and recycle urinary excreted nutrients. For instance, Karasawa [41; 42] indicated that urinary urea could be retrograded to the ceca and converted into ammonia by the microflora in chickens with ammonia being reabsorbed and subsequently utilized for synthesis of nonessential amino acids in the liver. Based on these reports [41; 42], we cannot exclude that a proportion of the urinary excreted Ca was recycled in the GIT of broilers fed Ca-deficient diets. This idea, however, needs confirmation in future studies.

Modulation of renal expression of P transporters in pigs and broilers

Surprisingly, urinary P excretion was substantially reduced with incremental Ca intake in pigs (Chapter 2), while renal mRNA expression of P-related transporters was largely unaffected by both dietary Ca and phytase inclusion (Table 2). This discrepancy between urinary P excretion and expression levels of renal P transporters may indicate the involvement of posttranscription regulation mechanisms. Indeed, several reports in mammals indicated that NaPi-IIa accounts for 70-90% of total renal P reabsorption, and its expression is regulated at the post-transcriptional level, i.e. trafficking of NaPi-IIa to and from the apical membrane via its PDZ binding motif [43; 44]. In particular, pigs receiving a lower Ca diet had lower serum Ca concentration, which might relieve the suppressive effect of CaSR on PTH transcription and secretion in the parathyroid gland of mammals [45; 46]. Besides its calciotropic role, PTH readily stimulates urinary P excretion via inducing removal of NaPi-IIa from the brush-border membrane of renal epithelial cells, independent of renal NaPi-IIa mRNA expression [47] or its endocytic motifs

[48]. However, we did not further investigate the posttranscription regulation of NaPi-IIa since it was beyond the scope of this study. Noteworthy, a pore-forming CLDN for P has not been identified yet, hence renal P reabsorption via the paracellular pathway in response to dietary Ca and phytase intake remains unknown in the kidney of pigs.

In contrast to mammals, incremental dietary Ca intake reduced the mRNA expression of P transporters (NaPi-IIa, PiT-1, PiT-2 and XPR1) in the kidney of broilers. In Chapter 4 and 6 it was found that dietary Ca intake decreased serum P concentration. Thus, expression of P-related transporters in the kidney of broilers appeared to be positively correlated to serum P concentration. Our results are in line with those of Ren *et al.* [49], who indicated that laying hens fed a reduced P diet had a lower serum P concentration, which was accompanied by a lower NaPi-IIa protein expression in the kidney. Renal NaPi-IIa expression is under tight control of $1,25(OH)_2D_3$ since its promoter contains a VDR responsive element [3]. It is conceivable that broilers fed a higher Ca diet had greater serum Ca content (Chapter 4 and 6), which might activate CaSR on the parathyroid gland and trigger a lower secretion of PTH, thereby, reducing serum $1,25(OH)_2D_3$ concentration [50]. Taken together, dietary Ca and phytase intake result in a decrease in the expression of P transporters in the kidney of broilers.

Modulation on expression of other CLDN in pigs

We also observed a lower impact of phytase inclusion on renal CLDN-7 and -10 mRNA expression in pigs. However, the physiological function of both CLDN is under debate. A study using the LLC-PK1 cell line indicated that CLDN-7 forms a barrier for chloride but a paracellular channel for sodium [14]. By contrast, a murine kidney cell culture indicated that genetic deletion of CLDN-7 decreases permeability for both sodium and chloride [51]. A similar debate exists for CLDN-10, as it may confer a channel for sodium or chloride in the LLC-PK1 cell line depending on the spliced variants [52]. These results indicated that paracellular permeability to traverse certain minerals across the renal epithelial layer might be changed. However, it remains unknown which specific minerals are involved.

Conclusions

The Ca-related transporters and Ca-forming CLDN are downregulated in the kidney of pigs, but upregulated in the kidney of broilers, in response to incremental dietary Ca and/or microbial phytase content. Insights obtained from studies in mammals should not be extrapolated to poultry and *vice versa*. Unlike pigs where the kidneys play a central role in maintaining whole body Ca homeostasis, in chickens the intestine may be far more important for Ca homeostasis than the kidneys.

Acknowledgement

This research was conducted by Wageningen Livestock Research, within the framework of the public private partnership "Feed4Foodure" and partially funded by the Ministry of Economic Affairs (Policy Support Research project number BO-31.03-005-00). Mr L. Kruijt assisted with RT-qPCR analysis, and Y.X. Hu received a scholarship form the Chinese Scholarship Council (CSC) to study at Wageningen University & Research.

Author contributions

P. Bikker, M.M. van Krimpen, J. van Baal, W.H. Hendriks and Y.X. Hu designed and conducted the research; Y.X. Hu analysed the samples and data; J. van Baal designed and validated the RT-qPCR primers. Y.X. Hu, J. van Baal, P. Bikker and W.H. Hendriks interpreted the data and wrote the manuscript. All authors with the exception of the late M.M. van Krimpen have read and approved the final manuscript and declare to have no conflict of interest.

References

1. Rigo J, Pieltain C, Viellevoye R et al. (2018) Calcium and phosphorus homeostasis: pathophysiology. In *Neonatology: A Practical Approach to Neonatal Diseases*, pp. 333-353 [Buonocore G, Bracci R and Weindling M, editors]. Milano: Springer.

2. Blaine J, Chonchol M & Levi M (2015) Renal control of calcium, phosphate, and magnesium homeostasis. *Clin J Am Soc Nephrol* **10**, 1257-1272.

3. Proszkowiec-Weglarz M & Angel R (2013) Calcium and phosphorus metabolism in broilers: effect of homeostatic mechanism on calcium and phosphorus digestibility. *J Appl Poult Res* **22**, 609-627.

4. González-Vega JC & Stein HH (2016) Calcium transporters and gene expression and absorption of calcium in pigs. In *Phytate Destruction-Consequences for Precision Animal Nutrition*, pp. 217-223 [Walk CL, Kühn I, Stein HH, Kidd MT and Rodehutscord M, editors]. Wageningen, the Netherlands: Wageningen Academic Publishers.

5. Moor MB & Bonny O (2016) Ways of calcium reabsorption in the kidney. *Am J Physiol Renal Physiol* **310**, F1337-F1350.

6. Yelshanskaya MV, Nadezhdin KD, Kurnikova MG *et al.* (2020) Structure and function of the calcium-selective TRP channel TRPV6. *J Physiol* **599**, 2673-2697.

7. Kiela PR & Ghishan FK (2018) Molecular mechanisms of intestinal transport of calcium, phosphate, and magnesium. In *Physiology of the Gastrointestinal Tract (Sixth Edition)*, pp. 1405-1449 [Said HM, Ghishan FK, Kaunitz JD, Merchant JL and Wood JD, editors]. Cambridge: Academic Press.

8. Hernando N, Gagnon KB & Lederer ED (2020) Phosphate transport in epithelial and nonepithelial tissue. *Physiol Rev* **101**, 1–35.

9. Giovannini D, Touhami J, Charnet P *et al.* (2013) Inorganic phosphate export by the retrovirus receptor XPR1 in metazoans. *Cell Rep* **3**, 1866-1873.

10. Otani T & Furuse M (2020) Tight junction structure and function revisited. *Trends Cell Biol* **30**, 805-817.

11. Alexander RT, Rievaj J & Dimke H (2014) Paracellular calcium transport across renal and intestinal epithelia. *Biochem Cell Biol* **92**, 467-480.

12. Hou J, Renigunta A, Yang J *et al.* (2010) Claudin-4 forms paracellular chloride channel in the kidney and requires claudin-8 for tight junction localization. *Proc Natl Acad Sci U S A* **107**, 18010-18015.

13. Thongon N & Krishnamra N (2012) Apical acidity decreases inhibitory effect of omeprazole on Mg^{2+} absorption and claudin-7 and -12 expression in Caco-2 monolayers. *Exp Mol Med* **44**, 684-693.

14. Alexandre MD, Lu Q & Chen Y-H (2005) Overexpression of claudin-7 decreases the paracellular Cl^-conductance and increases the paracellular Na^+ conductance in LLC-PK1 cells. *J Cell Sci* **118**, 2683-2693.

15. Breiderhoff T, Himmerkus N, Stuiver M *et al.* (2012) Deletion of claudin-10 (Cldn10) in the thick ascending limb impairs paracellular sodium permeability and leads to hypermagnesemia and nephrocalcinosis. *Proc Natl Acad Sci U S A* **109**, 14241-14246.

16. Colston K & Feldman D (1982) 1,25-Dihydroxyvitamin D_3 receptors and functions in cultured pig kidney cells (LLC PK_1). Regulation of 24,25-dihydroxyvitamin D_3 production. *J Biol Chem* **257**, 2504-2508.

17. Symeou V, Leinonen I & Kyriazakis I (2014) Modelling phosphorus intake, digestion, retention and excretion in growing and finishing pigs: model description. *Animal* **8**, 1612-1621.

18. Jendza JA, Dilger RN, Sands JS *et al.* (2006) Efficacy and equivalency of an *Escherichia coli*-derived phytase for replacing inorganic phosphorus in the diets of broiler chickens and young pigs. *J Anim Sci* **84**, 3364-3374.

19. Goldstein DL & Braun EJ (1989) Structure and concentrating ability in the avian kidney. *Am J Physiol* **256**, R501-R509.

20. Dantzler WH (2016) Renal morphology. In *Comparative Physiology of the Vertebrate Kidney (Second Edition)*, pp. 7-36. NY: Springer.

21. CVB (2018) *Voedernormen Varkens en Voederwaarden Voedermiddelen voor Pluimvee (Feeding Standards, Feeding Advices and Nutritional Values of Feeding Ingredients of Poultry)*. Wageningen, the Netherlands: Federatie Nederlandse Diervoederketen.

22. Nygard AB, Jørgensen CB, Cirera S *et al.* (2007) Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. *BMC Mol Biol* **8**, 67.

23. Yue H, Lei X-W, Yang F-L *et al.* (2010) Reference gene selection for normalization of PCR analysis in chicken embryo fibroblast infected with H5N1 AIV. *Virol Sin* **25**, 425-431.

24. Fleet JC (2018) Regulation of intestinal calcium and phosphate absorption. In *Vitamin D (Fourth Edition)*, pp. 329-342 [Feldman D, editor]. CA: Academic Press.

25. Riccardi D & Brown EM (2009) Physiology and pathophysiology of the calcium-sensing receptor in the kidney. *Am J Physiol Renal Physiol* **298**, F485-F499.

26. Hannan FM, Kallay E, Chang W *et al.* (2018) The calcium-sensing receptor in physiology and in calcitropic and noncalcitropic diseases. *Nat Rev Endocrinol* **15**, 33–51.

27. Riccardi D & Valenti G (2016) Localization and function of the renal calcium-sensing receptor. *Nat Rev Nephrol* **12**, 414-425.

28. Prot-Bertoye C & Houillier P (2020) Claudins in renal physiology and pathology. *Genes* **11**, 290.

29. Fujita H, Sugimoto K, Inatomi S *et al.* (2008) Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca^{2+} absorption between enterocytes. *Mol Biol Cell* **19**, 1912-1921.

30. Hou J, Renigunta V, Nie M *et al.* (2019) Phosphorylated claudin-16 interacts with Trpv5 and regulates transcellular calcium transport in the kidney. *Proc Natl Acad Sci U S A* **116**, 19176-19186.

31. Hou J, Rajagopal M & Yu AS (2013) Claudins and the kidney. *Annu Rev Physiol* **75**, 479-501.

32. Diaz R, Hurwitz S, Chattopadhyay N *et al.* (1997) Cloning, expression, and tissue localization of the calcium-sensing receptor in chicken (*Gallus domesticus*). *Am J Physiol* **273**, R1008-R1016.

33. Pavlos NJ & Friedman PA (2017) GPCR signaling and trafficking: the long and short of it. *Trends Endocrinol Metab* **28**, 213-226.

34. Breitwieser GE (2013) The calcium sensing receptor life cycle: trafficking, cell surface expression, and degradation. *Best Pract Res Clin Endocrinol Metab* **27**, 303-313.

35. Bar A, Shani M, Fullmer CS *et al.* (1990) Modulation of chick intestinal and renal calbindin gene expression by dietary vitamin D_3, 1,25-dihydroxyvitamin D_3, calcium and phosphorus. *Mol Cell Endocrinol* **72**, 23-31.

36. Rosenberg J, Hurwitz S & Bar A (1986) Regulation of kidney calcium-binding protein in the bird (*Gallus domesticus*). *Comp Biochem Physiol A Comp Physiol* **83**, 277-281.

37. Bar A (2009) Differential regulation of calbindin in the calcium-transporting organs of birds with high calcium requirements. *J Poult Sci* **46**, 267-285.

38. Hunziker W & Schrickel S (1988) Rat brain calbindin D28: six domain structure and extensive amino acid homology with chicken calbindin D28. *Mol Endocrinol* **2**, 465-473.

39. Nemere I, Leathers VL, Thompson BS *et al.* (1991) Redistribution of calbindin-D28k in chick intestine in response to calcium transport. *Endocrinol* **129**, 2972-2984.

40. Christakos S & Norman AW (1980) Vitamin D-dependent calcium-binding protein synthesis by chick kidney and duodenal polysomes. *Arch Biochem Biophys* **203**, 809-815.

41. Karasawa Y (1989) Effect of colostomy on nitrogen nutrition in the chicken fed a low protein diet plus urea. *J Nutr* **119**, 1388-1391.

42. Karasawa Y & Maeda M (1994) Role of caeca in the nitrogen nutrition of the chicken fed on a moderate protein diet or a low protein diet plus urea. *Br Poult Sci* **35**, 383-391.

43. Biber J, Hernando N & Forster I (2013) Phosphate transporters and their function. *Annu Rev Physiol* **75**, 535-550.

44. Levi M & Gratton E (2019) Visualizing the regulation of SLC34 proteins at the apical membrane. *Pflugers Arch* **471**, 533-542.

45. Goltzman D, Mannstadt M & Marcocci C (2018) Physiology of the calcium-parathyroid hormone-vitamin D axis. In: *Vitamin D in Clinical Medicine. Front of Hormone Research* pp. 1-13 [Giustina A and Bilezikian JP, editors]. Basel: Karger publisher.

46. Tfelt-Hansen J & Brown EM (2005) The calcium-sensing receptor in normal physiology and pathophysiology: a review. *Crit Rev Clin Lab Sci* **42**, 35-70.

47. Levi M, Gratton E, Forster IC *et al.* (2019) Mechanisms of phosphate transport. *Nat Rev Nephrol* **15**, 482-500.

48. Hernando N, Forgo J, Biber J *et al.* (2000) PTH-induced downregulation of the type IIa Na/P_i-cotransporter is independent of known endocytic motifs. *Clin J Am Soc Nephrol* **11**, 1961-1968.

49. Ren Z, Yan J, Hu Q *et al.* (2020) Phosphorus restriction changes the expression of fibroblast growth factor 23 and its receptors in laying hens. *Front Physiol* **11**, 85.

50. Bergwitz C & Juppner H (2010) Regulation of phosphate homeostasis by PTH, vitamin D, and FGF23. *Annu Rev Med* **61**, 91-104.

51. Fan J, Tatum R, Hoggard J *et al.* (2019) Claudin-7 modulates Cl^- and Na^+ homeostasis and WNK4 expression in renal collecting duct cells. *Int J Mol Sci* **20**, 3798.

52. Van Itallie CM, Rogan S, Yu A *et al.* (2006) Two splice variants of claudin-10 in the kidney create paracellular pores with different ion selectivities. *Am J Physiol Renal Physiol* **291**, F1288-F1299.

CHAPTER 8

General discussion

In most of the grains and cereals, phosphorus (P) is predominantly stored in the form of inositol phosphates (IP) with variable degrees of phosphorylation [(1)]. To improve IP degradation and thereby overall dietary P absorption, a lower dietary calcium (Ca) supply [(3)] and inclusion of microbial phytase [(4)] has been intensively investigated in pigs and broilers. On the basis of previous studies, my research aimed to further clarify the impact of dietary Ca level, limestone particle size and microbial phytase inclusion on Ca and P absorption along the gastrointestinal tract (GIT), bone mineralisation or breaking strength, urinary Ca and P excretion as well as gene expression of Ca- and P-related transporters and claudins (CLDN) in the intestinal mucosa and kidney of pigs and broilers (Chapter 1). To achieve this goal, I first investigated the interaction effect between dietary Ca content and microbial phytase inclusion on Ca and P absorption along the GIT, their deposition in the metacarpal bone, excretion in the kidney (Chapter 2), as well as gene expression of Ca- and P-related transporters and CLDN (Chapter 3) in the jejunal and colonic mucosa of growing pigs. Thereafter, I studied the impact of dietary Ca content supplied via coarse or fine limestone on Ca and P absorption along the GIT, characteristics of tibia breaking strength (Chapter 4) and gene expression of Ca- and P-related transporters and CLDN (Chapter 5) in the duodenal and jejunal mucosa of broilers. Subsequently, I investigated the impact of dietary Ca content in the presence or absence of microbial phytase on Ca and P absorption along the small intestine and duodenal expression of Ca- and P-related transporters and CLDN in broilers (Chapter 6). The similar experimental design of the above-mentioned studies in broilers and pigs enabled comparison of renal expression of Ca- and P-related transporters and CLDN between pigs and broilers, as modulated by dietary Ca level, limestone particle size, and microbial phytase inclusion (Chapter 7). Here, I will summarize the major findings obtained in these six studies and compare the results between broilers and pigs.

Fine limestone is equally digestible to coarse limestone in broilers and pigs

The morphology and digestive enzyme system of GIT is rather different between pigs and poultry. Unlike mammals, poultry have a crop to transiently store and wet the ingested items, crop and gizzard to grind the ingesta and function as a real stomach, and a pair of caeca to ferment the precaecal indigestible components

and reflux in various sections of the intestine [5]. In addition, compared to pigs, broilers seem to have a higher specific activity of alkaline phosphatases and greater ratio of villus height to crypt depth in the small intestine, indicating a higher intrinsic potential to degrade IP and larger surface area per unit volume to absorb P compared to pigs (Chapter 1). Even so, the studies described in this thesis indicate that limestone particle size has a similar impact on intestinal Ca and P absorption in pigs and broilers.

Impacts of limestone particle size on intestinal Ca and P absorption in broilers

In contrast to the study described in Chapter 4 showing a similar apparent distal ileal Ca digestibility between coarse and fine limestone (51 vs. 49%), Anwar *et al.* [6] showed that coarse limestone was significantly better digestible than fine limestone in the distal ileum of broilers (70 vs. 42%). Similar to my findings, these authors also observed that limestone was segregated from the other digesta in the GIT and retained in the gizzard, which was more pronounced for the coarse compared to fine limestone. Because limestone requires to be solubilised in the GIT prior to mucosal Ca absorption, Anwar *et al.* [6; 7] assumed that a greater segregation of coarse compared to fine limestone in the gizzard indicated a longer mean retention time, thereby, allowing more Ca to be solubilised, released and subsequently absorbed in the small intestine. Indeed, it has been reviewed that the selective retention of particles in the gizzard results in some fine particles having a short gizzard retention time, and the threshold size for being constrained from leaving the gizzard in chickens is between 500 and 1500 µm [8]. As such, it is possible that fine limestone particles (<500 µm) may transmit from the gizzard without adequate solubilisation. Since limestone solubility is much lower in the small intestine compared to the gizzard due to the higher pH, it is conceivable that fine limestone, with a shorter retention time in the gizzard compared to coarse limestone, may hamper Ca solubilisation and mucosal Ca absorption in the small intestine.

On the other hand, *in vitro* studies have shown that fine limestone is more soluble than coarse limestone. For instance, Anwar *et al.* [7] indicated that *in vitro* limestone solubility was almost twice for the fine compared to coarse limestone at 10 min of incubation in 0.2 N hydrochloric acid (0.60 vs. 0.33). Their finding is in

line with Kim *et al.* [9], who demonstrated that fine limestone was better soluble than coarse limestone within 20 min of incubation in 0.2 N HCl and 3M glycine buffer solution (**Figure 1**). Moreover, this difference in limestone solubility gradually diminished with time and disappeared after 20 min of incubation. It is conceivable that fine limestone may increase the surface area available to the gastric acid and stimulate Ca release per unit of time in the gizzard. As such, the effect of particle size on limestone solubilisation in the gizzard seems to be double-edged, since fine compared to coarse limestone can reduce gizzard retention time while enhance limestone solubilisation per unit of time.

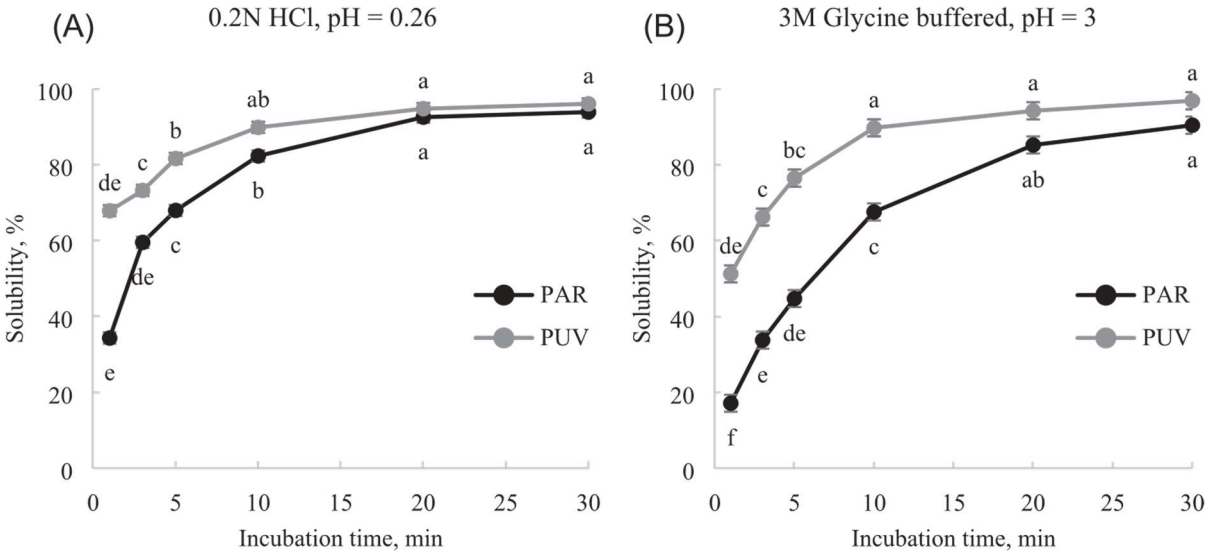


***Figure 1**. In vitro limestone solubility of two limestone samples (PAR and PUV) from the same origin but different particle sizes in two different solutions, (A) 0.2 N HCl and (B) pH3-buffered solution (mean±SEM) from Kim et al. [9]. Measured starting pH was 0.26 and 3, for 0.2 N HCl and pH3-buffered solution, respectively. [a-f] Data points with different superscript letters differ (P<0.05). PAR=particulate limestone (geometric mean diameter (GMD)=402 µm); PUV=pulverized limestone (GMD<75 µm).*

The discrepancy between the study described in Chapter 4 and Anwar *et al.* [6; 7] may be caused by the different coarse limestone used. Particle size was <500 vs. 500-2000 µm for the fine and coarse limestone in study of Chapter 4, and <500 vs. 1000-2000 µm in the study of Anwar *et al.* [6; 7]. It was conceivable that the larger particle size of coarse limestone (1000-2000 µm) in the latter studies might result in a greater retention of limestone particles in the gizzard, thereby, increasing limestone exposure to the gastric acid and stimulating mucosal Ca absorption in the small intestine. As such, Ca digestibility in the distal ileum of broilers was significantly higher for the coarse than fine limestone in their studies (70 vs. 42%). The coarse limestone used in the study of Chapter 4 was relatively smaller (500-2000 µm), which might reduce limestone retention in the gizzard and

stimulated limestone particles transmit into the small intestine. The distal ileal Ca digestibility of broilers fed coarse limestone, therefore, was lower in the study of Chapter 4 and not significantly different from the fine limestone particularly at a low Ca intake (51 vs. 49%).

It is noteworthy that diets were supplied in pellets in the study described in Chapter 4, while mash diets were used by Anwar *et al.* [6; 7]. As described in Chapter 4, in the pelleted feed, ash recovery of the coarse particles added to the diets was 76%, which confirmed the presence of coarse limestone after pelleting. To validate this conclusion, a small study was conducted where the fine, coarse and very coarse limestone (Faunacal®, Wülfrath, Germany) was incinerated at 550 °C for 9 h, either by itself or when included in a mash diet (**Textbox 1**). Results indicated 78 and 84% recovery of coarse and very coarse particles in the ash of the mash diets, similar to the 76% recovery of coarse particles in the ash of pelleted diets (Chapter 4). Thus, the observed recovery of 76% may be caused by the incineration process while pelleting as used in my study appears to have limited impact on limestone particle size.

***Textbox 1**: In the feed ash of the coarse limestone diets, 76% of the added particles could be recovered (Chapter 4). To clarify the impact of incineration on limestone particle size, fine, coarse and very coarse limestone (Faunacal®, Wülfrath, Germany) were incinerated in the oven at 550 °C for 9 h in pure form and in mash diets. Using dry sieve analysis [2], the determined geometric mean diameter (GMD) of limestone before and (after) incineration was (µm): fine 437 (418), coarse 1256 (1302), very coarse 1883 (1754). In the ash of the mash diets made of 35.4% maize, 30.0% wheat, 25.5% soybean meal, 3.6% soybean oil and supplemented with 2.2% limestone, recovery of the added fine, coarse and very coarse limestone particles was 90, 78 and 84%, respectively.*

Of note is that the determination of distal ileal Ca digestibility in broilers may be affected by the selective retention of particles in the gizzard. In particular, distal ileal Ca digestibility may be overestimated if digesta samples are collected prior to limestone particles transmit from the gizzard. Thus, steady-state condition may affect determination of Ca digestibility especially for the coarse limestone with a greater retention in the gizzard. In the study of Chapter 4, experimental diets were provided for 6 days with a continuous lighting schedule (24L:0D) on the last 3 days to ensure a steady-state passage of digesta in the GIT of broilers at dissection. The steady-state condition was also confirmed by that substantial digesta were collected from the crop.

In contrast to the limited impact of limestone particle size on distal ileal Ca digestibility, coarse compared to fine limestone significantly enhanced cumulative

P absorption in the distal ileum of broilers (60 vs. 57%, Chapter 4). As reported in Chapter 4, it was assumed that an acute and steep Ca release of fine limestone probably promoted Ca-IP complexation in the proventriculus and gizzard, thereby, hampering endogenous mucosal phosphatases to catalyse IP degradation in the small intestine. The cumulative P absorption in the distal ileum of broilers, therefore, was reduced in the presence of fine limestone. These findings are in line with an earlier *in vitro* study to determine the impact of limestone particle size (28-1306 µm) on Ca solubility and IP degradation [10], which indicated that the finest limestone (28 µm), with its higher Ca solubility, displayed the greatest inhibition on microbial phytase to catalyse *in vitro* IP hydrolysis.

Impact of limestone particle size on Ca and P absorption in pigs

Similar to broilers, study in pigs indicated that incremental limestone particle size had no impact on apparent total tract digestibility (ATTD) of Ca while it significantly increased ATTD of P (geometric mean diameter (GMD) of limestone particle size ranged 34-1883 µm, mash diets were used, Bikker *et al.* [11] unpublished data). Moreover, the latter authors also observed that incremental limestone particle size stimulated limestone particle segregation from the digesta and increased Ca retention in the stomach of pigs. It also significantly increased apparent Ca digestibility in the proximal and distal small intestine but not in the large intestine of pigs. It was conceivable that this fluctuation of Ca digestibility might be caused by a non-steady state passage of limestone through the GIT. In line with Bikker *et al.* [11], Merriman and Stein [12] reported that limestone particle size (200–1125 µm) had no impact on ATTD of Ca in growing pigs. On the other hand, the latter study [12] indicated that ATTD of P was also independent of limestone particle size hence conflicting Bikker *et al.* [11]. Of note is that Merriman and Stein [12] obtained their limestone sources by grinding four limestone batches, collected from the same limestone mine, to four different particle sizes. Hence there might be a distribution with some finer and coarser particles in each limestone source in the study of Merriman and Stein [12]. In contrast, limestone sources of different particle sizes were separated from the same limestone batch via sieving in the study of Bikker *et al.* [11].

Ca and P absorption in the GIT of broilers and pigs

The negative impact of a high dietary Ca intake on intestinal P absorption has been intensively studied in broilers and pigs. Moreover, this negative impact of Ca is generally attributed to Ca-IP and Ca-P complexation (for review see Wilkinson *et al.* [13]). Studies in pigs and poultry have demonstrated that microbial phytase inclusion increases dietary IP degradation (e.g. Kriseldi *et al.* [14] and Rosenfelder-Kuon *et al.* [15]) and enhances Ca and P absorption (e.g. Ravindran *et al.* [16] and Li *et al.* [17]). The improved Ca absorption may allow a lower dietary Ca supply, which in turn, may enhance phytase efficacy to catalyse IP degradation. Interestingly, the interaction effect between dietary Ca content and microbial phytase inclusion on Ca and P absorption was different between pigs and broilers.

Modulation of dietary Ca and phytase intake on intestinal Ca and P absorption

As described in Chapter 6, apparent P absorption in the distal ileum of broilers was linearly reduced with incremental dietary Ca intake. Moreover, this reduction was less pronounced in diets supplemented with microbial phytase. In line with these observations, Tamim *et al.* [18] reported that dietary limestone inclusion reduced ileal IP degradation to a lesser extent in broilers fed microbial phytase supplemented diets. Accordingly, apparent P absorption in the ileum of broilers was decreased less in the phytase-supplemented diet in the study of Tamim *et al.* [18]. Furthermore, dietary phytase inclusion also increased apparent Ca absorption in the distal ileum of broilers at a low Ca intake (i.e. Ca/rP of 1.3 and 1.8; Chapter 6). However, irrespective of microbial phytase inclusion, broilers fed the lowest Ca content diet had the lowest body weight gain and highest feed conversion ratio. As such, it can be concluded that dietary phytase inclusion alleviates the negative impact of incremental dietary Ca intake on intestinal P absorption but cannot restore the compromised growth performance in broilers fed a Ca-deficient diet.

In contrast to broilers, the negative impact of dietary Ca increment on intestinal P absorption was more pronounced in pigs fed diets supplemented with microbial phytase (Chapter 2). Thus, microbial phytase inclusion alleviates the negative impact of dietary Ca intake on apparent P absorption in broilers, while it enhances this impact in pigs. This difference may be caused by that broilers have a greater intrinsic potential to catalyse IP degradation and absorb IP-P compared to pigs. More specific, apparent P digestibility was high in the distal ileum of broilers

fed the lowest Ca diets without microbial phytase inclusion (64 and 79% in Chapter 4 and 6, respectively; dietary Ca content approximately 3 g/kg, P content 6 and 5 g/kg in Chapter 4 and 6, respectively). In contrast, apparent P digestibility was much lower in the distal small intestine of pigs fed the lowest Ca diet without microbial phytase inclusion (26% in Chapter 2; dietary Ca and P content 2 and 5 g/kg, respectively). Given that mineral P digestibility is rather similar between pigs and poultry (monosodium phosphate (MSP): 89 vs. 91%; monocalcium phosphate (MCP): 83 vs. 85% in pigs and broilers, respectively; CVB [19]), the much higher apparent P digestibility in the distal small intestine of broilers appears to indicate a greater intrinsic potential to degrade IP and absorb IP-P in broilers compared to pigs. This assumption is in line with a literature survey from Rodehutscord and Rosenfelder [20], showing that receiving diets based on corn and soybean meal with a low phytase activity, inositol hexakisphosphate (IP6) degradation was approximately 70% in the ileum of broilers while it hardly exceeded 40% in pigs.

A greater potential to release and absorb IP-P might have masked the impact of microbial phytase inclusion on P absorption in broilers. Indeed, microbial phytase inclusion markedly increased IP6 degradation from 6 to 79% in the distal small intestine of pigs (Chapter 2). Accordingly, apparent P digestibility in the distal small intestine of pigs was almost doubled in the presence of microbial phytase (24 vs. 54%, Chapter 2). By contrast, microbial phytase inclusion increased apparent P digestibility to a lesser extent in the distal ileum of broilers (62 vs. 80%, Chapter 6). Thus, microbial phytase inclusion seems to have a greater impact on P absorption in pigs than poultry, which is in line with systemic reviews and meta-analysis based on previously published results. In particular, meta-analysis studies indicated that microbial phytase inclusion (1000 FTU/kg) enhanced P absorption by 25.6% unit in pigs [21] and only by 8.6% unit in broilers [22].

Site of Ca and P absorption

Previous studies focused mainly on Ca and P absorption at the distal ileum of broilers or total tract of pigs, an approach which does not provide insight into Ca and P absorption kinetics along the GIT. Thus, I measured Ca and P absorption along the GIT of pigs and broilers under steady-state condition. Results indicated that Ca and P were predominantly absorbed in the proximal small intestine, i.e. anterior to the ileum in broilers (Chapter 4 and 6). By contrast, P was primarily absorbed in the

distal small intestine of pigs particularly in the absence of microbial phytase (Chapter 2). Moreover, dietary phytase supplementation increased apparent P absorption in the proximal small intestine of pigs (Chapter 2). On the other hand, dietary phytase inclusion reduced the amount of IP available for the microbiota residing in the colon to release Ca and P ions from the IP complex for colonic absorption. Consequently, post-ileal Ca and P absorption was greater for pigs without microbial phytase inclusion (Chapter 2). The Ca- and P-related uptake and efflux transporters were abundantly expressed in the colon of pigs, confirming that Ca and P could be absorbed in the colon probably via a transcellular pathway (Chapter 3). Thus, other GIT segments besides the small intestine may also substantially contribute to Ca and P absorption. Post-ileal Ca and P absorption in broilers was not measured in the studies described in this thesis due to cloacal Ca and P secretion.

Intestinal expression of Ca- and P-related transporters and CLDN

Interestingly, incremental dietary Ca intake reduced apparent P digestibility in the distal ileum of broilers, while P solubility along the GIT (gizzard, jejunum, ileum) was not significantly affected by dietary Ca content or limestone particle size (Chapter 4). This finding indicates that Ca-IP and Ca-P complexation might not be the only mechanism by which Ca reduces P absorption in the GIT of broilers. Indeed, the dietary Ca-induced downregulation of P transporter (NaPi-IIb) in the duodenal and jejunal mucosa of broilers was another important mechanism for dietary Ca reducing mucosal P absorption (Chapter 5 and 6). An explanation behind this downregulation might be that incremental dietary Ca intake elevated serum Ca concentration, which could activate extracellular Ca sensing receptor (CaSR) in the parathyroid gland and signal a reduction in parathyroid hormone (PTH) expression and secretion, thereby, decreasing serum 1,25-dihydroxycalciferol ($1,25(OH)_2D_3$, the bioactive form of vitamin D_3) concentration [23; 24]. Expression of both Ca- and P-related transporters in the duodenal and jejunal mucosa of broilers, therefore, was downregulated since the promoter of these genes contains a vitamin D_3 receptor (VDR) responsive element [25]. Thus, modulation on expression of P-related transporters is, at least in part, an important mechanism of dietary Ca reducing P absorption in the GIT of broilers.

Unlike broilers which displayed abundant level of NaPi-IIb mRNA in both duodenal and jejunal mucosa, NaPi-IIb mRNA in the porcine GIT was below the

limit of detection (Chapter 3). In addition, irrespective of dietary Ca content and microbial phytase inclusion, soluble inorganic P concentration in the serum of pigs was much lower than that in the GIT lumen (Chapter 3), allowing mucosal P uptake down an electrochemical gradient. Thus, it is tempting to speculate that P might be predominantly absorbed via the paracellular pathway in the GIT of pigs. This assumption is in line with an earlier pig study from Stein *et al.* [26], indicating that ATTD of P in MCP was independent on dietary MCP inclusion level (**Table 1**). Furthermore, incremental dietary Ca intake reduced ATTD of P in pigs, accompanied by a reduction in ileal IP degradation and inorganic P solubility (Chapter 2). Thus, it can be concluded that a higher Ca intake reduces P absorption via reducing dietary IP degradation and precipitation of P in the GIT of pigs.

Table 1. Apparent total tract digestibility (ATTD) of phosphorus (P) as affected by incremental dietary monocalcium phosphate (MCP) inclusion in pigs [1]

Item	Dietary MCP inclusion, g/kg						*P*-value
	0.0	3.4	6.8	10.2	13.6	17.0	
Dietary P content, g/kg	2.6	3.2	3.9	4.7	5.3	6.4	-
Dietary Ca content, g/kg	4.2	4.7	5.5	6.9	7.2	8.4	-
ATTD of P, %	38.4	48.8	54.6	58.9	60.1	65.2	<0.001
MCP digestibility, %	-	88.5	85.0	82.8	79.5	83.5	0.35

[1] Data from Stein *et al.* [26].
Diet consisted of corn (54%), corn starch (23%) and soybean meal (20%).

Paracellular Ca and P absorption is less well documented compared to their transcellular route. Recent studies have shown that CLDN can form pores selectively permeable to small ions and may be involved in paracellular ion absorption [27; 28]. Although there are a few studies investigating the gene expression of tight junction proteins (e.g. occludin, zonula occludens-1, -2 and -3) in the GIT of pigs [29-31] and broilers [32; 33], regulation of their expression, as modulated by dietary intervention, has not been reported in pigs and broilers before. I investigated the intestinal expression levels of CLDN-2, -12 and -16 in the pig and broiler studies, since it was postulated that these CLDN members are involved in paracellular absorption of Ca in the intestine and/or kidney of mice [34; 35]. Results indicate that the mRNA expression of CLDN-2 and -12 was downregulated with incremental dietary Ca intake in the duodenal and jejunal mucosa of broilers, which was accompanied by a downregulated expression of Ca-

related transporters (CaSR, CaBP-D28k and PMCA1, Chapter 5 and 6). These observations on Ca-related transporters and CLDN are in agreement with the measured apparent Ca digestibility in the GIT of broilers (Chapter 4 and 6), indicating that these transporters and CLDN contribute to the observed effect of dietary Ca level and phytase on Ca absorption in broilers.

Unlike broilers, incremental dietary Ca intake and microbial phytase inclusion increased expression of CLDN-2 and -12 and downregulated TRPV5, TRPV6 and CaBP-D9k in the jejunal and/or colonic mucosa of pigs (Chapter 3). The concentration gradient of soluble inorganic Ca across the porcine enterocyte was negative upon low but positive with high dietary Ca intake. Thus, it appears that Ca absorption might be switched from a transcellular to paracellular pathway with increasing dietary Ca content in the GIT of pigs (Chapter 3). It is conceivable that in pigs fed a low Ca diet, creating a negative concentration gradient of Ca ion across the porcine enterocyte, expression of CLDN-2 was downregulated to prevent Ca efflux via tight junction complexes. In reverse, a high Ca diet upregulated the expression of CLDN-2 to favour the mucosal crossing of luminal Ca along a positive electrochemical gradient (Chapter 3). Furthermore, the downregulation of Ca transporters (TRPV5, TRPV6 and CaBP-D9k) in the GIT of pigs fed high dietary Ca was as expected, since a higher Ca intake also reduced serum $1,25(OH)_2D_3$ (Chapter 3), probably via the above described parathyroid–kidney axis [36]. Mucosal expression of Ca transporters (TRPV5, TRPV6 and CaBP-D9k), therefore, was downregulated since their promoters contain VDR elements [37].

Expression of Ca and P transporters and CLDN in kidney

As mentioned above, a dilemma for the animals is that the surplus of absorbed Ca and P above requirement cannot be retained in the soft tissue or form hydroxyapatite in the bone. Rather, this surplus needs to be eliminated via urine by downregulating selective reabsorption processes in the kidney. The kidney is generally considered to play a central role in the maintenance of whole body Ca and P homeostasis [38].

Renal expression of Ca- and P-related transporters in poultry

Because of the difficulty to separate urine from faeces in poultry, digestible P content in feed ingredients is determined and expressed as retainable P or distal ileal digestible P in some feed composition databases (e.g. CVB [19]). Although there are a few studies investigating urinary Ca and P excretion using surgically colostomized broilers fed diets varying in limestone particle size or inclusion level (e.g. Manangi *et al.* [39; 40]), these studies are restrictive by the very small number of replications (one replicate bird per treatment group). Since both Ca and P are reabsorbed via transporters and CLDN in the kidney, the expression of Ca- and P-related transporters and CLDN in the kidney of broilers was quantified as an indicator of urinary Ca and P excretion. Of note is the upregulation of the Ca-related transporters and CLDN with incremental dietary Ca intake in the kidney of broilers (Chapter 7). This observation is in line with Rosenberg *et al.* [41] and Bar *et al.* [42], who reported that a higher Ca intake increased protein expression of CaBP-D28k in the kidney of chickens. Thus, an increase in dietary Ca intake enhanced renal expression of Ca-related transporters and CLDN in broilers.

Whole body Ca homeostasis is maintained via altering Ca absorption in the GIT, deposition and resorption in the bone and urinary excretion by the kidney [43]. In case of a higher Ca intake in broilers accompanied with a decreased intestinal P absorption, surplus of serum Ca could not be retained in bone due to limited P availability or above Ca requirement, nor could it be eliminated via urine in the kidney as indicated by the elevated renal expression of Ca-related transporters and CLDN at a higher Ca intake. The reduced apparent Ca digestibility in the distal ileum (Chapter 4 and 6) and downregulated expression of Ca-related transporters and CLDN in the duodenal and jejunal mucosa (Chapter 5 and 6), therefore, appears to play a critical role to maintain serum Ca concentration within the normal physiological range in broilers. This is in line with an earlier study of Christakos and Norman [44] showing that in chickens, vitamin D_3 treatment stimulated CaBP-D28k mRNA expression eight times more in the duodenal mucosa than kidney. These findings suggest that the GIT has a more important role than previously considered in controlling whole body Ca homeostasis in this avian species (**Figure 2**).

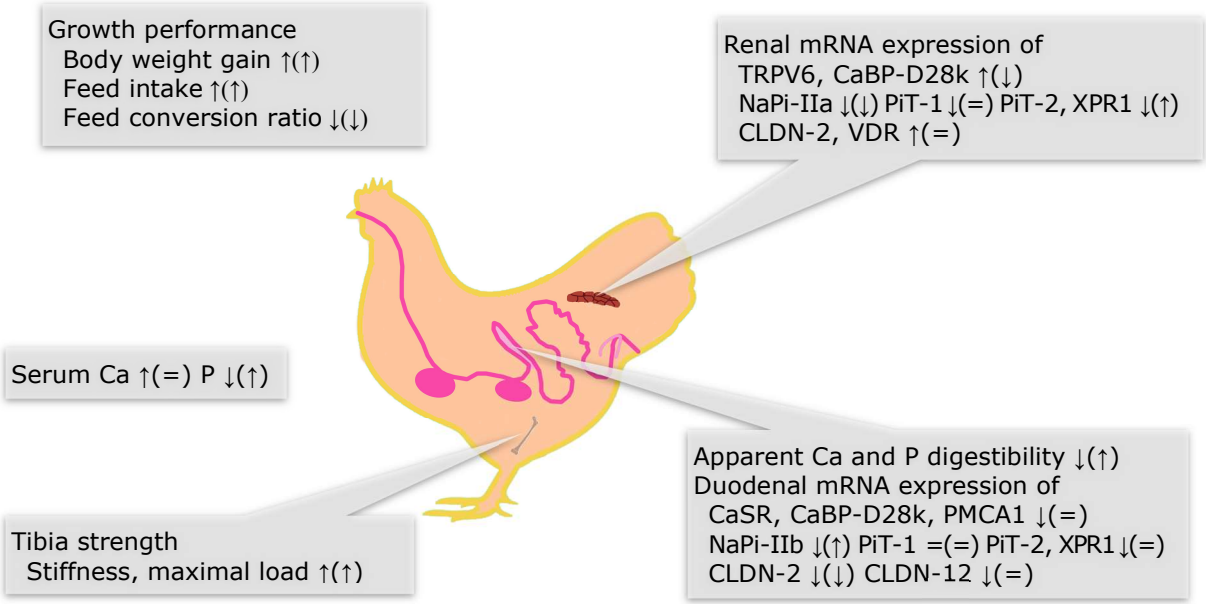


***Figure 2**. Schematic illustration of the effects of increasing dietary Ca intake and microbial phytase inclusion on growth performance, characteristics of tibia breaking strength, apparent Ca and P digestibility, mRNA expression of Ca- and P-related transporters and CLDN in the intestinal mucosa and kidney of broilers. Results are shown as a positive "↑", negative "↓" or no "=" effect of incremental dietary Ca intake (and microbial phytase inclusion in parenthesis) on the measured parameters. Ca, calcium; CaBP-D28k, calbindin D28k; CLDN, claudin; NaPi-IIa, sodium dependent phosphate transporter type IIa; NaPi-IIb, sodium dependent phosphate transporter type IIb; PMCA1, plasma membrane Ca-ATPase 1; P, phosphorus; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; TRPV6, transient receptor potential cation channel subfamily V member 6; XPR1, xenotropic and polytropic retrovirus receptor 1.*

Incremental dietary Ca intake decreased serum P concentration (Chapter 4 and 6), which was accompanied by a reduced expression of P-related transporters in the intestinal mucosa (Chapter 5 and 6) and kidney (Chapter 7) of broilers. Thus, expression of P transporters in the GIT and kidney was positively correlated to serum P concentration in broilers. This finding is in line with previous studies, showing a positive correlation between serum P concentration and NaPi-IIb mRNA expression in the GIT [45] or NaPi-IIa protein expression in the kidney [46] in laying hens. A mechanism behind this positive correlation might be that incremental dietary Ca intake, with the elevated serum Ca concentration, reduced serum $1,25(OH)_2D_3$ concentration probably via the above described parathyroid-kidney axis in broilers. Expression of P-related transporters in the GIT and kidney, therefore, was downregulated since their promoter contains a VDR responsive element [25].

Renal expression of Ca- and P-related transporters in pigs

In contrast to broilers, pigs showed a negative correlation between incremental dietary Ca intake and expression of Ca-related transporters and CLDN in their

kidney (Chapter 7). A lower renal expression of Ca-related transporters and CLDN presumably reduced Ca reabsorption, which is in line with the increased urinary Ca excretion in pigs fed a higher Ca diet (Chapter 2). In support of these findings, Gonzalez-Vega *et al.* [47] reported a linear reduction of TRPV6 and PMCA1 mRNA expression in the kidney of pigs fed diets with a higher Ca content. This regulation of Ca transporters and CLDN expression may be 1,25$(OH)_2D_3$ and PTH dependent, via the above described parathyroid-kidney axis. In addition to the systemic regulations via 1,25$(OH)_2D_3$, a higher Ca intake might also directly downregulate expression of Ca-related transporters and CLDN via activating the extracellular Ca sensing receptor (CaSR), a G-protein-coupled transmembrane receptor which is abundantly expressed in the renal tubules [48]. Thus, in line with many previous studies in rodents (e.g. Renkema *et al.* [49]), the kidneys play a pivotal role in whole body Ca and P homeostasis in pigs (**Figure 3**).

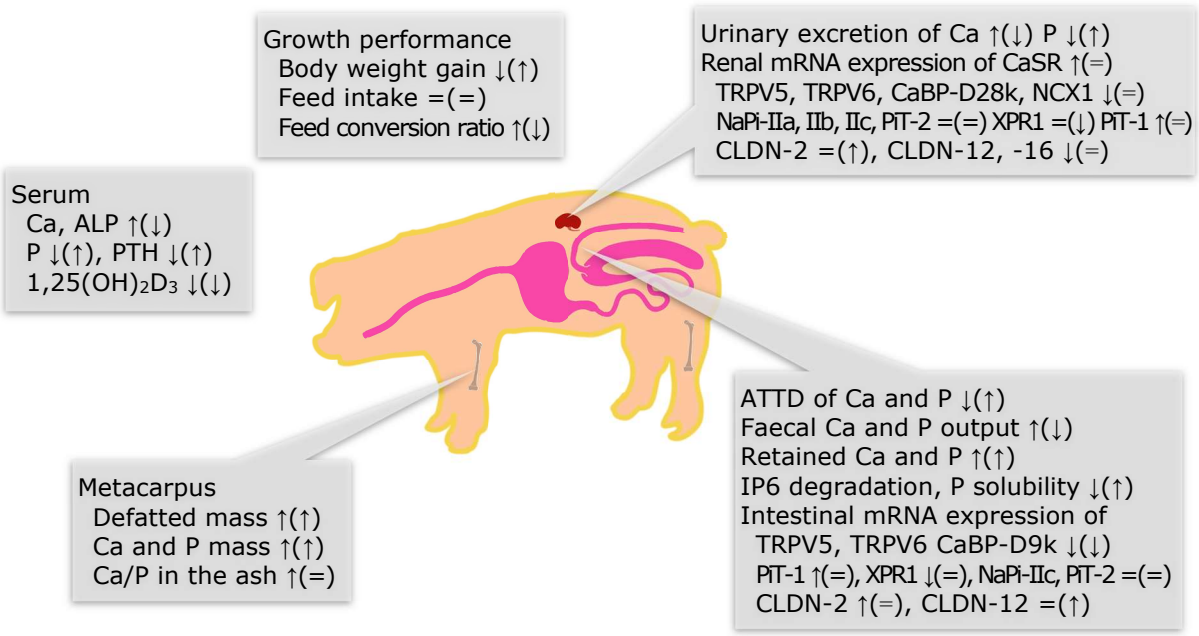


***Figure 3**. Schematic illustration of the effects of increasing dietary Ca intake and microbial phytase inclusion on growth performance, total tract Ca and P absorption and retention, IP6 degradation and inorganic P solubility, metacarpal composition, serum characteristics and mRNA expression of Ca and P-related transporters and CLDN in the intestinal mucosa and kidney cortex of pigs. Results are shown as a positive "↑", negative "↓" or no "=" effect of incremental dietary Ca intake (and microbial phytase inclusion in parenthesis) on the measured parameters. 1,25$(OH)_2D_3$, 1,25-dihydroxycalciferol; ALP, alkaline phosphatases; ATTD, apparent total tract digestibility; Ca, calcium; CaBP-D9k, calbindin D9k; CaBP-D28k, calbindin D28k; CaSR, Ca sensing receptor; CLDN, claudin; IP6, inositol hexakisphosphate; NaPi-IIa, sodium dependent phosphate transporter type IIa; NaPi-IIb, sodium dependent phosphate transporter type IIb; NaPi-IIc, sodium dependent phosphate transporter type IIc; NCX1, sodium-Ca exchanger; P, phosphorus; PiT-1, inorganic phosphate transporter 1; PiT-2, inorganic phosphate transporter 2; PTH, parathyroid hormone; TRPV5, transient receptor potential cation channel subfamily V member 5; TRPV6, transient receptor potential cation channel subfamily V member 6; XPR1, xenotropic and polytropic retrovirus receptor 1.*

Summary

- Limestone particle size has limited impact on distal ileal Ca absorption in broilers. Coarse compared to fine limestone improves intestinal P absorption in broilers, although this impact is relatively small.
- Dietary phytase inclusion alleviates the negative impact of incremental Ca intake on intestinal P absorption in broilers, but enhances this impact in pigs.
- Calcium induced downregulation of P-related transporters in the duodenal and jejunal mucosa is an important mechanism by which dietary Ca can reduce intestinal P absorption in broilers. In pigs, dietary Ca intake reduces intestinal P absorption via decreasing IP degradation and lowering P solubility.
- Incremental dietary Ca intake reduces expression of Ca transporters and CLDN in the duodenal and jejunal mucosa of broilers. In pigs, increasing Ca intake reduces expression of Ca transporters but increases Ca permeable CLDN in the jejunal mucosa, indicating a switch from the transcellular to paracellular Ca absorption.
- Serum Ca concentration is positively correlated to renal expression of Ca-related transporters and CLDN in broilers, and negatively correlated in pigs, indicating that the GIT plays a preeminent role in maintaining whole body Ca homeostasis in broilers, while this role is granted to the kidneys in pigs.
- In general, insights obtained in pig studies cannot be extrapolated to poultry and *vice versa* at least when it comes to dietary Ca and P metabolism and homeostasis.

References

1. Rodehutscord M, Ruckert C, Maurer HP *et al.* (2016) Variation in chemical composition and physical characteristics of cereal grains from different genotypes. *Arch Anim Nutr* **70**, 87-107.

2. ASAE (2008) Method of determining and expressing fineness of feed materials by sieving. In: *ASABE Standards (Standard No. S319.4)*. MI: St. Joseph.

3. Selle PH, Cowieson AJ & Ravindran V (2009) Consequences of calcium interactions with phytate and phytase for poultry and pigs. *Livest Sci* **124**, 126-141.

4. Lei XG, Weaver JD, Mullaney E *et al.* (2013) Phytase, a new life for an "old" enzyme. *Annu Rev Anim Biosci* **1**, 283-309.

5. Svihus B (2014) Function of the digestive system. *J Appl Poult Res* **23**, 306-314.

6. Anwar MN, Ravindran V, Morel PC *et al.* (2016) Effect of limestone particle size and calcium to non-phytate phosphorus ratio on true ileal calcium digestibility of limestone for broiler chickens. *Br Poult Sci* **57**, 707-713.

7. Anwar MN, Ravindran V, Morel PC *et al.* (2017) Effect of calcium source and particle size on the true ileal digestibility and total tract retention of calcium in broiler chickens. *Anim Feed Sci Technol* **224**, 39-45.

8. Svihus B (2011) The gizzard: function, influence of diet structure and effects on nutrient availability. *World Poultry Sci J* **67**, 207-224.

9. Kim SW, Li W, Angel R *et al.* (2019) Modification of a limestone solubility method and potential to correlate with *in vivo* limestone calcium digestibility. *Poult Sci* **98**: 6837-6848.

10. Manangi MK & Coon CN (2007) The effect of calcium carbonate particle size and solubility on the utilization of phosphorus from phytase for broilers. *Int J Poult Sci* **6**, 85-90.

11. Bikker P, Mens A, He Z *et al.* (2021) Influence of limestone particle size on calcium and phosphorus utilisation in pigs. Wageningen UR Livestock Research, Wageningen, the Netherlands (unpublished report).

12. Merriman LA & Stein HH (2016) Particle size of calcium carbonate does not affect apparent and standardized total tract digestibility of calcium, retention of calcium, or growth performance of growing pigs. *J Anim Sci* **94**, 3844-3850.

13. Wilkinson SJ, Selle PH, Bedford MR *et al.* (2011) Exploiting calcium-specific appetite in poultry nutrition. *Worlds Poult Sci J* **67**, 587-598.

14. Kriseldi R, Johnson JA, Walk CL *et al.* (2021) Influence of exogenous phytase supplementation on phytate degradation, plasma inositol, alkaline phosphatase, and glucose concentrations of broilers at 28 days of age. *Poult Sci* **100**, 224-234.

15. Rosenfelder-Kuon P, Klein N, Zegowitz B *et al.* (2020) Phytate degradation cascade in pigs as affected by phytase supplementation and rapeseed cake inclusion in corn-soybean meal-based diets. *J Anim Sci* **98**, skaa053.

16. Ravindran V, David LS, Abdollahi MR *et al.* (2020) True ileal calcium digestibility in soybean meal and canola meal, and true ileal phosphorous digestibility in maize-soybean meal and maize-canola meal diets, without and with microbial phytase, for broiler growers and finishers. *Br Poult Sci* **62**, 293-303.

17. Li W, Angel R & Plumstead PW *et al.* (2020) Effects of limestone particle size, phytate, calcium source, and phytase on standardized ileal calcium and phosphorus digestibility in broilers. *Poult Sci* **100**, 900–909.

18. Tamim NM, Angel R & Christman M (2004) Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. *Poult Sci* **83**, 1358-1367.

19. CVB (2018) *Chemische Samenstellingen en Nutritionele Waarden van Voedermiddelen (Chemical Compositions and Nutritional Values of Feed Ingredients).* Wageningen, the Netherlands: Federatie Nederlandse Diervoederketen.

20. Rodehutscord M & Rosenfelder P (2016) Chapter 1 Update on phytate degradation pattern in the gastrointestinal tract of pigs and broiler chickens. In *Phytate Destruction-Consequences for Precision Animal Nutrition*, pp. 15-28 [Walk CL, Kühn I, Stein HH, Kidd MT and Rodehutscord M, editors]. Wageningen, the Netherlands: Wageningen Academic Publishers.

21. Rosenfelder-Kuon P, Siegert W & Rodehutscord M (2020) Effect of microbial phytase supplementation on P digestibility in pigs: a meta-analysis. *Arch Anim Nutr* **74**, 1-18.

22. Bougouin A, Appuhamy JA, Kebreab E *et al.* (2014) Effects of phytase supplementation on phosphorus retention in broilers and layers: a meta-analysis. *Poult Sci* **93**, 1981-1992.

23. Crenshaw TD, Rortvedt LA & Hassen Z (2011) Triennial Growth Symposium: A novel pathway for vitamin D-mediated phosphate homeostasis: implications for skeleton growth and mineralization. *J Anim Sci* **89**, 1957-1964.

24. Chang AR & Anderson C (2017) Dietary phosphorus intake and the kidney. *Annu Rev Nutr* **37**, 321-346.

25. Proszkowiec-Weglarz M & Angel R (2013) Calcium and phosphorus metabolism in broilers: effect of homeostatic mechanism on calcium and phosphorus digestibility. *J Appl Poult Res* **22**, 609-627.

26. Stein HH, Kadzere CT, Kim SW *et al.* (2008) Influence of dietary phosphorus concentration on the digestibility of phosphorus in monocalcium phosphate by growing pigs. *J Anim Sci* **86**, 1861-1867.

27. Günzel D (2017) Claudins: vital partners in transcellular and paracellular transport coupling. *Pflugers Arch* **469**, 35-44.

28. Zihni C, Mills C, Matter K *et al.* (2016) Tight junctions: from simple barriers to multifunctional molecular gates. *Nat Rev Mol Cell Biol* **17**, 564-580.

29. Lagos LV, Lee SA, Fondevila G *et al.* (2019) Influence of the concentration of dietary digestible calcium on growth performance, bone mineralization, plasma calcium, and abundance of genes involved in intestinal absorption of calcium in pigs from 11 to 22 kg fed diets with different concentrations of digestible phosphorus. *J Anim Sci Biotechnol* **10**, 47.

30. Metzler-Zebeli BU, Mann E, Ertl R *et al.* (2015) Dietary calcium concentration and cereals differentially affect mineral balance and tight junction proteins expression in jejunum of weaned pigs. *Br J Nutr* **113**, 1019-1031.

31. Lu H, Shin S, Kuehn I *et al.* (2020) Effect of phytase on nutrient digestibility and expression of intestinal tight junction and nutrient transporter genes in pigs. *J Anim Sci* **98**, 1-12.

32. Gloux A, Le Roy N, Brionne A *et al.* (2019) Candidate genes of the transcellular and paracellular calcium absorption pathways in the small intestine of laying hens. *Poult Sci* **98**, 6005-6018.

33. Proszkowiec-Weglarz M, Schreier LL, Kahl S *et al.* (2020) Effect of delayed feeding post-hatch on expression of tight junction- and gut barrier-related genes in the small intestine of broiler chickens during neonatal development. *Poult Sci* **99**, 4714-4729.

34. Yu A (2015) Claudins and the kidney. *J Am Soc Nephrol* **26**, 11-19.

35. Hou J, Renigunta V, Nie M *et al.* (2019) Phosphorylated claudin-16 interacts with Trpv5 and regulates transcellular calcium transport in the kidney. *Proc Natl Acad Sci U S A* **116**, 19176-19186.

36. Torres PAU & De Brauwere DP (2011) Three feedback loops precisely regulating serum phosphate concentration. *Kidney Int* **80**, 443-445.

37. Schröder B & Breves G (2006) Mechanisms and regulation of calcium absorption from the gastrointestinal tract in pigs and ruminants: comparative aspects with special emphasis on hypocalcemia in dairy cows. *Anim Health Res Rev* **7**, 31-41.

38. González-Vega JC & Stein HH (2016) Chapter 14 Calcium transporters and gene expression and absorption of calcium in pigs. In *Phytate Destruction-Consequences for Precision Animal Nutrition*, pp. 533-585 [Walk CL, Kühn I, Stein HH, Kidd MT and Rodehutscord M, editors]. Wageningen, the Netherlands: Wageningen Academic Publishers.

39. Manangi MK, Maharjan P & Coon CN (2018) Effect of different concentrations of dietary P and Ca on plasma inorganic P and urinary P excretion using noncolostomized and colostomized broilers. *Poult Sci* **97**, 522-530.

40. Manangi MK, Maharjan P & Coon CN (2018) Calcium particle size effects on plasma, excreta, and urinary Ca and P changes in broiler breeder hens. *Poult Sci* **97**, 2798-2806.

41. Rosenberg J, Hurwitz S & Bar A (1986) Regulation of kidney calcium-binding protein in the bird (*Gallus domesticus*). *Comp Biochem Physiol A Comp Physiol* **83**, 277-281.

42. Bar A, Shani M, Fullmer CS *et al.* (1990) Modulation of chick intestinal and renal calbindin gene expression by dietary vitamin D_3, 1,25-dihydroxyvitamin D_3, calcium and phosphorus. *Mol Cell Endocrinol* **72**, 23-31.

43. Berndt T & Kumar R (2009) Novel mechanisms in the regulation of phosphorus homeostasis. *Physiology* **24**, 17-25.

44. Christakos S & Norman AW (1980) Vitamin D-dependent calcium-binding protein synthesis by chick kidney and duodenal polysomes. *Arch Biochem Biophys* **203**, 809-815.

45. Huber K, Hempel R & Rodehutscord M (2006) Adaptation of epithelial sodium-dependent phosphate transport in jejunum and kidney of hens to variations in dietary phosphorus intake. *Poult Sci* **85**, 1980-1986.

46. Ren Z, Yan J, Hu Q *et al.* (2020) Phosphorus restriction changes the expression of fibroblast growth factor 23 and its receptors in laying hens. *Front Physiol* **11**, 85.

47. Gonzalez-Vega JC, Liu Y, McCann JC *et al.* (2016) Requirement for digestible calcium by eleven- to twenty-five-kilogram pigs as determined by growth performance, bone ash concentration, calcium and phosphorus balances, and expression of genes involved in transport of calcium in intestinal and kidney cells. *J Anim Sci* **94**, 3321-3334.

48. Riccardi D & Valenti G (2016) Localization and function of the renal calcium-sensing receptor. *Nat Rev Nephrol* **12**, 414-425.

49. Renkema KY, Nijenhuis T, van der Eerden BC *et al.* (2005) Hypervitaminosis D mediates compensatory Ca^{2+} hyperabsorption in TRPV5 knockout mice. *J Am Soc Nephrol* **16**, 3188-3195.

Summary

Due to an increasing global population and growing wealth and income, pork and chicken meat consumption has been increasing rapidly over the past decades. Phosphorus (P), an essential macro element for all animal species, is predominantly stored in various forms of inositol phosphate (IP) in most cereal grains. These various forms of IP are poorly digestible by pigs and poultry. Thus, mineral P, an expensive, finite and non-renewable resource, is routinely added to pig and poultry diets. Phosphorus not deposited in body tissues is excreted via urine and faeces which contributes to environmental pollution including surface water eutrophication. As such, insights into P digestion, absorption and metabolism can substantially contribute to reduce feed costs, conserve natural P resources and alleviate environmental pollution.

It has been demonstrated that reduction of dietary calcium (Ca) supply and supplementation of exogenous microbial phytase significantly improve dietary P absorption and reduce fecal P excretion. There are a few studies that investigated the interaction effects between dietary Ca content and microbial phytase supplementation on Ca and P absorption and excretion. The results, however, are inconsistent and a firm conclusion cannot be drawn, yet. On the basis of previous studies, this thesis aimed to provide further insights into the interacting effects between dietary limestone particle size and inclusion level, and microbial phytase supplementation on absorption of Ca and P along the GIT, their deposition in bone and excretion via the kidney, as well as expression of Ca- and P-related transporters and claudins (CLDN) in the GIT and kidney of pigs and poultry (Chapter 1).

Dietary Ca and phytase interaction in the GIT of pigs

Impact of dietary Ca content (2.0, 5.8 and 9.6 g/kg) in the presence (500 FTU/kg) or absence of microbial phytase on dietary Ca and P absorption in different GIT segments of growing pigs was investigated in Chapter 2. Dietary P was fixed at 4.7 g/kg for all dietary treatment groups. Results indicate that incremental dietary Ca content reduced apparent total tract digestibility (ATTD) of P, which was more pronounced in pigs fed phytase-supplemented diets. Moreover, dietary Ca also significantly reduced the cumulative IP degradation in the distal small intestine and decreased inorganic P solubility. As such, it can be concluded that incremental

dietary Ca content reduces intestinal apparent P digestibility via hampering IP degradation and precipitation of P, with a larger impact in microbial phytase-supplemented diets. In addition, microbial phytase supplementation significantly increased ATTD of Ca and P. Measurement in different GIT segments further indicated that microbial phytase increased apparent P absorption in the proximal small intestine and enhanced cumulative IP degradation in the distal small intestine. Consequently, less IP would be available for the microbiota residing in the colon to release Ca and P from the IP complex for colonic absorption, and apparent P absorption in the colon seemed to be lower in the presence of microbial phytase. However, colonic Ca and P absorption remains elusive, which was addressed in Chapter 3.

The mRNA expression level of Ca and P transporters and CLDN in the colon and jejunum of pigs, as modulated by dietary Ca content and microbial phytase supplementation were studied in Chapter 3. Results indicate that mRNA of the transporters involved in Ca (TRPV5, TRPV6, CaBP-D9K, NCX1) and P (PiT-1 and PiT-2) absorption was abundantly expressed in the pig colon, confirming the existence of colonic Ca and P absorption via a transcellular pathway. Moreover, mRNA expression level of Ca transporters (TRPV5, TRPV6 and CaBP-D9k) in the colon of pigs was significantly reduced with a high dietary Ca content (9.6 g/kg) and microbial phytase inclusion (500 FTU/kg), indicating that colonic Ca absorption might be flexible and contribute to whole body Ca homeostasis. A similar treatment effect was observed in the jejunum, i.e. mRNA expression level of Ca transporters was downregulated upon high Ca intake (TRPV5) and microbial phytase supplementation (TRPV5 and TRPV6). Surprisingly, jejunal mRNA expression level of Ca-permeable CLDN (CLDN-2 and -12) was increased with a high Ca intake and/or microbial phytase supplementation. Furthermore, the concentration gradient of soluble inorganic Ca across the porcine enterocyte was negative upon low but positive with high dietary Ca intake. Altogether, jejunal Ca absorption switches from the transcellular to paracellular route with increasing dietary Ca intake in pigs.

Limestone particle size and inclusion level in the GIT of poultry

Chapter 4 clarified the impact of limestone particle size (coarse vs. fine) and inclusion level (expressed as Ca/P) on Ca and P absorption, characteristics of tibia breaking strength in broilers. Unlike previous reports showing a greater Ca digestibility for the coarse compared to fine limestone, the study described in Chapter 4 indicates that apparent Ca digestibility was significantly higher for the fine compared to coarse limestone in the jejunum of broilers. However, this difference disappeared in the distal ileum particularly at a low Ca intake. In addition, growth performance and characteristics of tibia breaking strength (maximal compressive load, stiffness and energy to fracture) was also independent of limestone particle size. As such, limestone particle size has a limited impact on apparent distal ileal Ca absorption in broilers.

Incremental dietary Ca content gradually reduced apparent P digestibility but enhanced growth performance and characteristics of tibia breaking strength. Moreover, coarse compared to fine limestone improved apparent distal ileal P digestibility in broilers, but this impact was relatively small (60 vs. 57%, Chapter 4). The optimal dietary Ca/P to optimize apparent P digestibility while maintaining growth performance and tibia development was 1.00-1.25 for both fine and coarse limestone. Interestingly, P solubility in all measured GIT segments (crop, jejunum and ileum) was not affected by limestone particle size or inclusion level, indicating that Ca-IP and Ca-P complexation may not be the only mechanism by which Ca reduces P absorption in broilers. As such, it was hypothesized that dietary Ca might reduce P absorption via downregulating expression levels of P transporters. This hypothesis was tested in Chapter 5.

The mRNA expression level of Ca and P transporters and CLDN in the duodenum and jejunum of broilers, as affected by limestone particle size and inclusion level was studied in Chapter 5. Results indicate that incremental dietary Ca intake reduced mRNA expression level of P transporter NaPi-IIb in both the duodenum and jejunum of broilers, confirming that Ca-induced downregulation of P transporters is an important mechanism by which Ca reduces intestinal P absorption. Similarly, mRNA expression level of Ca transporters and CLDN was also downregulated with incremental dietary Ca intake in the duodenum (CaSR, CaBP-D28k, PMCA1, CLDN-2) and jejunum (CaSR, CaBP-D28k and CLDN-2) of broilers. Moreover, this downregulation of Ca and P transporters was greater for

fine limestone when increasing dietary Ca/P from 0.5 to 1.0, while it was greater for coarse limestone from 1.0 to 1.75. As such, it can be concluded that dietary Ca reduces expression of duodenal and jejunal mucosa Ca and P transporters in a limestone particle size dependent manner.

Ca and phytase interaction in the GIT of broilers

Chapter 6 investigated the impact of dietary Ca content (expressed as Ca to retainable P ratio, Ca/rP) in the presence (1000 FTU/kg) or absence of microbial phytase on Ca and P absorption, duodenal expression of Ca and P transporters and CLDN in broilers. In contrasts to the greater reduction impact of dietary Ca on intestinal P absorption in the phytase-supplemented diets in growing pigs (Chapter 2), broilers showed a lesser reduction of dietary Ca on P absorption in the presence of microbial phytase. This difference can be attributed to that broilers may have a greater intrinsic potential to release dietary IP than pigs. In addition, unlike pigs which showed a downregulation of Ca transporters (TRPV5, TRPV6, CaBP-D9k) with an upregulation of Ca-permeable CLDN (CLDN-2) upon higher Ca intake, mRNA expression of both Ca transporters (CaSR, CaBP-D28k and PMCA1) and CLDN (CLDN-2 and -12) was downregulated at a higher Ca intake in the duodenum of broilers. The differences in modulating intestinal expression of Ca transporters and CLDN presumably are related to a difference in Ca and P post-absorptive metabolism and excretion via urine between pigs and broilers.

Renal mRNA expression of Ca and P transporters and CLDN in pigs vs. broilers

The surplus of absorbed Ca and P is not retained in the bone since hydroxyapatite ($Ca_5(PO_4)_3(OH)$) has a constant Ca/P of approximate 2.1. They are eliminated via urine in the kidney, and the kidney is generally considered to play a pivotal role in whole body Ca and P homeostasis. Urinary Ca and P excretion in broilers is more difficult to measure. Thus, the mRNA expression level of Ca and P transporters and CLDN in the kidney of pigs and broilers was measured as an indicator of urinary Ca and P excretion (Chapter 7). As expected, mRNA expression level of Ca transporters (CaSR, TRPV5, TRPV6, CaBP-D28k and NCX1) and CLDN (CLDN-12

and -16) were downregulated at a higher Ca intake in the kidney of pigs, confirming that the kidneys play a pivotal role in whole body Ca homeostasis in pigs. By contrast, mRNA expression level of Ca transporters and CLDN (TRPV6, CaBP-D28k and CLDN-2) were upregulated with dietary Ca intake in the kidney of broilers. This study shows that insights obtained in the studies with poultry should not be extrapolated to pigs and *vice versa*.

Conclusions

The results presented in this thesis indicate that microbial phytase supplementation enhances the negative impact of incremental dietary Ca content on apparent P absorption in growing pigs, while it alleviates this negative impact in broilers. Calcium-induced downregulation of P transporter is an important mechanism by which Ca reduces P absorption in the GIT of broilers. In pigs, incremental dietary Ca content reduces intestinal P absorption via hampering IP degradation and precipitation of P. Limestone particle size has a limited impact on apparent distal ileal Ca absorption, while slightly improves apparent distal ileal P absorption in broilers. Insights obtained in studies with poultry should not be extrapolated to pigs and *vice versa* at least when it comes to dietary Ca and P metabolism and homeostasis.

Acknowledgements

Finally, my PhD study is coming to an end. Completing this PhD thesis was by no means an easy job for me, and I would have never accomplished it without the help and support I received from a number of people during this journey.

Firstly, I would like to express my gratitude to my promoter Wouter. In spite of your packed agenda, you always made some time for me and were actively involved in our regular meetings. I really appreciate that you always tried to find an easier understandable way to explain your ideas and impart your knowledge to me. Your input into the project in the past years, particularly in the last stage of my PhD study is key to the completion of my thesis and graduating on time. Paul, as my daily supervisor, you have been very supportive during my PhD study. Although you had many research projects running at the same time, you always made some time for me whenever I visited your office. I cannot be more grateful for your detailed guidance and instant feedback whenever I had a question or problem. Jurgen, it goes without saying that you substantially contributed to my project particularly to gene expression work. I cannot imagine how to have completed this project without your expertise on cell signalling and animal physiology. Your excellent writing skills and detailed comments on my manuscripts was critical to improve the legibility of my thesis. Lastly, to all of my supervisors, thanks a lot for your patience and kindness in the past years.

My special thanks to Marinus, who invited me to do a PhD in the Netherlands and picked me up at Ede-Wageningen station on arrival. I will always be grateful for your encouragement and comfort when I had a hard time in a relationship. You were much more than a supervisor to me. It's my deepest sorrow that you suddenly passed away on New Year's Eve of 2020. It saddens me that I cannot talk to you anymore. I hope that I met your expectations when you offered me the PhD position.

I would like to acknowledge the staff members of Wageningen Livestock Research (Jan, Leo and Piet) and Animal Nutrition Laboratory (Leon, Saskia, Xuan-Huong, Erika, Jane-Martine and Michel). Jan, I really appreciate your coordination with ForFarmers in arranging my second broiler trial after Marinus had passed away. Saskia, thanks a lot for organizing practical aspects of my lab work. I understand that your duty commands you to be somewhat strict with PhD students particularly at the beginning of their lab work. Without your organisation, my lab work would have gone much slower. Xuan-Huong, thanks a lot for your precise and detailed instruction in the lab. It was a great pleasure to have lunch with you

over the past years, your Asian foods to some extent alleviated my homesick. I still have a vivid memory about the very delicious dumplings that you prepared for me to cheer me up at times of frustration. Leo, thanks a lot for helping me collect the samples for gene expression analysis, and instructing me to do the RT-qPCR analysis in the lab. It was a great pleasure to work with you. I also want to express my gratitude to Yvonne and Betty, who were always very kind and ready to help me over the past years. The generous and instant help from you both made my studies and living in Netherlands much easier.

I would like to thank the industry partners involved in my PhD project: De Heus (Masja), Nutreco (Jan-Willem and Carlijn) and ForFarmers (Albert and Jan). Thank you all for your arrangement in conducting my animal trials in your facilities. I am also very grateful to the farm staff who took care of the experimental animals and helped me collect my samples.

To my awesome ANU colleagues, it was a great pleasure to spend the past four years with you. Thanks very much for your suggestions when I had a hard time. Your company definitely made this journey much more enjoyable. I wish you all good luck and great succus with your research projects and personal life. Special thanks to Sholeha for being my buddy and helping me at the beginning of my PhD study. I also want to thank the MSc student I supervised, Yuan Cao. You helped me to collect my samples and have been a very hardworking student. Hopefully the things you learned during your MSc thesis can be helpful to your PhD application and future research.

I would also like to thank the Feed4Future project for funding my research and the Chinese Scholarship Council (CSC) for supporting my living in Netherlands. Many thanks to Prof. Xugang Luo (my MSc supervisor), Mrs Liyang Zhang, Dr Xiudong Liao and Dr Lin Lyu. Without your help, it was impossible for me to obtain the CSC scholarship and start my PhD study in Netherlands. Thank you all for your instructions and tremendous help during my MSc study.

最后，谢谢我的爸爸妈妈把我抚养长大，虽然你们没能给我一个安全稳定的学习成长环境，但却给了我一个温馨充满回忆的童年。谢谢我的兄长，在我出国留学的这些年里，一直陪伴在父母身边照顾他们。另外，感恩在农大西校区田径场遇到的好心大哥，我们素未平生，你惠赠的300块确实让我感受到了这落寞人间的温暖。最后，谢谢陪伴我的亲人朋友，和你们一起经历过的点滴构成了我的人生，感谢你们的出现。

About the author

CURRICULUM VITAE

Hu Yixin was born in Hunan Province, China on April 23rd, 1991. He started his BSc study in the Department of Animal Science and Technology at Hunan Agriculture University in 2009. After that he conducted a MSc study at the Institute of Animal Science, Chinese Academy of Agricultural Sciences from 2013 under supervision of Prof. Xugang Luo. For his MSc thesis, he investigated the impact of dietary phosphorus content on gene expression of phosphorus related transporters in the small intestine of broilers. Yixin obtained his MSc degree in 2016, thereafter he worked as a technician trainee in an international feed company from Germany (Sano China) for half a year. On September 8th, 2017, Yixin started as a PhD candidate at Wageningen University & Research. During his PhD study, Yixin continued to focus on the absorption and metabolism of calcium and phosphorus in pigs and poultry. The results of his PhD work are presented in this thesis.

LIST OF PUBLICATIONS

Liu SB, *YX Hu*, XD Liao, L Lu, SF Li, LY Zhang, HZ Tan, L Yang, HQ Suo and XG Luo. 2016. Kinetics of phosphorus absorption in ligated small intestinal segments of broilers. J Anim Sci 94:3312-3320.

Liao XD, HQ Suo, L Lu, *YX Hu*, LY Zhang and XG Luo. 2017. Effects of sodium, 1,25-dihydroxyvitamin D_3 and parathyroid hormone fragment on inorganic P absorption and Type IIb sodium-phosphate cotransporter expression in ligated duodenal loops of broilers. Poult Sci 96:2344–2350.

Hu YX, XD Liao, Q Wen, L Lu, LY Zhang and XG Luo. 2018. Phosphorus absorption and gene expression levels of related transporters in the small intestine of broilers. Br J Nutr 119:1346-1354.

Hu YX, P Bikker, M Duijster, WH Hendriks, J van Baal and MM van Krimpen. 2020. Coarse limestone does not alleviate the negative effect of a low Ca/P ratio diet on characteristics of tibia strength and growth performance in broilers. Poult Sci 99:4978–4989.

Hu YX, J van Baal, WH Hendriks, M Duijster, MM van Krimpen and P Bikker. 2021. Mucosal expression of Ca and P transporters and claudins in the small intestine of broilers is altered by dietary Ca/P in a limestone particle size dependent manner. Submitted to PLoS One.

Hu YX, J van Baal, WH Hendriks, M Duijster, MM van Krimpen and P Bikker. 2021. Dietary microbial phytase supplementation enhances the impact of dietary Ca content on P absorption and retention in growing pigs. Submitted to Br J Nutr.

Hu YX, J van Harn, WH Hendriks, J van Baal, MA Dijkslag, MM van Krimpen and P Bikker. 2021. Low-Ca diets increase duodenal mRNA expression of Ca and P transporters and claudins but compromise growth performance irrespective of microbial phytase inclusion in broilers. Submitted to Poult Sci.

TRAINING AND SUPERVISION PLAN

Completed in fulfilment of the requirements for the education certificate of the Wageningen Institute of Animal Science (WIAS)

The Basic Package (3 ECTS [1])	**Year**
Course on Essential Skills	2018
WIAS Introduction Day	2018
Scientific Integrity & Ethics in Animal Science	2020
Disciplinary Competences (15 ECTS)	
WIAS Research Proposal	2018
Feed Technology course (ANU 31306)	2018
Advanced Statistics course Design of Experiments	2018
Statistics for Life Science	2019
Professional Competences (6 ECTS)	
WIAS course High-Impact Writing in Science	2019
Effective Behaviour in Your Professional Surroundings	2019
Research Data Management	2019
Project and Time Management	2020
Introduction to R	2021
The Essentials of Scientific Writing & Presenting	2021
Societal Relevance (1 ECT)	
Making an Impact	2021
Presentation Skills (3 ECTS)	
43th Animal Nutrition Research Forum, Wageningen, Netherlands, oral	2018
44th Animal Nutrition Research Forum, Gembloux, Belgium, oral	2019
45th WIAS Annual Conference, Wageningen, Netherlands, poster	2020
Teaching competences (3 ECTS)	
Introduction to Animal Science	2019
Introduction to Animal Science	2020
Supervising MSc theses (Yuan Cao, minor thesis)	2020
Total ECTS	**31**

[1] One ECTS credit equals a study load of approximately 28 hours

Colophon

This research was conducted within the framework of the public private partnership "Feed4Foodure" (AF16123), which is supported by the "Vereniging Diervoederonderzoek Nederland (VDN)" and the Ministry of Agriculture, Nature and Food Quality (LNV, project number BO-31.03-005). The scholarship for the author was provided by the China Scholarship Council.

Cover design: Hu Yixin

Layout: Hu Yixin

Printed by: ProefschriftMaken || www.proefschriftmaken.nl