Universität Rostock Traditio et Innovatio

Institute of Genome Biology

Research Institute for Farm Animal Biology (FBN), Dummerstorf

Effects of dietary phosphorus on endogenous mechanisms of phosphorus homeostasis in pigs

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Cumulative Dissertation

to partial fulfillment of the requirements for the academic degree

Doctor rerum naturalium (Dr. rer.nat.)

Faculty of Mathematics and Natural Sciences

of University Rostock

Submitted by

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Rostock, September 2021

https://doi.org/10.18453/rosdok_id00003800

Day of Submission: 13.09.2021

Day of Doctoral Defense: 27.05.2022

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Abbreviations

ATP ..... Adenosine triphosphate

ADP ..... Adenosine diphosphate

ALP ..... Alkaline phosphatase

BBM ..... Brush border membrane

BPG ..... Bisphosphoglycerate

C ..... Cytosine

Ca ..... Calcium

CaSR ..... Calcium sensing receptor

CLDN ..... Claudins

DNA ..... Deoxyribonucleic acid

DBP ..... Vitamin binding protein

ERK ..... Extracellular signal-regulated kinase

FGF23 ..... Fibroblast growth factor 23

FGFR1 ..... Fibroblast growth factor receptor 1

G ..... Guanine

insP5 ..... Inositol-5 phosphate

insP6 ..... Inositol-6 phosphate

mRNA ..... Messenger ribonucleic acid

NF-κB ..... Nuclear factor kappa B

OCLN ..... Occludins

P ..... Phosphorus

PTH ..... Parathyroid hormone

PTG ..... Parathyroid gland

PH ..... Potential of hydrogen

PKA ..... Protein kinase A

PKC ..... Protein kinase C

RNA ..... Ribonucleic acid

RXR ..... Retinoid X receptor

RANK …….. Receptor activator of nuclear factor kappa B

SLC …… Solute carrier

VDR ……. Vitamin D receptor

VDREs ……. Vitamin D responsive elements

Summary

Although P is indispensable for cellular functions, animal growth and body development, excessive intake of inorganic P as a dietary supplement for animals disrupt the natural P cycle and cause serious environmental pollution by excessive excretion of the consumed P into the environment. The commercial swine production is considered a major source of P pollution to the environment. Body's P homeostasis is achieved through close coordination between many organs and tissues including the intestine, kidney, bone and PTG through several hormones and regulatory factors such as PTH, vitamin D, and FGF23. There is also evidence that the utilization of P in livestock animals is influenced by genetics. Considering these animal-intrinsic factors, the study of the underlying physiological mechanism of P homeostasis of pigs could help to re-evaluate strategies for improved P utilization in livestock production and reduce the environmental impact. In this context, aim of this dissertation is to investigate the effects of different dietary P intake on the endogenous mechanism of P homeostasis in a wide range of organs and tissues of pigs.

The sodium-dependent phosphate co-transporters are considered the major P transporters in mammals. To elucidate the particular contributions of sodium-dependent phosphate co-transporters to P homeostasis, the regulatory role of the currently annotated nine sodium-dependent phosphate co-transporters is investigated in pigs with divergent dietary P supplies. Two major P transporters, *SLC34A1* and *SLC34A3*, showed higher gene expression in the low P diet group than in the high P diet group in kidney and intestine. This result suggests that the gene expression of transcellular P transporters is tissue-specific and that dietary interventions changes the gene expression levels of P transporters in relevant tissues to maintain P homeostasis in the animal. Our data also indicate the effects of age differences on the transcriptional activity of sodium-dependent P co-transporters. Data from the literature search suggest that the intestine, with its high sensitivity to P concentrations in the intestinal lumen, is likely at the center of regulating P homeostasis and attempts to maintain P levels in the body in balance by releasing specific signaling molecules into the circulation when there is nutritional deficiency.

Analysis of the blood parameters and transcriptional responses in the kidney of sows showed that different dietary P intake effect on the renal expression of *CYP27B1* gene and serum calcitriol levels. However, serum parameters showed maintained P homeostasis, as evidenced by the unchanged inorganic P and calcium levels in the low and high P groups. These results suggest that the variable P-containing diet may influence vitamin D metabolism in animals to maintain bone and mineral homeostasis by regulating *CYP27B1* gene expression in the kidney and altering other regulators like calcitriol, PTH and FGF23 levels.

Finally, the effects of varying dietary P intake on gut microbiota composition and its influence on P bioavailability were also investigated. Analysis of the microbial composition and profile of inositolphophates in the large intestine of pigs identified several microbial families that affect P availability in the large intestine through their phytase activity. Our results show that low P diets for pigs trigger microbial mediated compensation through phytate cleavage and P release, thereby increasing the availability of P to the host and gut microbiota. These results suggest that targeting the intestinal microbiota through feeding interventions offers opportunities to optimize intestinal phytate and P utilization.

Zusammenfassung

Obwohl P für die Zellfunktionen, das Wachstum der Tiere und die Entwicklung des Körpers unentbehrlich ist, wird durch die übermäßige Zufuhr von anorganischem P als Nahrungsergänzungsmittel für Tiere der natürliche P-Kreislauf gestört und die Umwelt durch übermäßige Ausscheidung des aufgenommenen P in die Umwelt stark belastet. Die kommerzielle Schweinehaltung gilt als eine der Hauptquellen für die P-Belastung der Umwelt. Die P-Homöostase des Körpers wird durch eine enge Koordinierung zwischen vielen Organen und Geweben, einschließlich Darm, Niere, Knochen und PTG, über verschiedene Hormone und Regulierungsfaktoren wie PTH, Vitamin D und FGF23 erreicht. Es gibt auch Hinweise darauf, dass die Verwertung von P bei Nutztieren durch die Genetik beeinflusst wird. In Anbetracht dieser tierimmanenten Faktoren könnte die Untersuchung der zugrundeliegenden physiologischen Mechanismen der P-Homöostase von Schweinen dazu beitragen, Strategien für eine verbesserte P-Verwertung in der Tierhaltung neu zu bewerten und die Auswirkungen auf die Umwelt zu verringern. In diesem Zusammenhang sollen in dieser Dissertation die Auswirkungen einer unterschiedlichen P-Aufnahme über die Nahrung auf den endogenen Mechanismus der P-Homöostase in einer Vielzahl von Organen und Geweben von Schweinen untersucht werden.

Die Natrium-abhängigen Phosphat-Co-Transporter gelten als die wichtigsten P-Transporter in Säugetieren. Um die besonderen Beiträge der Natrium-abhängigen Phosphat-Co-Transporter zur P-Homöostase zu klären, wird die regulatorische Rolle der derzeit neun annotierten Natrium-abhängigen Phosphat-Co-Transporter bei Schweinen mit unterschiedlicher P-Versorgung durch die Nahrung untersucht. Zwei wichtige P-Transporter, SLC34A1 und SLC34A3, zeigten in der Gruppe mit niedrigem P-Gehalt im Futter eine höhere Genexpression als in der Gruppe mit hohem P-Gehalt in einigen Nieren- und Darmgeweben. Die Ergebnisse zeigen, dass die Expression von Genen, die für transzelluläre P-Transporter kodieren, gewebespezifisch ist und dass die Ernährungsweise die Expression einiger P-Transporter in den relevanten Geweben verändert, um die P-Homöostase im Tier aufrechtzuerhalten. Unsere Daten zeigen auch die Auswirkungen von Altersunterschieden auf die Transkriptionsaktivität von natriumabhängigen P-Co-Transportern. Die Daten aus der Literaturrecherche deuten darauf hin, dass der Darm mit seiner hohen Empfindlichkeit gegenüber P-Konzentrationen im Darmlumen wahrscheinlich im Zentrum der Regulierung der P-Homöostase steht und versucht, den P-Spiegel im Körper im Gleichgewicht zu halten, indem er bei einem Nährstoffmangel spezifische Signalmoleküle in den Blutkreislauf freisetzt.

Die Analyse der Blutparameter und der Transkriptionsreaktionen in der Niere von Sauen zeigte, dass sich die unterschiedliche P-Aufnahme über die Nahrung auf die renale Expression des CYP27B1-Gens und den Calcitriol-Spiegel im Serum auswirkt. Die Serumparameter zeigten jedoch eine gleichbleibende P-Homöostase, wie die unveränderten anorganischen P- und Kalziumwerte in den Gruppen mit niedrigem und hohem P-Gehalt belegen. Diese Ergebnisse deuten darauf hin, dass die variable P-haltige Ernährung den Vitamin-D-Stoffwechsel bei Tieren beeinflussen kann, um die Knochen- und Mineralstoffhomöostase aufrechtzuerhalten, indem sie die CYP27B1-Genexpression in der Niere reguliert und andere Regulatoren wie Calcitriol-, PTH- und FGF23-Spiegel verändert.

Schließlich wurden auch die Auswirkungen einer unterschiedlichen P-Aufnahme über die Nahrung auf die Zusammensetzung der Darmmikrobiota und deren Einfluss auf die Bioverfügbarkeit von P untersucht. Bei der Analyse der mikrobiellen Zusammensetzung und des Profils der Inositolphophaten

im Dickdarm von Schweinen wurden mehrere Mikrobenfamilien identifiziert, die die P-Verfügbarkeit im Dickdarm durch ihre Phytaseaktivität beeinflussen. Unsere Ergebnisse zeigen, dass die Ernährung von Schweinen mit niedrigem P-Gehalt eine mikrobiell vermittelte Kompensation durch Phytatspaltung und P-Freisetzung auslöst, wodurch die Verfügbarkeit von P für den Wirt und die Darmmikrobiota erhöht wird. Diese Ergebnisse deuten darauf hin, dass die gezielte Beeinflussung der Darmmikrobiota durch Fütterungsmaßnahmen Möglichkeiten zur Optimierung der Phytat- und P-Verwertung im Darm bietet.

1. General introduction

Along with many other necessary elements, P is essential for the existence of life as it constitutes about 1% of the body mass. The majority of P (~85%) in the body is existed in hard tissues (bone, teeth) and the rest is in soft tissues (cell membrane phospholipids, carbohydrates) and extracellular fluid (Goretti Penido and Alon 2012, Villa-Bellosta and Egido 2017). Although primarily used for skeletal growth, P plays an important role in metabolism in various parts of the body and is essential for all stages of growth and development. In mammalian organisms, P participate in many biological and cellular processes. In the cells, P is an important constituent of the cell membranes, high-energy phosphate esters (ATP), DNA (25% by mass), RNA, and intracellular signaling proteins (Pavlov, Aschar-Sobbi et al. 2010, Maffeo, Yoo et al. 2014). As a part of a small molecule, phosphorylated biomolecules also play important role in multiple enzymatic reactions within the cells, e.g., glycolysis and ammonia genesis. In the blood, inorganic P also plays an important role in blood oxygen-transport by regulating the formation of 2,3-bisphosphoglycerate (2,3-BPG), a modulator of hemoglobin oxygen affinity (MacDonald 1977). In addition, P plays an important role in energy transduction mechanism by being an important part of the ATP/ADP processes, in which the ATP molecule splits off one of its three phosphates, becoming ADP + P, and releases the energy holding that phosphate molecule. Moreover, extracellular P is also involved in regulating gene expression by triggering signal transduction (Kimata, Michigami et al. 2010, Yamazaki, Ozono et al. 2010). In addition to these roles, in the internal fluids of all cells, P is part of a major buffer system, which consists of hydrogen/dihydrogen phosphate ions.

As described above, although P is necessary for every living organism, its resources on earth are limited. In livestock production, especially in commercial swine production, more than the required P content is often supplied to ensure maximum growth and bone development of the animals. However, if used inefficiently, the excess P consumed by the animals can not only have a negative impact on normal growth processes, but can also be excreted in manure and cause severe environmental pollution (Fernandez, Poulsen et al. 1999, Poulsen, Jongbloed et al. 1999). In addition, normal P requirements are related to the age and growth stage of the animals. P utilization and homeostasis in monogastric animals is regulated via a feedback loop from many organs and tissues such as the intestinal tract, kidney, bone, and parathyroid gland (PTG) by various hormones and factors such as parathyroid hormone (PTH), calcitriol (1,25(OH)2-vitamin D3), and fibroblast growth factor-23 (FGF23) and is influenced by genetics (Berndt and Kumar 2009). Thus, a deeper understanding of the endogenous mechanisms of P homeostasis in pigs could help to find better nutritional strategies for adequate P supply and thus improve the efficiency of P utilization in pig farming and reduce the environmental impact.

1.1. The physiology and metabolism of P in monogastric animals

1.1.1 Physiologic factors and endocrine control of P homeostasis

It has been demonstrated that the body's P balance is strongly regulated by some hormonal factors such as PTH, FGF23 and calcitriol (Bergwitz and Juppner 2010). Calcitriol plays a key role in maintaining Ca and P homeostasis in the body by controlling intestinal absorption, renal excretion, and bone

remodeling (DeLuca 1980, Bacchetta, Sea et al. 2013). Calcitriol needs to bind the vitamin D receptor (VDR)/retinoid X receptor (RXR) heterodimer, which binds to specific binding sites in the genome called vitamin D response elements (VDREs) to regulate renal and intestinal handling of P (Bikle 2014). Their regulatory function is also influenced by the amount of dietary P intake (Just, Oster et al. 2018). Indeed, the level of dietary P intake has been shown to influence gene expression of transcellular P transporters (Wubuli, Reyer et al. 2019). In addition, numerous other genes were found to be involved in the regulation of P homeostasis with immunomodulatory effects in the kidney and intestine of growing pigs (Just, Oster et al. 2018). It is controversial whether dietary P directly regulates P homeostasis or is indirectly controlled by downstream signaling cascades of the Ca-sensing receptor (CaSR) (Ba, Brown et al. 2003, Oster, Just et al. 2016). However, a recent study has found that dietary P acts directly on the Ca-sensing receptor in the PTG to stimulate PTH secretion (Centeno, Herberger et al. 2019). In addition, FGF23 regulates P homeostasis via complex feedback mechanisms by closely interacting with other regulatory factors including the circulating Ca and P concentration, PTH and calcitriol. Increased serum P concentration stimulates the secretion of FGF23 in osteoblasts and osteocytes, which affects directly or indirectly on the kidney to suppress synthesis of calcitriol by downregulating the expression of 1α-hydroxylase (CYP27B1) and to downregulate the expression of SLC34A1 and SLC34A3 sodium-dependent P co-transporters (Chande and Bergwitz 2018). Inhibition of calcitriol and sodium-dependent P transporters reduces intestinal absorption and renal reabsorption of inorganic P and mobilization of P from bone mineral. Furthermore, there are conflicted findings regarding the effect of FGF23 on the PTG. It was initially proposed that the FGF23 would also acts on the PTG to inhibit secretion of PTH through the activation of Klotho–FGFR1 receptor complexes (Ben-Dov, Galitzer et al. 2007). However, recent genetic studies suggested that FGF23 induces secretion of PTH (Kawakami, Takeshita et al. 2017).

1.1.2 Organs involved in maintaining P homeostasis

Body's P homeostasis is modulated mainly by several organs and tissues, absorption in the small intestine (especially the jejunum), retention/excretion in the kidney, and P reserving in the bone (Figure 2) (Prasad and Bhadauria 2013). Relevant studies have shown that a variety of tissues, including the gastrointestinal tract (stomach, duodenum, jejunum, ileum, caecum and colon), kidney, liver, bone, muscle, lung and vascular tissue are also important for the physiological homeostasis of body P (Wubuli, Reyer et al. 2019).

1.1.2.1 Contribution of the gastrointestinal tract (jejunum)

The gastrointestinal tract plays an important role in maintenance of P homeostasis by coordinating with other main organs and tissues including the kidney and bone. Normally, the vast majority of inorganic P in the diet will be absorbed through the entire length of the intestine. In the intestinal tract, P absorption occurs mainly through passive para-cellular diffusion and active transcellular transport (Figure 1). The P-specific passive para-cellular transport occurs through specific tight junction proteins of neighboring cells. Claudins (CLDN) and occludins (OCLN) are two types of important tight-junction-specific integral membrane proteins. Previous studies suggest that, the passive para-cellular transport might predominate in the intestinal P transport under normal dietary conditions (Walton and Gray 1979, Danisi and Straub 1980). Growing evidences suggested that environmental clues, i.e. the dietary

mineral supply, is further directed through active transcellular transport mechanisms, which is mainly mediated by the sodium-dependent phosphate co-transporters (Eto, Tomita et al. 2006). According to studies, the small intestine, in particular the jejunum and duodenum has a significantly higher P absorption capability compared to large intestine (e.g., colon) (Walling 1977, Fang, Xiang et al. 2012). However, studies also have revealed important differences in the role of each segment of small intestine in the handling of P between rodent species, as the ileum predominates in mice, whereas maximal P absorption occurs in the duodenum of the rat (Marks, Srai et al. 2006). The sodium-dependent P co-transporters are located on the apical luminal sides of small intestinal epithelial cells. Several members of the Type II and Type III sodium-dependent P co-transporters families were identified in the small intestine of various mammalian species. Among others, the SLC34A2 is considered as the most important P transporter in the intestinal tract in human. Similarly in mice, a knockout study demonstrated that SLC34A2, a member of the type III sodium/phosphate co-transporter family, is the major player in intestinal P absorption (Sabbagh, O'Brien et al. 2009). However, the SLC34A3, another one member of the type II sodium-dependent P transporters family, is predominated in the small intestine of pigs (Wubuli, Reyer et al. 2019). Furthermore, the type III sodium-dependent P co-transporters (SLC20A1 and SLC20A2) are also expressed in the small intestine of rat and pigs with a high absorptive capacity for P (Giral, Caldas et al. 2009, Candeal, Caldas et al. 2014, Wubuli, Reyer et al. 2019). On the other hand, pigs and humans share similarities in anatomy, pathophysiology, nutrient requirements, and functional genomics, which highlights the pig as an animal model in biomedical research (Lunney 2007, Guilloteau, Zabielski et al. 2010). However, subtle changes in e.g. gene activity need to be considered in order to explore the molecular responses in P homeostasis and its consequences for health and well-being and non-rodent animal models. The regulatory role of type II and type III sodium-dependent P co-transporters in maintaining P homeostasis has also been shown to be mediated by the major phosphaturic hormone FGF23 and its co-receptor αKlotho (Bon, Frangi et al. 2018, Hu, Shi et al. 2019). Recent studies have also pointed to the possible regulatory role of vitamin D in the adaptation of intestinal active transcellular P transport to varying concentrations of dietary P. Thus, a number of studies have investigated the distinct regulatory mechanisms of SLC34A2 expression and Intestinal absorption of P, which are believed to be regulated by several other factors including dietary P and calcitriol. It has been described previously that the action of calcitriol on P regulation is rely on a transcription factor, vitamin D receptor (VDR) (see section 1.1.1). However, several studies also reported that, low P diets stimulated SLC34A2 protein and the P transport activity without significant changes in mRNA expression (Hattenhauer, Traebert et al. 1999, Katai, Miyamoto et al. 1999). Moreover, other studies in VDR knockout and wild-type mice have shown that a low-p diet significantly increases protein expression of SLC34A2 in the intestinal BBM of VDR knockout mice, whereas calcitriol has no effect on sodium-dependent p-transport activity or protein expression of SLC34A2 (Segawa, Kaneko et al. 2004). These results suggest that the adaptation of intestinal P transport mediated by sodium phosphate P cotransporters to different levels of dietary P is independent of calcitriol and regulated by post-transcriptional mechanism (Capuano, Radanovic et al. 2005).

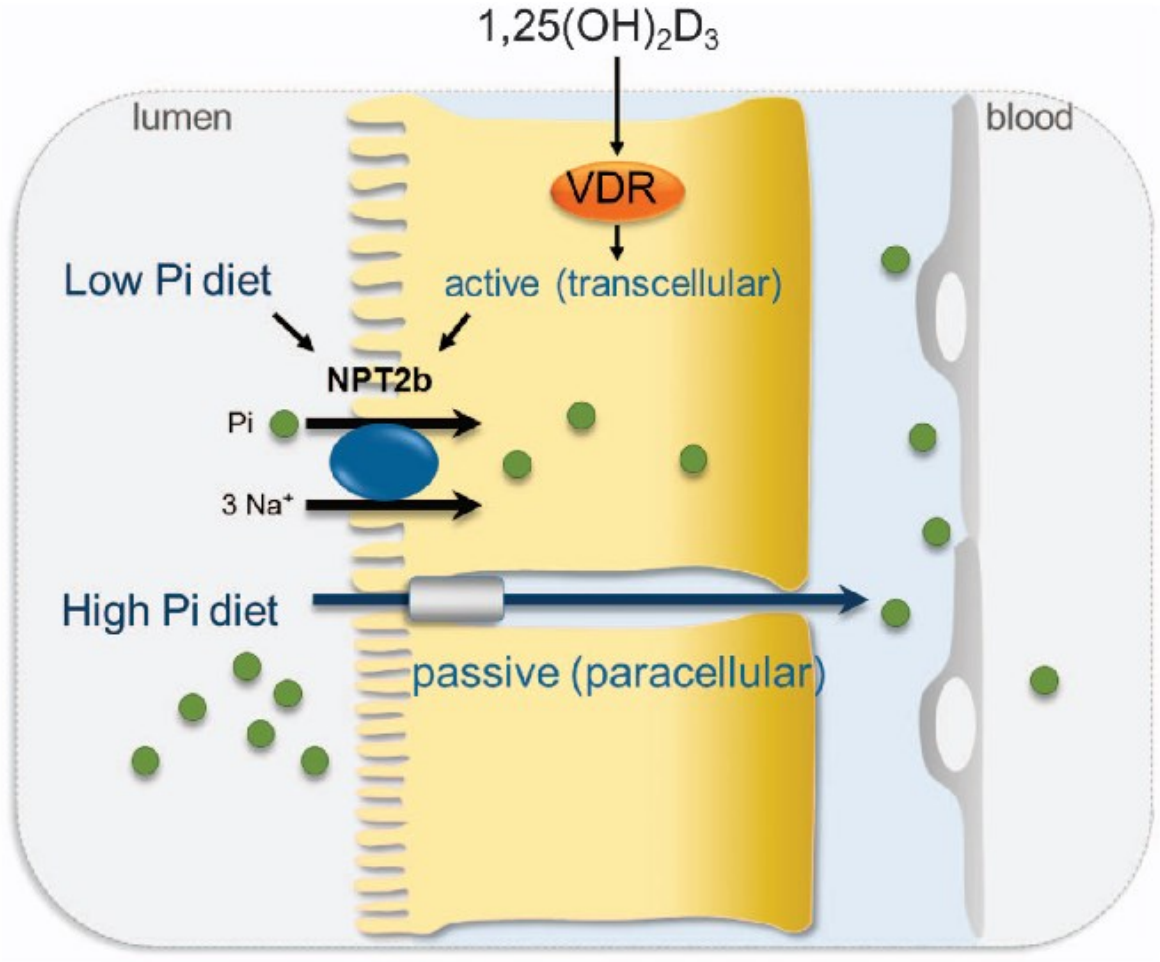

Figure 1. Intestinal P transportation. In the intestinal tract, inorganic P is transported by passive paracellular and active transcellular transport. Paracellular transport occurs through specific tight junction proteins of neighboring cells such as claudins and occludins. Under normal nutritional conditions, the majority of dietary P is likely to be transported via the passive paracellular pathway. Active transcellular transport is mediated mainly by sodium-dependent P co-transporters such as SLC34A2, which are localized at the intestinal brush border membrane. Expression of sodium-dependent P co-transporters is induced by calcitriol under lower dietary P conditions. Adapted from "Vitamin D endocrine system and the intestine" by Christakos S, 2014, *Bonekey Rep*, 3, p. 496.

1.1.2.2 Contribution of the kidney

Together with other main organs and tissues, the kidney also has a pivotal role in maintaining P homeostasis. The kidney contributes to maintaining normal serum P levels by reabsorbing or excreting excess P. Normally, P is filtrated daily at the glomerulus, then the vast majority of P (~80%) will be reabsorbed by the renal proximal tubule and a small amount of P reabsorbed in the distal tubule (Amiel, Kuntziger et al. 1970, Tenenhouse, Gauthier et al. 1998). The excess P is excreted into the urine. Reabsorption of the filtered P at the renal proximal tubules is achieved by the kidney specific members (*SLC34A1* and *SLC34A3*) of the type II sodium-dependent P co-transporters family (Figure 2). The *SLC34A1* and *SLC34A3* are expressed at the proximal tubules of kidney and are considered the main contributor of reabsorption of P in the kidney (Beck, Karaplis et al. 1998, Madjdpour, Bacic et al. 2004).

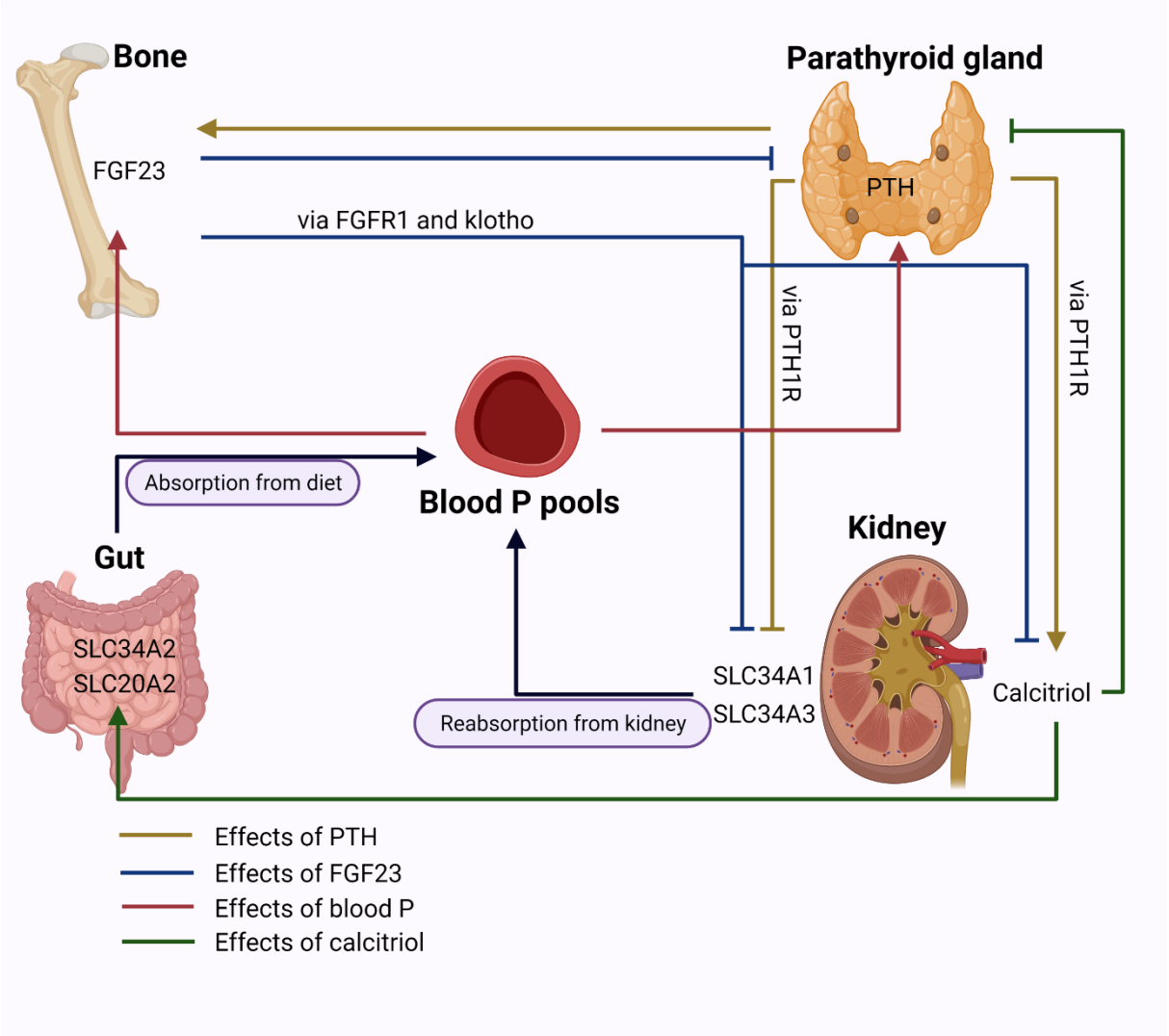

Figure 2. Endocrine regulation of P homeostasis. Blood P induces secretion of FGF23 in osteoblasts and osteocytes in bone (red arrow), which affects directly or indirectly on the kidney to suppress synthesis of calcitriol and to downregulate the expression of *SLC34A1* and *SLC34A3* P co-transporters (blue arrows). Inhibition of calcitriol synthesis will decreases P absorption in the intestinal tract (green arrow) and mobilization of P from bone minerals. FGF23 suppresses PTH in rodents (blue arrow). FGF23 also suppresses P co-transporters expression and calcitriol synthesis in the kidney (blue arrows). Downregulation of *SLC34A1* and *SLC34A3* expression in the proximal tubules of kidney decreases renal P reabsorption (black arrow) and induce urinary P excretion, thereby lower blood levels of P. Similar to FGF23, PTH also downregulates *SLC34A1* and *SLC34A3* in the kidney (yellow arrow) and reduces renal P reabsorption. However, different from FGF23, PTH stimulates the synthesis of calcitriol in the kidney (yellow arrow) and thereby increases intestinal P absorption (green arrow) and the release of P by bone resorption, which increase blood P (black arrows). However, the net effect of PTH is to decrease blood P concentration. About PTG: there is only one pair of PTGs in pigs which are embedded in the cranial part of the thymus near the carotid bifurcation and not close to the thyroid gland. Figure was created with BioRender.com.

A knockout mice study has demonstrated that *SLC34A1* is responsible for approximately 70% of renal P reabsorption (Beck, Karaplis et al. 1998). Similar studies have also suggested the important role of

SLC34A3 in renal P reabsorption in mice as it may account for ~30% of P absorption in the kidney (Ohkido, Segawa et al. 2003, Tenenhouse, Martel et al. 2003). Interestingly, it has been found that the *SLC34A3* is highly expressed in the kidney of rodents during weaning (Segawa, Kaneko et al. 2002). In addition, genetic studies in humans have also identified a key role of *SLC34A3* in the regulation of P homeostasis in human (Bergwitz, Roslin et al. 2006, Jaureguiberry, Carpenter et al. 2008). However, the expression levels and P handling function of these two renal P transporters are regulated by other regulatory factors such as FGF-23, PTH and dietary P. PTH and FGF23 decrease renal P reabsorption by downregulating the *SLC34A1* and *SLC34A3* expression in the proximal tubules of kidney and induce urinary P excretion (Bacic, LeHir et al. 2006, Gattineni, Bates et al. 2009). The amount of dietary P has also changed the gene expression level of the *SLC34A1* and *SLC34A3* in pigs (Pokharel, Regassa et al. 2017). In addition, kidney is the place where the calcitriol is synthesized by the help of *CYP27B1* (Bikle 2014). In the kidney, *CYP27B1* is inhibited by FGF23 and high level of Ca and P. The FGF23 suppresses the synthesis of calcitriol in the proximal tubules of kidney by repressing the expression of *CYP27B1* (Perwad and Portale 2011). In contrast, the PTH induces *CYP27B1* and stimulates the synthesis of calcitriol in the kidney, which increases intestinal P absorption and the release of P by bone resorption (Chande and Bergwitz 2018).

1.1.2.3 Contribution of the skeletal system

The skeletal system plays an important role in P homeostasis, as the vast majority of the P in the body (~85%) are stored in the mineral deposits of bone and teeth (Herman and Dallemagne 1961). Together with Ca, P plays an important role in the mineralization of bone, as the majority of bone mass is composed mainly of a calcium phosphate-based mineral named hydroxyapatite (Hughes, Robinson et al. 2019). The bone matrix vesicles acquire P either by hydrolysis of phosphoric esters to inorganic P by alkaline phosphatase (ALP) or by uptake of extracellular P via Type II sodium-phosphate cotransporters to form hydroxyapatite crystals, which mineralize the extracellular matrix of the bone. As such, the skeletal system greatly contribute to the maintenance of P homeostasis by keeping balances between bone formation and resorption. Type I collagen, encoded by COL1A1 and COL1A2 in osteoblasts, is the major component of the organic bone matrix and therefore plays a key role in bone stability. Therefore, any defect in these two genes or other genes involved that regulate the post-translational modification, secretion and processing of type I collagen inhibits the production of type I collagen in osteoblasts and disrupts the normal osteogenesis process (Tauer, Robinson et al. 2019). However, a recent study has shown that only trabecular bone in pigs is sensitive to low dietary P levels (Gerlinger, Oster et al. 2020).

Bone plays as an important endocrine regulator of the body's P homeostasis via endocrine and metabolic regulation mechanisms through the secretion of FGF23 (Figure 2). FGF23 is an important phosphaturic hormone secreted primarily by osteoblasts and osteocytes in bone. FGF23 favors the reduction of P reabsorption in the kidney by downstream signaling events (Erben 2016, Kawai, Kinoshita et al. 2016). Specifically, FGF23 need to bind the receptor FGFR1 by the help of the co-receptor αKlotho to regulate P reuptake in the kidney through activation of the ERK1- ERK2 signaling pathway (Andrukhova, Zeitz et al. 2012). As such, FGF23 coordinates bone mineral metabolism with renal P handling by forming the FGF23/FGFR1/αKlotho complex in vertebrates.

Osteoblasts and osteoclasts are the two important contributing cell types in bone responsible for bone formation and resorption processes, respectively, in a very complex system involving the function of hormones (e.g. e.g., PTH, calcitriol), P-transporters (e.g., SLC20A1, SLC20A2), ERK1-ERK2 signaling,

RANK (receptor activator of NF-κB)-RANKL (RANK ligand) signaling, and osteoprotegerin (OPG) are involved via feedback mechanisms (Chande and Bergwitz 2018). These processes are also closely linked to other aspects of the complex regulatory network of Ca and P homeostasis. The RANKL/RANK/OPG system has been shown to play an important role in bone modeling and remodeling by acting on osteoclasts and regulating their formation and function (Boyce and Xing 2008). The formation and proper function of osteoclasts appears to require RANK-RANKL signaling. Mice in which either the RANK or RANKL gene was knocked out appear to have impaired osteoclast formation (Li, Sarosi et al. 2000). In osteoclasts, high P concentration inhibits RANK-RANKL signaling and NF-κB expression, which then blocks osteoclast formation, survival, and bone resorption (Kanatan, Sugimoto et al. 2003, Mozar, Haren et al. 2008). On the other hand, osteoprotegerin (OPG), an inhibitory receptor, blocks RANK-RANKL signaling by binding to RANKL and preventing it's binding to RANK, thereby protecting bone from excessive resorption (Boyce and Xing 2008).

1.1.2.4 Contribution of the PTG

PTGs is an important contributor of mineral homeostasis and bone mineralization in both human and animals. In pigs, there is only two small sized PTGs which are embedded in the cranial part of the thymus near the carotid bifurcation and not close to the thyroid gland (Oster, Keiler et al. 2018). The main role of the PTG is to produce and release PTH that regulate blood calcium levels, which is closely related to P homeostasis (Figure 2). There are two major cell types in the PTG, small sized chief cells and larger oxyphil cells. The chief cells are the dominant functional cell type in PTG with their function to synthesize and release PTH (Cozzolino, Monciino et al. 2020). The level of PTH secretion in the PTG is also influenced by many other hormonal and non-hormonal factors including vitamin D (e.g., calcitriol), FGF23, and dietary P levels (Centeno, Herberger et al. 2019). As such, PTH also plays a pivotal role in P homeostasis by tightly regulating the excretion of P from kidney, and reabsorption of P from the intestine and bones (Underland, Markowitz et al. 2020). It has been demonstrated that secretion of PTH in the PTG is highly sensitive to the amount of dietary P. A higher level of dietary P immediately increased PTH secretion (Martin, Ritter et al. 2005), whereas PTH lowers serum P concentration by inducing P excretion (see section 1.1.2.2). Regarding the underlying molecular mechanisms of the direct effect of dietary P on PTH secretion, further studies have shown that stimulation of PTH secretion by higher P levels was accompanied by increased PTH gene expression through a post-transcriptional mechanism via parathyroid protein-PTH mRNA interactions that regulate PTH mRNA stability (Canalejo, Rodríguez et al. 2020). In addition, PTH secretion in the PTG in response to changes in Ca and P is also directly involved with some specific miRNAs (Canalejo, Rodríguez et al. 2020). Furthermore, studies have shown that calcitriol reduces PTH secretion by acting directly on VDRE in PTH promotor region and indirectly via serum calcium and the calcium-sensing receptor (CaSR) (Silver, Russell et al. 1985, Canaff and Hendy 2002). However, PTH stimulates calcitriol synthesis in the kidney (see section 1.1.2.2). Moreover, regulation of P homeostasis by the PTH also relies on other receptors and factors such as FGFR1, PTH1R and SPP1 (Maeda, Okazaki et al. 2013, Gattineni, Alphonse et al. 2014, Yuan, Jiang et al. 2014, Fan, Bi et al. 2016). Taken together, the overall net effect of PTH is to decrease serum P levels.

1.1.2.5 Contribution of the liver

Together with the kidney, the liver also contributes significantly to the maintenance of the body's P homeostasis, as it plays an important role in vitamin D metabolism. Specifically, liver expresses the vitamin D binding protein (GC; also named DBP), where vitamin D metabolites bind to be transported within the body. The liver is the primary site of production of 25-hydroxyvitamin D (25OHD, also known as calcidiol), the major circulating form of vitamin D. The natural form of vitamin D is transported to the liver by DBP and converted to 25-hydroxyvitamin D by a series of 25-hydroxylases such as CYP2R1 and CYP27A1. 25-hydroxylation can occur in both the mitochondrial and microsomal fractions of the liver. CYP2R1 is the major 25-hydroxylase located mainly in the endoplasmic reticulum of the liver and 25-hydroxylates vitamin D, whereas CYP27A1 is the mitochondrial 25-hydroxylase (Zhu, Ochalek et al. 2013). Knockout studies suggested that these two 25-hydroxylases compensate for each other, and there are also other enzymes with 25-hydroxylase activity (e.g., CYP3A4, CYP2C11) that may contribute to the necessary levels that reflect metabolic demands of 25-hydroxyvitamin D (Bikle 2014). 25-hydroxyvitamin D (biologically inactive form of vitamin D) is then transported to the kidney by DBP and metabolized (1a-hydroxylation) to 1, 25(OH)2D (Calcitriol), the active form of vitamin D, via the responsible enzyme encoded by CYP27B1 (see section 1.1.2.2). Calcitriol need to bind a transcription factor, the vitamin D receptor (VDR), to mediate renal P homeostasis (see section 1.1.1). As such, vitamin D may directly and indirectly regulate P homeostasis and contribute to relevant developmental processes including bone development and remodeling. In fact, numerous studies have shown the importance of healthy liver function in maintaining P homeostasis. Evidences from studies of P homeostasis in hepatectomy rat model suggest that, injuries in liver could effect on the levels of renal and intestinal sodium-dependent P transporters and the PTH, and causes hypophosphatemia due to abnormal urinary nicotinamide (NAM) metabolism that not associated with known hormonal factors such as FGF23 and PTH (Nomura, Tatsumi et al. 2014). This evidence indicate that liver also play a significant role in P homeostasis through the NAM metabolism pathway of the liver-kidney axis. In addition, high-P diets were also shown to affect lipid metabolism in rat liver through utilization of free fatty acids (Chun, Bamba et al. 2016).

1.1.2.6 Contribution of the other organs/tissues

In addition to the main organs and tissues described in above, some other organs and tissues are might also involve in P homeostasis. It has been shown that the important phosphate-regulatory hormone, FGF23, is also expressed in many other tissues including brain, thymus, lung, lymph node, spleen, stomach, and testis (Bergwitz, Roslin et al. 2006, Liu, Zhou et al. 2006, Ramon, Kleynen et al. 2010). In addition, we have also found that the sodium-dependent P transporter genes were also present in other tissues such as lung, muscle, vascular tissue and stomach (Wubuli, Reyer et al. 2019). Moreover, it has been suggested that abnormal phosphate homeostasis is associated with cardiovascular disease (Holden, Hétu et al. 2019).

1.1.2.7 Contribution of the microbiota

The majority of P in the fodder plant is present in the form of phytate-bound P, although the percentage of phytate-bound P in total P is varies greatly among species of plants (Eeckhout and De

Paepe 1994). Phytate or phytic acid (myo-inositol hexaphosphate, $C_6H_{18}P_6O_{24}$) is a complex form of six P groups and inositol found in many plant tissues (Figure 3). Although plants have some intrinsic phytase, it is less active in the intestine than microbial phytase because phytases of plant origin are more susceptible to the internal body environment (e.g., PH, peptic digestion) (Phillippy 1999, Schlemmer, Jany et al. 2001, Zimmermann, Lantzsch et al. 2002). In addition, phytate has very low digestibility (20∼30%) and utilization in the monogastric animals because they have very low activity of the endogenous intestinal phosphatases in the stomach and intestinal tract for phytate hydrolysis and release inorganic P Into the gut lumen which could be subsequently transferred by paracellular and transcellular transport mechanisms (Eeckhout and Depaepe 1994, Pallauf and Rimbach 1997, Herrmann, Ruff et al. 2019). Although phytases can be produced by the microbiota in the digestive tract, phytase activity in pigs is very limited due to the lack of significant endogenous and intestinal microbial phytases (Schlemmer, Jany et al. 2001). As a result, more than half (50∼80%) of the plant-resourced P intake in pigs is excreted as waste when they are fed diets without added phytase because they cannot digest phytate (Kornegay, Harper et al. 1997, Schlemmer, Jany et al. 2001). Therefore, improving the bioavailability and digestibility of P from plant origin is important to improve P efficiency in livestock production. In practice, exogenous bacterial phytases has traditionally been used as a dietary supplements in industrial livestock production to increase bioavailability of plant sourced P and reduce the environmental impact of excreted P. Commercial bacterial phytases showed higher efficacy with a high release rate of phytate-bound P in pigs (Augspurger, Webel et al. 2003, Adeola, Olukosi et al. 2006). Studies have also suggested that, the addition of exogenous microbial phytase supplementation to the diet of monogastric animals improves the digestibility of P and Ca from phytate and enhances the intestinal absorption of minerals, greatly reduces the need for supplemental inorganic P and the excretion of P into the environment (Selle and Ravindran 2007, Selle, Cowieson et al. 2009, Almeida, Sulabo et al. 2013). A study showed that the addition of *E. faecium* to the diet increased P utilization and bone mineralization in broilers by improving intestinal P absorption, increasing alkaline phosphatase concentration, and altering intestinal microbial composition (increased abundance of *Alistipes, Eubacterium, Rikenella* and *Ruminococcaceae*) (Wang, Cai et al. 2020). In monogastric animals, however, the microbial community in the intestinal tract, e.g., *Bifidobacteria, Lactobacillus, Pediococcus,* and *Pseudomonas spp.* contribute to nutrient digestibility and phytate degradation (release of P from inositol phosphates) by producing phytase to increase the bioavailability of P from plant sources (Haros, Bielecka et al. 2005, Hosseinkhani, Emtiazi et al. 2009, Raghavendra and Halami 2009, Asghar, Arif et al. 2016). This evidence implies the broad potential ability of the intestinal microbiota to degrade phytate and provide P in the gastrointestinal tract of monogastric animal species.

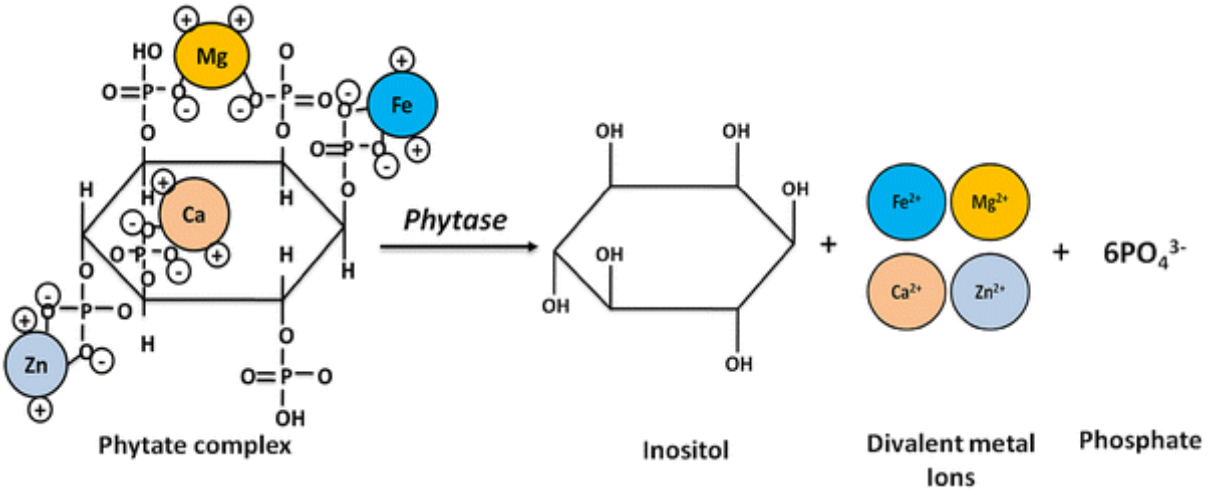

Figure 3. Schematic representation of phytate hydrolysis reaction. Phytase cleaves the scissile phosphoester bonds of phytate molecules to release P groups, myo-inositol, and metal ions. Adapted from "Cereal phytases and their importance in improvement of micronutrients bioavailability" by Vashishth A, 2017, *3 Biotech*, 7, p. 42.

1.2. Phosphorus efficiency in the animal husbandry and environment

1.2.1. Biological importance of improving physiological P efficiency in livestock

As we know it, P has the same fundamental importance for the life as water, carbon and oxygen. If use inefficiently, the certain amount of P consumed by farm animals will be wasted by excreted via manure and causes serious environmental burden. Studies suggested that, the normal P demand tend to decrease with age and it is higher in young individuals than adults because the young individuals need more P for their growth and development. Therefore, an adequate supply of nutritional P is necessary to ensure maximum growth and healthy skeletal development in farm animals. In practice, however, it often goes beyond the age-specific requirements in intensive livestock production. An excessive supply of inorganic P might not only has negative effects to the normal growth processes, but also breaks the natural P cycle through excessive excretion of the consumed P to the environment (Fernandez, Poulsen et al. 1999, Poulsen, Jongbloed et al. 1999). Rapid growth and intensification of the livestock industry, especially the commercial pig productions, is considered as important sources of environmental P loads (Oster, Reyer et al. 2018). Thus, study the physiological mechanism of P homeostasis of pigs might help to improve efficiency of P utilization in pig husbandry and reduce its environmental impact. Body's P homeostasis is achieved through close coordination between many organs and tissues including the intestinal tract, kidney, bone and PTG through several hormones and regulatory factors such as PTH, calcitriol, and FGF23 (Berndt and Kumar 2009). Deeply understanding of the underlying physiological mechanisms of P homeostasis in livestock could lead to improve efficiency of P utilization and reduce excess excretion of consumed P to the environment (Elser and Bennett 2011).

1.2.2. Current approaches in improving P efficiency

Because of the multiple functional and structural roles of P in both plants and animals, P use efficiency is a complex feature to decipher. Thus, managing P to minimize environmental impact in farm animal husbandry depends on numerous factors such as bio-availability, microbiota, genetics, P utilization and recycling (Oster, Reyer et al. 2018). Hence, interdisciplinary experimental approaches are required to investigate efficiency of P usage within the animal husbandry and identify possible strategies to improve P use efficiency. Feed supply appropriate to species, age and developmental stage has a major positive impact on P efficiency in livestock production. In order to reduce the environmental impact of high P excretion levels in the modern farming system, multiple measures, e.g., liquid feeding, phase feeding, phytase super dosing, or feed pre-treatment, have been applying towards improving P use efficiency. In this context, phase feeding is a widely used practice in livestock production to improve P efficiency and reduce P excretion by adjusting P requirements for specific genetic lines at different stages of the animal life cycle to accurately meet the nutrient needs of the animal compared to a feeding practice during the growing and finishing stages (Pomar, Hauschild et al. 2009). Liquid feeding is also used in pig production worldwide, which increases the total tract digestibility of phytate-bound P of cereals compared to traditional dry feeding by pre-digesting the feed (Blaabjerg, Jørgensen et al. 2010). In addition, other approaches such as feeding low phytate cereals and/or improving phytate digestibility through the strategic use and evaluation of various hydrolytic enzymes (e.g., microbial phytase) are also used to improve P utilization (Augspurger, Webel et al. 2003, Selle and Ravindran 2007, Hill, Sutton et al. 2009). Furthermore, since the P homeostasis and individual P demand in farm animals have been shown to be influenced by genetic factors (Alexander, Qu et al. 2008, Pokharel, Regassa et al. 2017, Reyer, Oster et al. 2019), genetic approaches may provide opportunity to improve P utilization by developing/selecting animals with enhanced P utilization such as phytase transgenic pig (Golovan, Meidinger et al. 2001). In addition, the reuse of P from animal manure also helps to reduce the P load on the environment.

1.2.3. Nutritional programming

Numerous studies have demonstrated that, the nutritional challenges (e.g., malnutrition or over-nutrition) during the fetal development or immediate postnatal period can induce long-term relatively nonreversible changes in the gene expression and phenotype of the offspring (Weaver, Cervoni et al. 2004, Gallou-Kabani, Vigé et al. 2007, Godfrey, Lillycrop et al. 2007). This process is known as nutritional or metabolic programming. The consequent epigenetic modifications in organs and metabolism induced by nutritional programming are could be inherited trans-generationally and offer the possibility of inducing different and stable phenotypes (Gonzalez-Bulnes, Astiz et al. 2014, Chamorro-Garcia, Diaz-Castillo et al. 2017). There are many underlying mechanisms of nutritional programming such as DNA methylation, non-coding RNA (e.g., miRNA, siRNA), histone modification, and genomic imprinting that regulates gene expression at a transcriptional and posttranscriptional level. DNA methylation is a critical underlying mechanism of nutritional programming that can permanently change the organism's genetic activity and metabolism (Huypens, Sass et al. 2016, Miska and Ferguson-Smith 2016). DNA methylation is an epigenetic mechanism by which methyl (CH_3) groups are added to 5- carbon of the cytosine bases at the cis-acting element (e.g., promoter, enhancer) of the DNA molecule by DNA methyltransferase (Dnmt), thereby modify the function and expression status of the genes without changing the DNA sequence. In mammals, DNA methylation occurs almost

exclusively at cytosine bases in CpG sites, with the exception of promoters, where CG sites tend to be protected from methylation (Lister, Pelizzola et al. 2009). However, methylation in the promoter region usually downregulate (or silence) gene expression by blocking the access of transcription factors (Suzuki and Bird 2008). It has been shown that maternal dietary methyl supplements increased the level of DNA methylation in the *agouti* long terminal repeat (LTR) and changed the phenotype of the offspring (Cooney, Dave et al. 2002). Although numerous studies have been conducted to date to investigate P utilization and its interrelation with bone metabolism and immune parameters from the perspective of traditional genetics and transcriptional regulations (Hittmeier, Grapes et al. 2006, Alexander, Qu et al. 2008, Reyer, Oster et al. 2019), however, the epigenetic mechanisms behind the P utilization in mammals is yet to be elucidated. The identification of epigenetic markers in DNA sequences relevant for the maintenance of P homeostasis could contribute to the understanding of the intrinsic transformation of environmental factors in higher mammals.

1.2.4. Metabolic specifications during gestation, lactation and development (growth)

Numerous studies investigating P utilization and its relationship to bone metabolism during a period of high nutrient demand for anabolic processes have been conducted in growing pigs (Kebreab, Schulin-Zeuthen et al. 2007, Alexander, Qu et al. 2008, Hill, Sutton et al. 2009). In sows, however, the requirement for the mineral P differs considerably from that of growing pigs and is mentioned in the relevant feeding recommendations (2006). During pregnancy and farrowing, the body of sows undergoes many critical metabolic changes that have lasting effects on the nutritional and metabolic demands of the organism. Reproductive sows can adjust their metabolism during pregnancy and lactation to make the best use of available nutrient resources for pre- and postnatal care of fetuses and offspring. Thus, for pregnant and lactating sows, the feed is prepared to ensure an adequate supply of nutrients for maintenance, fetal and uterine development, and milk production during lactation (Mahan 1990). If the dietary energy intake is insufficient to meet the energy requirements, lactating sows can compensate for the nutrient deficiency by mobilizing their nutrient reserves, especially protein or fat (Eastwood, Leterme et al. 2016). In this way, small amounts of P can be mobilized to complement the dietary intake P (Bikker and Blok 2017). In addition, as is known in mammals, the animal can compensate for fetal and early postnatal mineral requirements by increasing intestinal minerals absorption (Kovacs 2016). However, the digestibility of dietary mineral P sources is generally lower in reproductive sows than in growing pigs (Kemme, Jongbloed et al. 1997, Lee, Casas et al. 2018), resulting in increased excretion of ingested P content into the environment (Poulsen 2000). Therefore, a better understanding of the specific endogenous mechanisms of P homeostasis during growth, gestation, and lactation through specific dietary supplementation could help to develop a feeding strategy for more sustainable P utilization in the swine industry.

1.2.5. Genetic factors of P homeostasis

Numerous studies have shown that the utilization of P in livestock animals is, together with other factors, influenced by genetics. The sodium-dependent P transporter genes, which are considered the most important P transporters in mammals, have been shown to play an important role in the body's P homeostasis by affecting the intestinal absorption and renal retention, storage and usage of P in skeletal tissues (Beck, Karaplis et al. 1998, Ichikawa, Sorenson et al. 2006, Iwaki, Sandoval-Cooper et al. 2008, Sabbagh, O'Brien et al. 2009). In addition, it has been demonstrated that certain P characteristics (e.g., P utilization, bone mineralization) in monogastric animal species varies within and between different genetic breeds and is thus influenced by genetic and transcriptional variations (Hittmeier, Grapes et al. 2006, Alexander, Qu et al. 2008, Beck, Stratz et al. 2016, Rothammer, Bernau et al. 2017, Reyer, Oster et al. 2019). Therefore, it is important to re-evaluate strategies for improved P utilization in livestock production considering animal-intrinsic factors such as genetics and specific metabolic mechanisms of modern genetic breeds.

1.2.6. Sodium-dependent P co-transporters

The sodium-dependent P co-transporters are known as the main P transporters in mammals that consist of three structurally independent subfamilies, type I (SLC17 family), type II (SLC34 family) and type III (SLC20 family) sodium-dependent P co-transporters (Lederer and Miyamoto 2012). Under normal physiological condition, all the sodium-dependent P co-transporters transport P ions exclusively sodium-dependent manner. So far, nine sodium-dependent P co-transporters from three subfamilies, including *SLC17A1* (Solute Carrier Family 17 Member 1), *SLC17A2* (Solute Carrier Family 17 Member 2), *SLC17A3* (Solute Carrier Family 17 Member 3), *SLC17A4* (Solute Carrier Family 17 Member 4), *SLC20A1* (Solute Carrier Family 20 Member 1), *SLC20A2* (Solute Carrier Family 20 Member 2), *SLC34A1* (Solute Carrier Family 34 Member 1), *SLC34A2* (Solute Carrier Family 34 Member 2), and *SLC34A3* (Solute Carrier Family 34 Member 3), were annotated in pigs (Table 1). Type I family members are predominantly expressed in the kidney and liver, but are also present individually in many other tissues (Reimer 2013, Wubuli, Reyer et al. 2019). Although the type I sodium-dependent P co-transporters were originally identified as P transporters, they have been shown to also function as transporters for organic anions (e.g. urate) in the normal physiological environment (Reimer and Edwards 2004, Jutabha, Endou et al. 2012, Togawa, Miyaji et al. 2012). in addition, according to studies, the P transport activity of the P transporters from the type I family (*SLC17A1, SLC17A2, SLC17A3* and *SLC17A4*) is relatively weak compared to the P transporters of other families (Werner, Moore et al. 1991). P transporters of the type II family (*SLC34A1, SLC34A2,* and *SLC34A3*) are considered to be the most important players in P homeostasis and are known to be expressed in a variety of tissues including kidney and intestinal tract (Murer, Forster et al. 2004). Members of this family have over 80% sequence similarity with differences mainly in the intracellular C- and N-terminal regions (Forster, Köhler et al. 2002). In this family, *SLC34A1* and *SLC34A3* are mainly expressed in the kidney, whereas *SLC34A2* is predominantly expressed in the intestine. Type II sodium-dependent P co-transporters are located at the apical membrane of epithelial cells in renal proximal tubules and enterocytes, and serve as the main actor for active transcellular transport of P. The type III sodium-dependent P co-transporters (*SLC20A1* and *SLC20A2*) were originally identified as retroviral receptors Glvr-1 and Ram-1, respectively. Their function of sodium-dependent P transportation was confirmed later. Members of this family are ubiquitously expressed in all kind of mammalian tissues and cell types, thereby considered as housekeeping genes necessary for intracellular P homeostasis and supplying cells with inorganic P for normal cellular functions (e.g., endoplasmic reticulum homeostasis, DNA replication) (Kavanaugh and Kabat 1996). They are expressed at very low levels in the kidney and intestinal tract and may also contribute only partially to active transcellular P transport (Tenenhouse, Gauthier et al. 1998, Tenenhouse, Roy et al. 1998). However, recent studies have reported some specific physiological roles of this family members. For example, SLC20A1 appeared to play an important role in bone P homeostasis and bone metabolism (Palmer, Zhao et al. 1999, Collins, Bai et al. 2004). A recent study showed that P-dependent secretion of FGF23 is also modulated independently from other endocrine regulatory circuits by the SLC20A2 (Bon, Frangi et al. 2018). Furthermore, SLC20A1 may also play a role in P induced vascular physiology and effects of p on parathyroid function (Tatsumi, Segawa et al. 1998, Lau, Festing et al. 2010).

Table 1. Gene information of sodium/phosphate co-transporters.

Gene	Gene Synonyms	Ensembl ID (v. 91)	Description
SLC17A1	*NAPI-1, NPT1*	ENSSSCG00000001107	Solute carrier family 17 member 1

SLC17A2	*NPT3*	ENSSSCG00000036191	Solute carrier family 17 member 2
SLC17A3	*NPT4*	ENSSSCG00000037547	Solute carrier family 17 member 3
SLC17A4	*NPT5*	ENSSSCG00000031944	Solute carrier family 17 member 4
SLC34A1	*NaPi-2a, NPT2a*	ENSSSCG00000037535	Solute carrier family 34 member 1
SLC34A2	*NaPi-2b, NPT2b*	ENSSSCG00000008758	Solute carrier family 34 member 2
SLC34A3	*NaPi-2c, NPT2c*	ENSSSCG00000040105	Solute carrier family 34 member 3
SLC20A1	*GLVR1, Glvr-1, PiT1*	ENSSSCG00000032288	Solute carrier family 20 member 1
SLC20A2	*GLVR2, Glvr-2, MLVAR, PiT2, Ram-1*	ENSSSCG00000007027	Solute carrier family 20 member 2

2. Aim of the thesis

Animal husbandry, especially the commercial pig productions, is considered as important sources of environmental P loads. The aim of the current thesis is to investigate the animal-intrinsic mechanisms of P homeostasis in pigs fed with divergent levels of dietary P supply to identify P relevant characteristics and mechanism from the molecular perspective that might contribute to improving P utilization/efficiency in pig production and thus reduce the environmental impact of excess P loads. Therefore, we first wanted to explore how sodium-dependent P transporters, considered the major P transporters in mammals, contribute to P homeostasis and respond to different levels of P feeding in different tissues of growing pigs. Secondly, we aimed to investigate the comprehensive effect of deviant dietary P levels on reproductive pigs and what endogenous mechanisms are called upon in the kidney and jejunum for this purpose. Blood metabolites and hormones related to P homeostasis, as well as transcriptional responses in kidney cortex and jejunum mucosa, were analyzed by qRT-PCR and RNA sequencing.

3. Publications

3.1. Publications summary

The central theme of this dissertation is the intrinsic mechanisms of P homeostasis. Each study in itself reveals important molecular and genetic aspects that contribute to the complex regulatory mechanisms of P homeostasis. The results of the divergent dietary P feeding trial of the first study showed that the sodium/phosphate co-transporter responds to divergent level of dietary P supply in a broad range of pig tissues. In this study, *SLC34A1* and *SLC34A3*, two major active P transporters in mammals, showed higher gene expression levels in the low P diet group than in the high P diet group in kidney and intestine of pigs. This suggests that dietary interventions alter gene expression of key P transporters in relevant tissues that respond to aberrant dietary P uptake to maintain P homeostasis in animals (International Journal of Molecular Sciences 20 (22): 5576, 1-12).

In addition, the transcriptional responses in kidney of sows to different level of dietary P supplies were also investigated. The different amount of dietary P supply to sows during pregnancy and lactation triggered endocrine adaptation. Differential P intake revealed transcriptional responses in the kidney in the regulation of related to vitamin D metabolism. These results indicated that renal responses to a continuous dietary P reduction could trigger rather complicated molecular mechanisms to maintain balanced P level. (BMC Genomics 21: 626, 1-11).

Moreover, to estimate the microbial contribution to metabolize P sources, samples from caecum and colon digesta of piglets fed diets containing divergent levels of P were analyzed for microbial composition and phytate degradation. In this study, a low P diet triggers microbial-mediated phytate cleavage and P release. Further analysis has identifies a number of microbial species that might have potential to provide additional phytase activity and increase the bioavailability of P in the large intestine of animals fed a low P diet. (Microorganisms 9, no. 6: 1197).

3.2. Own contribution paper I & pdf

Tissue-wide gene expression analysis of sodium/phosphate co-transporters in pigs.

Wubuli, Aisanjiang, Henry Reyer, Eduard Muráni, Siriluck Ponsuksili, Petra Wolf, Michael Oster, and Klaus Wimmers. 2019. Tissue-Wide Gene Expression Analysis of Sodium/Phosphate Co-Transporters in Pigs. *International Journal of Molecular Sciences* 20, no. 22: 5576. (https://doi.org/10.3390/ijms20225576)

I hereby declare that my contribution in this publication summarized in this dissertation is as follows:

- Tissue sampling and processing
- Gene expression analysis
- Statistical analysis and visualization of all gene expression data
- Discussion and interpretation of the data
- Writing the manuscript

Abstract:

Sodium/phosphate co-transporters are considered important mediators of phosphorus (P) homeostasis. The expression of specific sodium/phosphate co-transporters is routinely used as an immediate response to dietary interventions in different species. However, a general understanding of their tissue-specificity is required to elucidate their particular contribution to P homeostasis. In this study, the tissue-wide gene expression status of all currently annotated sodium/phosphate co-transporters were investigated in two pig trials focusing on a standard commercial diet (trial 1) or divergent P-containing diets (trial 2). A wide range of tissues including the gastrointestinal tract (stomach, duodenum, jejunum, ileum, caecum, and colon), kidney, liver, bone, muscle, lung, and aorta were analyzed. Both trials showed consistent patterns in the overall tissue-specific expression of P transporters. While SLC34A2 was considered as the most important intestinal P transporter in other species including humans, SLC34A3 appeared to be the most prominent intestinal P transporter in pigs. In addition, the P transporters of the SLC17 family showed basal expression in the pig intestine and might have a contribution to P homeostasis. The expression patterns observed in the distal colon provide evidence that the large intestine may also be relevant for intestinal P absorption. A low dietary P supply induced higher expressions of *SLC20A1, SLC20A2, SLC34A1*, and *SLC34A3* in the kidney cortex. The results suggest that the expression of genes encoding transcellular P transporters is tissue-specific and responsive to dietary P supply, while underlying regulatory mechanisms require further analyses.

International Journal of
Molecular Sciences

MDPI

Article

Tissue-Wide Gene Expression Analysis of Sodium/Phosphate Co-Transporters in Pigs

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Received: 13 September 2019; Accepted: 5 November 2019; Published: 8 November 2019

Abstract: Sodium/phosphate co-transporters are considered to be important mediators of phosphorus (P) homeostasis. The expression of specific sodium/phosphate co-transporters is routinely used as an immediate response to dietary interventions in different species. However, a general understanding of their tissue-specificity is required to elucidate their particular contribution to P homeostasis. In this study, the tissue-wide gene expression status of all currently annotated sodium/phosphate co-transporters were investigated in two pig trials focusing on a standard commercial diet (trial 1) or divergent P-containing diets (trial 2). A wide range of tissues including the gastrointestinal tract (stomach, duodenum, jejunum, ileum, caecum, and colon), kidney, liver, bone, muscle, lung, and aorta were analyzed. Both trials showed consistent patterns in the overall tissue-specific expression of P transporters. While SLC34A2 was considered as the most important intestinal P transporter in other species including humans, SLC34A3 appeared to be the most prominent intestinal P transporter in pigs. In addition, the P transporters of the SLC17 family showed basal expression in the pig intestine and might have a contribution to P homeostasis. The expression patterns observed in the distal colon provide evidence that the large intestine may also be relevant for intestinal P absorption. A low dietary P supply induced higher expressions of *SLC20A1*, *SLC20A2*, *SLC34A1*, and *SLC34A3* in the kidney cortex. The results suggest that the expression of genes encoding transcellular P transporters is tissue-specific and responsive to dietary P supply, while underlying regulatory mechanisms require further analyses.

Keywords: phosphorus homeostasis; pig; tissue-specific transcript rate; transcellular Na+/Pi co-transporter

1. Introduction

Phosphorus (P) is essential for all living beings. It plays an important role in bone formation, energy metabolism (i.e., ATP), cell signaling (i.e., phosphorylation), cell membranes, blood buffering, and any processes involving nucleic acids [1]. In fact, monogastric farm animals need sufficient nutritional P supply for growth processes and to develop a healthy skeletal system. However, an inefficient and excessive supplementation of mineral P interferes with the natural P-cycle and might cause high environmental P loads [2,3]. Considering the importance of pig for the global meat production, strategies to balance environmental issues and animal welfare have to be identified.

Therefore, an improvement in global P efficiency including farm animal husbandry is desirable to achieve a sustainable P-cycle. A deeper understanding of animal-intrinsic P flows can contribute to this matter.

The utilization of P in livestock species is influenced by several factors, one of which is genetics [4,5]. Specifically, heritability estimates indicate that 42% of phenotypic variations in hematological P values of pigs are explained by genetics [6]. In particular, underlying genes might affect the gastrointestinal absorption of P, processes within the organism and cells, storage and usage of P in skeletal tissues, and the retention of P in the kidney [7,8]. Accordingly, promising candidate genes, which are frequently the subject of analyses in studies focusing on the regulation and maintenance of the P homeostasis, are sodium/P co-transporters [9,10].

Phosphate absorption is mediated by passive paracellular and active transcellular mechanisms. The latter serve e.g., for the directed enteral absorption and renal excretion of P, which is achieved via P transporting proteins. Various isoforms of these P transporters are known to be tissue-specifically expressed. In intestinal tissues, P transporters are located on the apical luminal sides of epithelial cells. In the kidney, they occur mainly in the proximal tubules. It has been shown that their gene expression is controlled by hormones such as parathyroid hormone (PTH), fibroblast growth factor-23 (FGF23), and calcitriol, which ensure transcellular P transport depending on dietary supply [11,12].

So far nine genes from three independent subfamilies, including *SLC17A1*, *SLC17A2*, *SLC17A3*, *SLC17A4*, *SLC20A1*, *SLC20A2*, *SLC34A1*, *SLC34A2*, and *SLC34A3* were annotated as sodium-dependent P co-transporters in pigs (Table 1). The members of the SLC17 family (also called type I sodium/phosphate co-transporter) were initially identified as sodium-dependent P co-transporters. However, recent evidence showed that their P transport activity is relatively weak compared to the other P transporter families [13]. Co-transporter of the SLC34 family (also called type II sodium/phosphate co-transporter) are considered to be major contributors to P homeostasis and are known to be expressed in a wide set of tissues [14]. The co-transporter of the SLC20 family (also called type III sodium/phosphate co-transporter) was initially identified as retroviral receptors, but later studies showed that they act as sodium-dependent P co-transporter [15]. All these P transporters were found in primate and rodent species, including humans and mice with relatively high sequence homology. However, according to current genome database only a part of them were found in *Sauropsida* (reptile and bird species). Although great efforts have been made to uncover molecular mechanisms of P homeostasis, a comparative tissue-wide gene expression profile of these transporters is still lacking in pigs. This will lead to a better understanding of how these P transporters contribute to P homeostasis and respond to different levels of nutritional P feeding conditions in different tissues.

Table 1. Gene information of sodium/phosphate co-transporters.

Gene	Gene Synonyms	Ensembl ID (v. 91)	Description
SLC17A1	*NAPI-1, NPT1*	ENSSSCG00000001107	Solute carrier family 17 member 1
SLC17A2	*NPT3*	ENSSSCG00000036191	Solute carrier family 17 member 2
SLC17A3	*NPT4*	ENSSSCG00000037547	Solute carrier family 17 member 3
SLC17A4	*NPT5*	ENSSSCG00000031944	Solute carrier family 17 member 4
SLC34A1	*NaPi-2a, NPT2a*	ENSSSCG00000037535	Solute carrier family 34 member 1
SLC34A2	*NaPi-2b, NPT2b*	ENSSSCG00000008758	Solute carrier family 34 member 2
SLC34A3	*NaPi-2c, NPT2c*	ENSSSCG00000040105	Solute carrier family 34 member 3
SLC20A1	*GLVR1, Glvr-1, PiT1*	ENSSSCG00000032288	Solute carrier family 20 member 1
SLC20A2	*GLVR2, Glvr-2, MLVAR, PiT2, Ram-1*	ENSSSCG00000007027	Solute carrier family 20 member 2

This study was designed to provide new insights into the tissue-specific gene expression of sodium/phosphate co-transporters in pigs. The analysis focuses on the transcriptional level as the primary control site for the expression of genes and as the basis for the repertoire and abundance of the corresponding gene products. The regulatory role of the transporters in P homeostasis is addressed in pigs with different dietary P supplies. Accordingly, two independent pig experiments were analyzed.

The first trial focuses on a standard commercial diet (Trial 1), whereas in the second trial the responses to low and high P diets (Trial 2) were investigated.

2. Results and Discussion

This study comprised of two datasets in order to provide an overview of the tissue-specific abundance of all sodium/phosphate co-transporters currently annotated in pigs. Trial 1 serves as an explorative data set to elucidate the absolute expression of P transporters in 15 different tissues and tissue compartments under basal conditions. While almost all transporters were abundant in kidney cortex, kidney medulla, and liver, specific expression patterns occur in tissues of the gastrointestinal tract and peripheral tissues (Figure 1). Trial 2 was designed to examine the effects of different levels of dietary P on the mRNA expression of these P transporter genes in P absorbing and reabsorbing tissues of growing pigs to distinguish dietary responses from constitutive abundance. In addition, all data from trial 2 were integrated to get insights into putative age- and breed-related differences in the P transporter expression profiles (Figure 2). In general, the expression profiles of the two trials were basically consistent. In addition, some of the sodium/phosphate co-transporters showed different expression patterns between high and low P group (Figure 3).

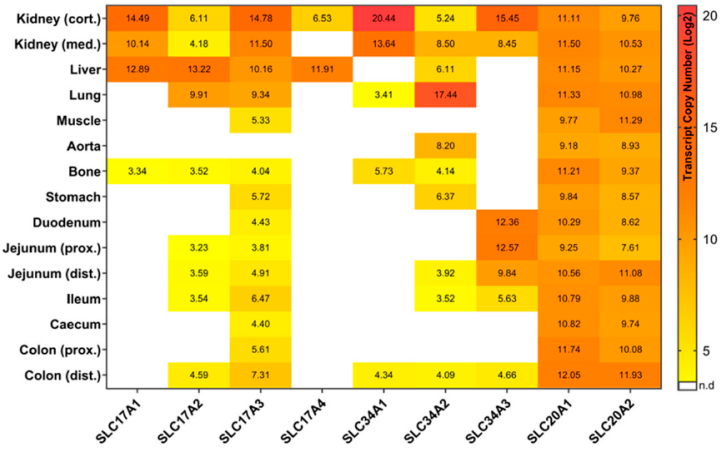

Figure 1. Heatmap of the gene expression of nine sodium/phosphate co-transporters in pigs on a standard commercial diet (Trial 1). In total five German Landrace pigs were fed a standard P diet for six months. Transcript copy numbers of all nine sodium/phosphate co-transporter were measured in 15 tissues by qRT-PCR. Copy numbers were displayed as log2 values.

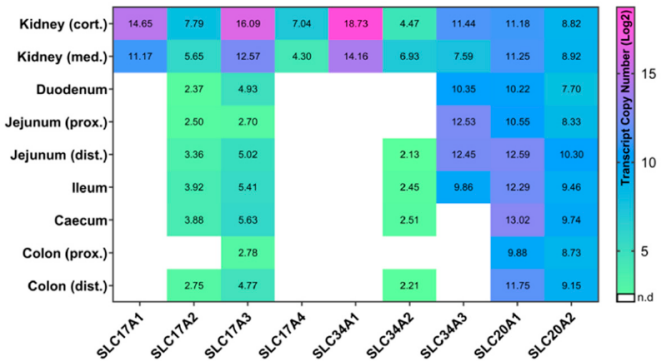

Figure 2. Heatmap of the integrated gene expression levels of nine sodium/phosphate co-transporters in pigs receiving divergent P-containing diets (Trial 2). In total 10 growing pigs were fed a high and low P diet for four month. Transcript copy numbers of all nine sodium/phosphate co-transporter were measured by qRT-PCR. In this heatmap, the average number of copies was calculated across all 10 pigs. Copy numbers were displayed as log2 values.

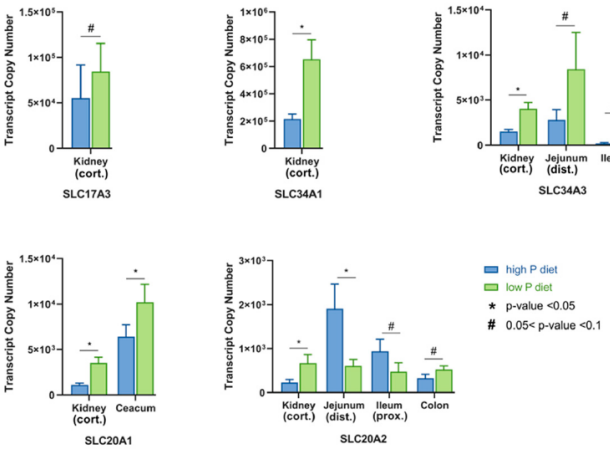

Figure 3. Transcript copy numbers of differentially expressed P transporters in pigs receiving divergent P-containing diets (Trial 2). Sodium/phosphate co-transporters differentially expressed between animals fed high and low P diets were identified. Superscripts indicate statistically significance (*, $p < 0.05$) and trend (#, $p < 0.1$).

2.1. Type I Sodium/Phosphate Co-Transporters Expression Analysis

Type I sodium-dependent P co-transporters are represented by four members: *SLC17A1*, *SLC17A2*, *SLC17A3*, and *SLC17A4*. In trial 1, *SLC17A1* has shown a limited expression profile in the 15 tissues, with the highest expression level in the kidney cortex and the lowest detectable expression in the bone (Figure 1). In trial 2, *SLC17A1* expression in the kidney was confirmed. For both trials no expression was measurable in the gastrointestinal tract. Previous studies revealed similar results, but also showed *SLC17A1* expression in brain samples, which were not included in the current study [16–18]. The expression of *SLC17A1* in bone tissue has not yet been described in other studies. The current results indicate that SLC17A1 might be responsible for the transport of uric acid from the liver to the serum and kidney. Indeed, *SLC17A1* expression was linked to renal urate export [19], and the transport of P across hepatic basolateral membranes [20,21]. Although SLC17A1 was initially identified as a sodium-dependent P co-transporter, this role is questioned due to the low level of its P binding affinity [22]. Current investigations point to other functions such as an organic anion transporter [23] or as a chloride-dependent P modulator [24,25]. However, in a knock-out mice study, changes in *SLC17A1* expression still seem to affect renal P homeostasis [26], requiring the clarification of the detailed mechanisms.

SLC17A2 showed a wide expression profile in both the peripheral and intestinal tissues in the two trials (Figures 1 and 2). Interestingly, its expression is considerably lower in the kidney compared to *SLC17A1* but showed relatively high expression levels in liver and lung tissue. In fact, previous studies also reported that the *SLC17A2* gene was expressed in a wide range of tissues including heart, muscle, kidney, liver, and lung [27]. It has multiple transport functions such as chloride-dependent anion transport, sodium-dependent P uptake, and poly-specific organic anion transport involved in urate circulation [27,28]. According to the expression profiles of both trials, SLC17A2 might further contribute to intestinal P absorption, albeit being low abundant in these compartments. Between the two trials some differences in expression profiles were observed, mainly due to lower detection limits.

The third type I sodium/phosphate co-transporter, *SLC17A3*, showed the broadest expression profile among the SCL17 family members in both trials (Figures 1 and 2). It showed relatively high expression values in kidney, liver, and lung and was solely not detectable in aorta. Overlapping findings are found in the Human Protein Atlas [29]. Due to its abundance in different tissues, SLC17A3 might act as a multi-specific organic anion transporter. SLC17A3 is known to be localized in the renal tubular cells and functions as a voltage-driven urate transporter [30]. Moreover, it seems to represent an excretory route for organic anionic agents as well as urate in vivo, which has been detected in hyperuricemia

patients with mutated SLC17A3 [31]. Interestingly, in both trials the *SLC17A3* gene is constitutively expressed in all segments of the gastrointestinal tract, which is known to play an important role in P uptake and transport (Figures 1 and 2). In addition, the expression of *SLC17A3* tended to be different in the kidney cortex of pigs fed divergent P-containing diets (Figure 3). Pigs receiving lower dietary P showed higher levels of renal (cortex) *SLC17A3* expression compared to the high P group.

The last member of Type I sodium/phosphate co-transporters, *SLC17A4*, showed a very limited expression in both trials. It was only expressed in the kidney and liver (Figures 1 and 2). Interestingly, this differs from findings in humans and rodents, where *SLC17A4* is specifically expressed in the gastrointestinal tract and acts as a polyspecific organic anion exporter with putative involvement in urea extrusion [29,32,33]. However, the results of the current study argue for very specific functions of SLC17A4 exclusively in the liver of pigs, although such a role is not yet described.

2.2. Type II Sodium/Phosphate Co-Transporters Expression Analysis

The Type II sodium/phosphate co-transporter family contains three members: *SLC34A1*, *SLC34A2*, and *SLC34A3*. They are considered as the major contributors of P homeostasis in mammals. To date, SLC34A1 and SLC34A3 have been regarded as important players in inorganic P homeostasis in the kidney, while SLC34A2 was suggested to be the most important inorganic P transporter in the intestine [8,34,35].

In the current study, *SLC34A1* was expressed in both trials mainly in the kidney and reached highest expression values among all tested genes (Figures 1 and 2). In mice, SLC34A1 has been shown to be responsible for up to 70% of renal P absorption [7]. Moreover, mutations of the *SLC34A1* gene are known to impaired P homeostasis in mice [9]. Interestingly, beside some abundance in lung and bone, *SLC34A1* was also detectable in the distal colon of trial 1, although only at a low level (Figure 1). The expression level of *SLC34A1* in the kidney cortex is different at a statistically significant level between high and low P groups, with more than three times higher values in the low P group compared to high P group (Figure 3). This observation is in accordance with a previous study focusing on divergent P supply in pigs [4]. Moreover, growing pigs on a low dietary P level exhibit lower serum P levels than those receiving a diet with recommended amounts of P [36]. Therefore, by increasing the renal *SLC34A1* mRNA abundance, the organism attempts to reabsorb P to maintain blood P levels.

SLC34A2 is expressed in both studies in different parts of the gastrointestinal tract and the periphery including the kidney (Figures 1 and 2). The degree of expression of *SLC34A2* in the kidney is considerably lower compared to the other two members of the SLC34 family. However, it is interesting that although SLC34A2 has been analyzed as the main actor of intestinal P absorption in rodents and humans [8,37], its expression levels in the intestinal tissue of pigs are relatively low in both trials. In addition, the *SLC34A2* gene is highly abundant in the lung, which is in accordance with other studies of rat and human [38,39]. Notably, it is abundant in stomach and aorta at relatively high levels.

In the two trials, the expression profiles of *SLC34A3* show specificity for segments of the small intestine as well as for the kidney (Figures 1 and 2). In intestine the expression values were highest in the duodenum and jejunum, decreased slightly in the ileum and were no longer detectable in the caecum and proximal colon, but were detectable again in the distal colon in trial 1. The small intestine, in particular the jejunum and duodenum are considered to be the main sites of P absorption in the intestinal tract [40,41]. Together, it seems that SLC34A3 may contribute for inorganic P absorption in both the renal and intestinal tract of pigs [42]. Similarly, mutations in *SLC34A3* suggested that this gene plays a key role in maintaining P homeostasis in humans [43,44]. In contrast, SLC34A3 in mice is exclusively expressed in the kidney and was not detected in the intestine [35,45], although similar renal expression profiles were found in mouse and pigs [4,46]. In addition, the expression of *SLC34A3* seems to be responsive to dietary P supply. Pigs from the low P group showed considerably higher *SLC34A3* expression in kidney, distal jejunum, and ileum compared to high P group (Figure 3). This is in accordance with an increased renal reabsorption and intestinal uptake of P with the aim of maintaining blood P values in pigs fed a low P diet.

2.3. Type III Sodium/Phosphate Co-Transporters Expression Analysis

The type III sodium/phosphate co-transporter family consists of two members: *SLC20A1* and *SLC20A2*. Both *SLC20A1* and *SLC20A2* genes are ubiquitously expressed in both trials of this study in all peripheral and intestinal tissues at relatively high levels (Figures 1 and 2). In fact, they are known as ubiquitously expressed genes in mammalian cells and were therefore regarded as "housekeeping" transporters of inorganic P to the cells [47,48]. It has been reported that type III sodium/phosphate co-transporters have dual functions both as viral receptors and as sodium/phosphate co-transporters [49–51]. Moreover, SLC20A1 and SLC20A2 are the major factors for P homeostasis in the brain of mice and humans, and SLC20A2 is also crucial for maintaining adequate P levels in the cerebrospinal fluid [52,53]. Recently, many studies have provided growing evidences that the SLC20A1 has multiple functions beyond its previously reported role as sodium/phosphate co-transporter. It has been shown that various cellular processes such as normal cell division and proliferation, cell density, cell apoptosis, and many other processes that are also independent of its P transport activity require a certain level of SLC20A1 [54–56]. Recently, Couasnay and co-workers identified functions of SLC20A1 independent from P transport for endoplasmic reticulum homeostasis, chondrocyte survival, and skeletal development [57]. In addition, Bon and colleagues have recently reported that SLC20A2, but not SLC20A1, is necessary for the corresponding P-dependent secretion of bone-derived fibroblast growth factor 23 (FGF23), which regulates serum P levels [58]. In trial 2 of the current study, *SLC20A1* and *SLC20A2* were differentially expressed in some tissues in the high and low P group (Figure 3). *SLC20A1* showed a higher expression in the kidney cortex and caecum in the low P group compared to the high P group. However, *SLC20A2* transcript rates were increased in the kidney cortex and proximal colon, but decreased in the distal jejunum and ileum when comparing low P and high P groups.

3. Materials and Methods

3.1. Animals

Animal trials in this study were approved by the Scientific Committee of the Leibniz Institute for Farm Animal Biology (FBN). The experimental setup was generally licensed and approved by the ethics committee of the federal state of Mecklenburg-Western Pomerania, Germany (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei). It was registered under the license LALLFM-V/TSD/7221.3–1-053–15 (16 Dec 2015). The tissue sets for expression analysis were generated in two trials. The first part of the study comprised five German Landrace fattening pigs, which were fed ad libitum a conventional diet according to recommendations [59] (Trial 1). The pigs (two females and three castrates) were housed for six month resulting in an average body weight of 118.4 ± 1.7kg. The second part of the study focused on P-divergent diets (Trial 2). In total 10 hybrids form a German Landrace × Large White × Pietrain cross were fed with P divergent diets from weaning (28 days postnatal) until slaughter (four months). The average body weight at slaughtering was 95.3 ± 10.0 kg. Five pigs (three males and two females) were fed with diets containing low mineral P (L) and five piglets (three males and two females) received diets containing high mineral P (H). From weaning to day 70, the achieved P and calcium levels were 5.2 g/kg and 9.8 g/kg (L) and 7.8 g/kg and 9.1 g/kg (H). In the finishing period, levels for P and calcium were 4.1 g/kg and 6.5 g/kg (L) and 7.0 g/kg and 6.7 g/kg (H). Neither phytase nor other phosphatases were added. Pigs had ad libitum access to pelleted feed and water.

3.2. Tissue Sampling

For trial 1, pigs were slaughtered at the age of six month in the experimental slaughterhouse of FBN. They were anaesthetized by electrical stunning and subsequently sacrificed by exsanguination. A total of 15 tissues were sampled according to Table 2 focusing on gastrointestinal tissues (stomach, duodenum, jejunum, ileum, caecum, and colon), kidney, liver, bone, muscle, lung, and aorta. Using the same procedure, the 10 pigs of trial 2 were slaughtered at the age of four month. Here nine

tissues with focus on P absorption (gastrointestinal tract) and P excretion (kidney) were sampled (Table 2). Gastrointestinal parts were washed with 0.9% NaCl to ensuring removal of residual digesta. Sampling positions are indicated in Table 2. All samples were cut in pieces and frozen in liquid nitrogen immediately. Samples were stored at −80 °C until downstream analysis.

Table 2. Tissue samples and their specifications taken for both trials.

Tissue	Short	Specification	Trial [1]
Kidney	Kidney cort	Cortex of left kidney	1, 2
Kidney	Kidney med	Medulla of left kidney	1, 2
Liver	Liver	Lobulus Spigelii	1
Stomach	Stomach	Fundus mucosa	1
Duodenum	Duod	Mucosa, 30–40 cm distal of pylorus	1, 2
Jejunum (prox.)	Jeju prox	Mucosa, 2 m distal of pylorus	1, 2
Jejunum (dist.)	Jeju dist	Mucosa, 2 m proximal of the ileocaecal junction	1, 2
Ileum	Ileum	Mucosa, 20 cm proximal of the ileocaecal junction	1, 2
Caecum	Caec	Mucosa	1, 2
Colon (prox.)	Colon prox	Mucosa, 50–60 cm distal of cecolic junction	1, 2
Colon (dist.)	Colon dist	Mucosa, 50–60 m proximal of rectum	1, 2
Bone	Bone	Calvarial bone, along the sagittal suture	1
Muscle	Muscle	Longissimus dorsi, between the 13th and 14th rib	1
Lung	Lung	Lower tip of the left lung lobe	1
Aorta	Aorta	Aorta, descending thoracic aorta	1

[1] Trial 1 represents pigs on a conventional standard diet and Trial 2 represents pigs on P divergent diets.

3.3. RNA Isolation and cDNA Synthesis

Total RNA was isolated from all tissues by TRI Reagent according to user guides (Sigma-Aldrich, Taufkirchen, Germany) and treated with Baseline-ZERO DNase mix (Biozym, Hessisch Oldendorf, Germany) for ensuring the removal of any genomic DNA residuals. The DNase treated RNA was purified with the column-based NucleoSpin RNA II-Kit (Macherey–Nagel, Düren, Germany). The concentration of final purified RNA was measured by the NanoDrop 2000 photo-spectrometer (PEQLAB, Erlangen, Germany). The existence of genomic DNA contamination was checked by PCR amplification of the ubiquitously expressed porcine ACTB (forward primer: 5′-GAGAAGCTCTGCTACGTCGC-3′; reverse primer: 5′-CCTGATGTCCACGTCGCACT-3′) and subsequent visualization on 2–3% agarose gel. For each sample, first-strand cDNA were synthesized from 1500 ng total RNA using random primers (Promega, Fitchburg, WI, USA) and oligo d(T) primers in the presence of Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). Existence of genomic DNA contamination in cDNA was also checked again by PCR amplification of porcine ACTB as mentioned above.

3.4. Quantitative Real-Time PCR (qRT-PCR)

Primers for all P transporter genes were designed using sequence information from the Ensembl database (accessed on February 2018) and the NCBI primer blast online tool (Supplementary Table S1). For each gene of interest primers for two amplicons were designed. Amplicons from a longer fragment were used to generate reference standard curves to allow the absolute quantification of copy numbers. The shorter nested fragment was intended for real-time PCR amplification of the respective P transporters. Performance of primers and an initial assessment of optimal amplification conditions were identified by end-point PCR. Standards were prepared by PCR amplification using SupraTherm Tag polymerase (GeneCraft, Lüdinghausen, Germany) and the following cycling conditions: An initial denaturation step at 95 °C for 3 min followed by 40 cycles consisting of denaturation at 95 °C for 15 s, annealing at corresponding annealing temperature for 60 s and extension at 72 °C for 60 s. PCR products of standards were checked by 2–3% agarose gel and purified using magnetic beads

(Beckmann Coulter, Krefeld, Germany). Concentrations of standards were measured using the NanoDrop 2000 photo-spectrometer.

Gene expression levels of all nine sodium/phosphate co-transporters and RPL32 as a housekeeping gene were quantified by qRT-PCR. Transcript copy numbers of every individual sample (two technical replicates per sample) were measured by the LightCycler 480 SYBR Green I Master system (Roche, Mannheim, Germany) according to the user guides. In detail, the reaction mix contained 6 µL of SYBR Green Master I mix, 0.6 µL of each primer, 2.8 µL of nuclease free water, and 2 µL cDNA. PCR were performed on the LightCycler 480 system.

The amplification program was set as follows: an initial denaturation at 95 °C for 5 min followed by 45 cycles consisting of denaturation at 95 °C for 10 s, annealing at the corresponding annealing temperature (Supplementary Table S1) for 15 s, and extension at 72 °C for 25 s. Transcript copy numbers for each sample were calculated based on standard curve method that uses the cycle threshold values of serial dilutions (10^7–10^0 copies) of the corresponding standard. Melting curve analysis was used to check amplified products.

3.5. Data Analysis

All data were analyzed by the open sourced R software (v.3.2.3; R Foundation for Statistical Computing, Vienna, Austria). Transcript copy numbers were normalized based on the expression of the housekeeping gene RPL32 and transformed by log2. The lower detection limits were adapted to the sample size. Accordingly, a mean copy number below 8 (log2 = 3) for trial 1 and a mean copy number below 4 (log2 = 2) for trial 2 were considered as non-detectable (n.d). For each tissue, transcripts should be detectable in at least 50 percent of the samples in order to be considered for subsequent data analysis. Tissue-specific numbers of transcript copies were averaged and represented as heatmap using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). For trial 2, the comparison of groups on a low and high P diet was performed using a linear regression model (R package stats v3.6) including dietary group as fixed effect and time of slaughter as covariate. Differences were considered as statistically significant at $p < 0.05$ and as trend at $0.05 < p < 0.10$. All figures were made by GraphPad Prism 8.

4. Conclusions

In the current study, according to the objective, the gene expression status of all nine known sodium/phosphate co-transporter genes was investigated in a broad range of pig tissues. In two independent pig trials, all P transporters exhibited a widely consistent expression pattern. Although the P transporters of the SLC17 family were not characterized as main actors in intestinal P absorption, *SLC17A2* and *SLC17A3* show a broad expression pattern in both peripheral and intestinal tissues of pigs. While SLC34A2 was considered as the most important intestinal P transporter in rodents and humans, *SLC34A3* showed a considerably higher abundance at the transcriptional level in the small intestine of pigs compared to *SLC34A2*. Therefore, the role of SLC34A2 in the intestines of pigs appears to be less pronounced compared to other species. However, further investigation of the protein expression level of the corresponding sodium/phosphate co-transporter might be of interest for further confirmation of these observations. Interestingly, seven and five out of nine sodium/phosphate transporters, including the important SLC34 family, were detectable in the distal colon of trial 1 and 2, respectively. Therefore, the distal colon might be also of relevance for intestinal inorganic P absorption. However, potential P transport functions of the distal colon still have to be confirmed experimentally. Regarding the responsiveness of P transporters to dietary P supply, two important P transporters, SLC34A1 and SLC34A3, showed higher gene expression in the low P group compared to the high P group in some kidney and intestinal tissues. Thus, it appears that the dietary regimen alters the level of expression of some P transporters in relevant tissues to maintain P homeostasis in the animal.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/22/5576/s1.

Author Contributions: Conceptualization, K.W., H.R.; methodology, A.W., H.R., M.O., S.P.; formal analysis, A.W., H.R., M.O.; investigation, A.W., H.R., M.O., E.M.; resources, P.W., K.W.; data curation, A.W., H.R., M.O.; writing—original draft preparation, A.W.; writing—review and editing, A.W., H.R., E.M., S.P., P.W., M.O., K.W.; visualization, A.W.; supervision, H.R., M.O., E.M., K.W.; project administration, K.W.; funding acquisition, K.W.

Funding: This study has received funding from the European Research Area Network on Sustainable Animal Production (ERA-NET SusAn) as part of the Pegasus Project (2817ERA02D). It was supported by the Leibniz Science Campus Phosphorus Research Rostock and matched funding from the Leibniz Institute for Farm Animal Biology (FBN).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

P	Phosphorus
qRT-PCR	Quantitative real time polymerase chain reaction
SLC	Solute carrier

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3.3. Own contribution paper II & pdf

Reduced phosphorus intake throughout gestation and lactation of sows is mitigated by transcriptional adaptations in kidney and intestine.

Aisanjiang Wubuli, Christian Gerlinger, Henry Reyer, Michael Oster, Eduard Muráni, Nares Trakooljul, Siriluck Wimmers, Klaus Wimmers. *BMC Genomics* 21: 626, 1-11. (doi: 10.1186/s12864-020-07049-0)

I hereby declare that my contribution in this publication summarized in this dissertation is as follows:

- Tissue sampling and processing
- Gene expression analysis
- Statistical analysis and visualization of gene expression data
- Discussion and interpretation of the data
- Writing the manuscript

Abstract:

The environmental impact of pig farming need to be reduced, with phosphorus (P) being of particular interest. Specified dietary regimens and management systems contribute to meet environmental concerns and reduce economic constrains. However, pregnant and lactating sows represent vulnerable individuals, whose reproductive potential and metabolic health status relies on adequate supply of macro- and micronutrients. The aim of this study was to investigate, whether sows fed with a dietary P content that is below or above current recommendations are capable to maintain mineral homeostasis during the reproduction cycle and which endogenous mechanisms are retrieved therefore in kidney and jejunum. Nulliparous gilts were fed iso-energetic diets with recommended (M), reduced (L), or high (H) amounts of mineral P supplements throughout gestation and lactation periods. Blood metabolites and hormones referring to the P homeostasis were retrieved prior to term (110 days of gestation) and at weaning (28 days of lactation). Transcriptional responses in kidney cortex and jejunal mucosa were analyzed using RNA sequencing. The variable dietary P content neither led to an aberration on fertility traits such as total weaned piglets nor to an effect on the weight pattern throughout gestation and lactation. Serum parameters revealed a maintained P homeostasis as reflected by unaltered inorganic P and calcium levels in L and H fed groups. The serum calcitriol levels were increased in lactating L sows. The endocrine responses to the dietary challenge were reflected at the transcriptional level. L diets led to an increase in CYP27B1 expression in the kidney compared to the H group and to an altered gene expression associated with lipid metabolism in the kidney and immune response in the jejunum. Our results suggest that current P requirements for gestating and lactating sows are sufficient and over supplementation of mineral P is not required. Shifts in renal and jejunal expression patterns between L and H groups indicate an affected intermediate metabolism, which long-term relevance needs to be further clarified.

Wubuli et al. BMC Genomics (2020) 21:626
https://doi.org/10.1186/s12864-020-07049-0

BMC Genomics

RESEARCH ARTICLE Open Access

Reduced phosphorus intake throughout gestation and lactation of sows is mitigated by transcriptional adaptations in kidney and intestine

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Abstract

Background: The environmental impact of pig farming need to be reduced, with phosphorus (P) being of particular interest. Specified dietary regimens and management systems contribute to meet environmental concerns and reduce economic constrains. However, pregnant and lactating sows represent vulnerable individuals, whose reproductive potential and metabolic health status relies on adequate supply of macro- and micronutrients. The aim of this study was to investigate, whether sows fed with a dietary P content that is below or above current recommendations are capable to maintain mineral homeostasis during the reproduction cycle and which endogenous mechanisms are retrieved therefore in kidney and jejunum. Nulliparous gilts were fed iso-energetic diets with recommended (M), reduced (L), or high (H) amounts of mineral P supplements throughout gestation and lactation periods. Blood metabolites and hormones referring to the P homeostasis were retrieved prior to term (110 days of gestation) and at weaning (28 days of lactation). Transcriptional responses in kidney cortex and jejunal mucosa were analyzed using RNA sequencing.

Results: The variable dietary P content neither led to an aberration on fertility traits such as total weaned piglets nor to an effect on the weight pattern throughout gestation and lactation. Serum parameters revealed a maintained P homeostasis as reflected by unaltered inorganic P and calcium levels in L and H fed groups. The serum calcitriol levels were increased in lactating L sows. The endocrine responses to the dietary challenge were reflected at the transcriptional level. L diets led to an increase in *CYP27B1* expression in the kidney compared to the H group and to an altered gene expression associated with lipid metabolism in the kidney and immune response in the jejunum.

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(Continued from previous page)
Conclusions: Our results suggest that current P requirements for gestating and lactating sows are sufficient and over supplementation of mineral P is not required. Shifts in renal and jejunal expression patterns between L and H groups indicate an affected intermediate metabolism, which long-term relevance needs to be further clarified.

Keywords: Dietary phosphorus, Mineral requirement, Monogastric farm animals, Phosphorus homeostasis, Gestational diets, Lactating sows

Background

Phosphorus (P) is present in every cell in the body and is involved in numerous biological processes like bone mineralization, energy metabolism, and intracellular signaling [1]. Consequently, P is also important for growth processes and bone development in livestock, whereby practically often more P is supplied in dietary formulations than is required for age-specific processes [2, 3]. An inefficient utilization and the over supplementation of inorganic P may not only interfere with the mineral homeostasis of the organism [4], but can also lead to imbalances in the agricultural cycle through excessive nutrient excretion [5, 6]. Therefore, an efficient utilization of dietary supplied P sources is essential for the sustainable use of the non-renewable resource P and the maintenance of an adequate organismal P homeostasis. Since monogastric livestock species, including pigs, account for the majority of global meat production, strategies for the efficient use of inorganic and plant-based P could contribute to improving global P efficiency and achieving a sustainable P cycle.

Body P homeostasis is mainly modulated through the P absorption by the small intestine (especially the jejunum), retention/excretion of minerals in the kidney, and P storage in the bone [7]. Hormonal factors including calcitriol ($1,25(OH)_2VitD_3$) are important regulators of the organism's P homeostasis [8]. Their regulatory activity is also being altered by the amount of dietary P intake [9]. In our previous studies, variable dietary P intake was shown to affect the expression levels of active P transporters [10], and numerous other genes involved in the regulation of mineral homeostasis with immunomodulatory effects in kidney and small intestine of growing pigs [11]. However, it is still under debate whether these effects of dietary P levels are directly sensed and mediated or indirectly orchestrated through downstream signaling cascades of the Ca sensing receptor (CaSR) [12, 13].

Previous studies have shown that P utilization also differs within and between different genetic pig breeds and thus, P homeostasis is influenced by genetics and transcriptional regulations [14–17]. Therefore, strategies towards improved P utilization should be re-evaluated considering animal-intrinsic factors such as genetics and specific metabolic requirements of modern pig breeds. To date, numerous studies have been conducted with growing pigs to investigate P utilization and its interrelation with bone metabolism and immune parameters in a period of high nutrient demand for anabolic processes [17–19]. In sows, the requirements of the organism differ considerably from those of growing pigs and are considered in the corresponding nutritional recommendations [20]. For gestating and lactating sows, the diets are formulated in order to ensure a sufficient supply of nutrients for maintenance, fetal development, and milk production [21]. Due to the metabolic changes occurring at the onset of milk production at farrowing, lactating sows can compensate by mobilizing their nutrient reserves, in particular protein or fat [22]. Thereby, small amounts of P (~ 0.97 g/d) can be mobilized to meet P requirements [23]. As known in mammals, the organism can further induce compensatory mechanisms for fetal and early postnatal P supply by increasing intestinal mineral absorption [24]. However, in general the microbial digestibility of dietary P sources in reproductive sows is lower compared to growing pigs [25], resulting in increased excretion of P into the environment [26].

A deeper understanding of the specific molecular mechanisms of P homeostasis in sows following dietary interventions could contribute to a more sustainable P utilization in pig husbandry. Gestating and lactating sows are of particular interest, as they have specific nutritional and metabolic demands and optimally utilize the available resources for the pre- and postnatal supply of the fetuses and offspring. In the current study, holistic expression patterns and blood phenotypes were analyzed to investigate the comprehensive effect of divergent levels of dietary P throughout gestation and lactation on sow physiology.

Results

Effects of a divergent dietary P supply on phenotype

The average body weight development and muscle characteristics in each experimental group are shown in Tables 1 and 2. The average body weight of sows at slaughtering was 201.1 ± 3.6 kg. No significant differences were observed in zoo-technical parameters and post mortem meat characteristics between the dietary groups. The different feeding had no effect on the health status of the animals.

Table 1 Average body weight of sows fed divergent amounts of dietary P (mean ± SE)

Body weight [kg]	L	M	H
0 day of gestation (insemination)	164.5 ± 2.9	168.7 ± 3.3	164.1 ± 4.7
30 days of gestation	193.8 ± 7.6	208.8 ± 8.0	195.0 ± 7.6
56 days of gestation	187.8 ± 8.7	177.8 ± 9.0	177.6 ± 10.7
84 days of gestation	219.4 ± 3.6	230.5 ± 5.1	220.0 ± 5.9
105 days of gestation	240.6 ± 2.8	250.5 ± 5.2	240.4 ± 7.5
28 days of lactation (weaning)	202.2 ± 5.3	212.75 ± 8.5	199.4 ± 5.4

Serum measurements comparing dietary groups are displayed in Fig. 1. Dietary treatments had no significant effects on serum inorganic P and Ca levels. However, lower alkaline phosphatase activity ($P = 0.042$) was observed in the H group compared to the group L at 110 days of gestation. A significantly higher level of serum calcitriol was observed in L fed animals compared to sows fed on M and H diets at 28 days of lactation (L vs. M, $P = 0.029$; L vs H, $P = 0.026$).

The reproductive performance of gestating sows was not impaired by modification of the dietary P supply ($P > 0.05$). The total litter size (mean ± SD) of sows was in average 15.0 ± 2.5 piglets for L, 14.8 ± 1.9 piglets for M and 13.8 ± 2.8 piglets for H. The respective average total number of born alive piglets per sow for L, M and H groups was 14.8 ± 2.5, 14.0 ± 2.2 and 13.0 ± 2.5, respectively. The average birth weights of piglets were 1.30 ± 0.27 kg (L), 1.25 ± 0.33 kg (M) and 1.22 ± 0.30 kg (H).

Effects of a divergent dietary P supply on transcriptional profiles in kidney and jejunum

A total of 28 RNA libraries from the kidney and jejunum of 14 sows fed medium ($n = 4$), lower ($n = 5$) and higher ($n = 5$) level of dietary P were sequenced and analyzed. Mapping of the processed sequences to the reference genome yielded per sample approximately 56 and 68 million high quality paired-end reads in kidney and jejunum, respectively. The average mapping efficiency was 98.5%. After filtering of absent and very low abundant genes, a total of 17,142 genes (13,995 annotated) in kidney and 16,693 genes (13,644 annotated) in jejunum were identified. To get an overview of the expression profile, a hierarchical clustering analysis of the selected variables (sPLS-DA) was performed (Fig. 2). Overall, the tissue effect dominated, which results in a clear separation of individual jejunum and kidney samples. Within each tissue, the expression profiles of the individuals of M and H groups showed lower distance than those of the L group.

In kidney, 57 genes (55 annotated; L vs. H), 63 genes (53 annotated; M vs. H) and 25 genes (24 annotated; L vs. M) were differentially expressed between sows ($q \leq 0.10$ corresponding to $P \leq 0.001$). In jejunum, 12 genes (L vs. H), three genes (M vs. H) and 67 genes (64 annotated, L vs. M) were altered significantly between groups ($q \leq 0.10$ corresponding to $P \leq 0.001$). The overlap of differently expressed genes between the different comparisons in the two tissues is shown in Fig. 3. The full list of differentially expressed genes is shown in Supplementary Table S1.

The complete list of DEGs in kidney and jejunum was subjected to KEGG pathway analysis to identify significantly affected biological pathways. Sixteen canonical pathways were observed among DEGs in kidney, of which the highest significantly enriched pathways are "metabolic pathways" (18 DEGs), "PPAR signaling pathways" (7 DEGs), "Protein processing in endoplasmic reticulum" (5 DEGs), "Fatty acid metabolism" (5 DEGs) and "Fatty acid degradation" (4 DEGs). In the jejunum, top three pathways, "metabolic pathways" (13 DEGs), "Viral protein interaction with cytokine and cytokine receptor" (3 DEGs) and "IL-17 signaling pathway" (3 DEGs) were revealed.

Table 2 Slaughter weight and meat characteristics of sows fed divergent amounts of dietary P (mean ± SE)

Trait	L	M	H
Live weight at slaughter (kg)	201.0 ± 4.9	208.3 ± 8.8	195.4 ± 5.3
Carcass weight (kg)	127.5 ± 7.8	131.2 ± 6.1	124.1 ± 5.3
MLD pH at 45 min	6.30 ± 0.10	6.18 ± 0.10	6.34 ± 0.04
MLD pH at 24 h	5.56 ± 0.05	5.53 ± 0.07	5.51 ± 0.03
MSM pH at 45 min	6.17 ± 0.19	5.89 ± 0.17	6.02 ± 0.15
MSM pH at 24 h	5.53 ± 0.05	5.51 ± 0.05	5.53 ± 0.06
MLD ash (%)	1.09 ± 0.01	1.11 ± 0.01	1.12 ± 0.01

MLD Musculus longissimus dorsi, MSM Musculus semimembranosus

Discussion

In order to gain a deeper understanding of the molecular mechanisms of P utilization in sows, respective blood parameters and transcriptional responses to divergent dietary P levels were investigated in this study. Concerning phenotypic characteristics, significant differences in serum ALP activity and calcitriol levels between the L and H groups were observed in gestating and lactating sows. The mRNA patterns illustrate the tissue-specific effects of dietary P and its contribution to maintain P homeostasis.

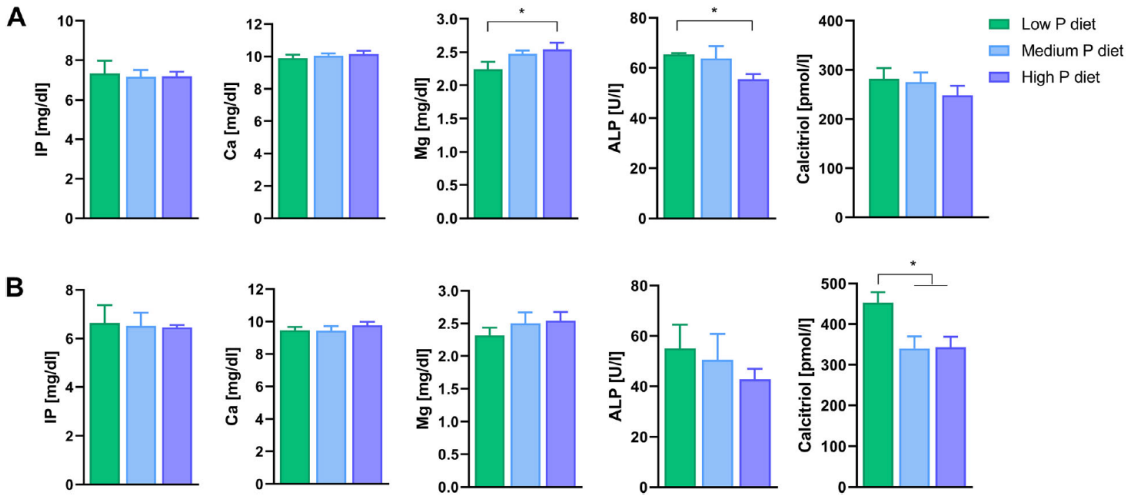

Fig. 1 Serum analytics of sows fed diets containing low, medium and high dietary P contents throughout gestation and lactation. Effects of divergent dietary P supply on serum inorganic P (IP), calcium (Ca), magnesium (Mg), alkaline phosphatase activity (ALP) and calcitriol (mean ± SE) at 110 days of gestation (**a**) and at 28 days of lactation (**b**). Superscripts indicate statistical significance between groups (*, $p < 0.05$)

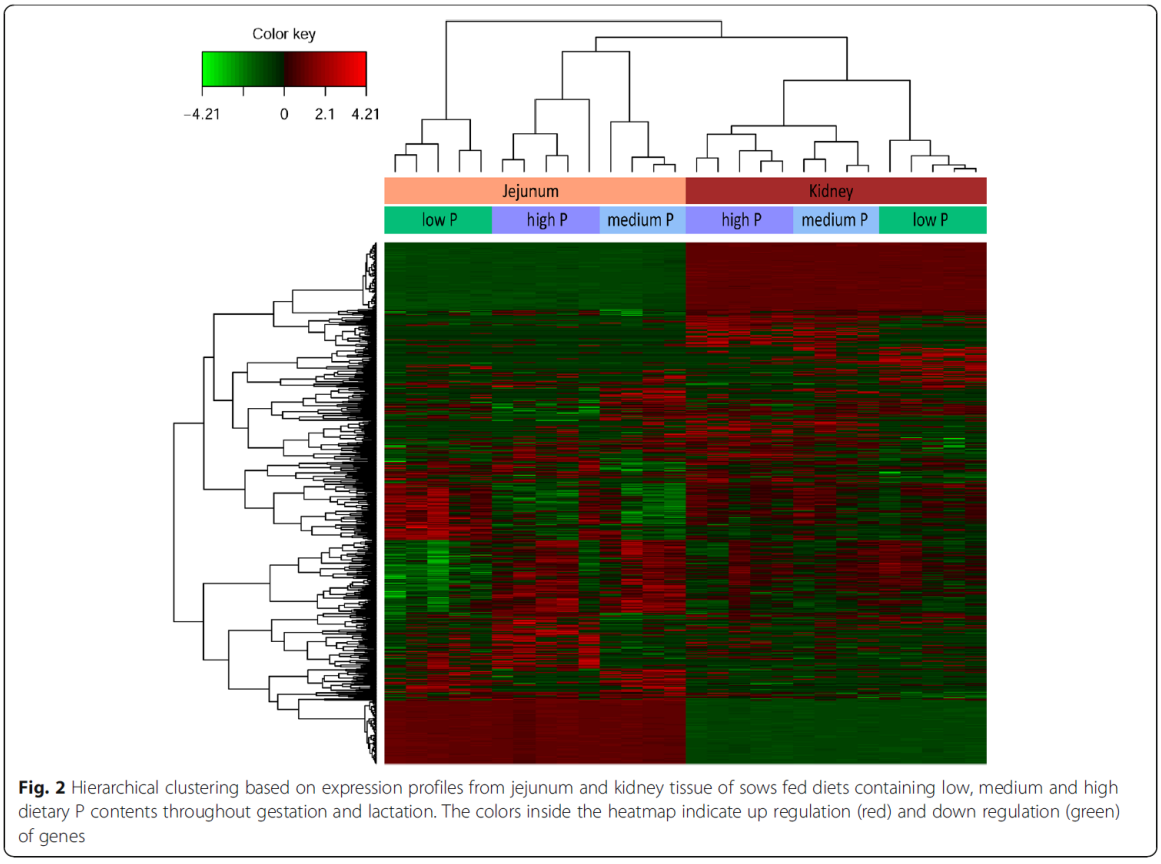

Fig. 2 Hierarchical clustering based on expression profiles from jejunum and kidney tissue of sows fed diets containing low, medium and high dietary P contents throughout gestation and lactation. The colors inside the heatmap indicate up regulation (red) and down regulation (green) of genes

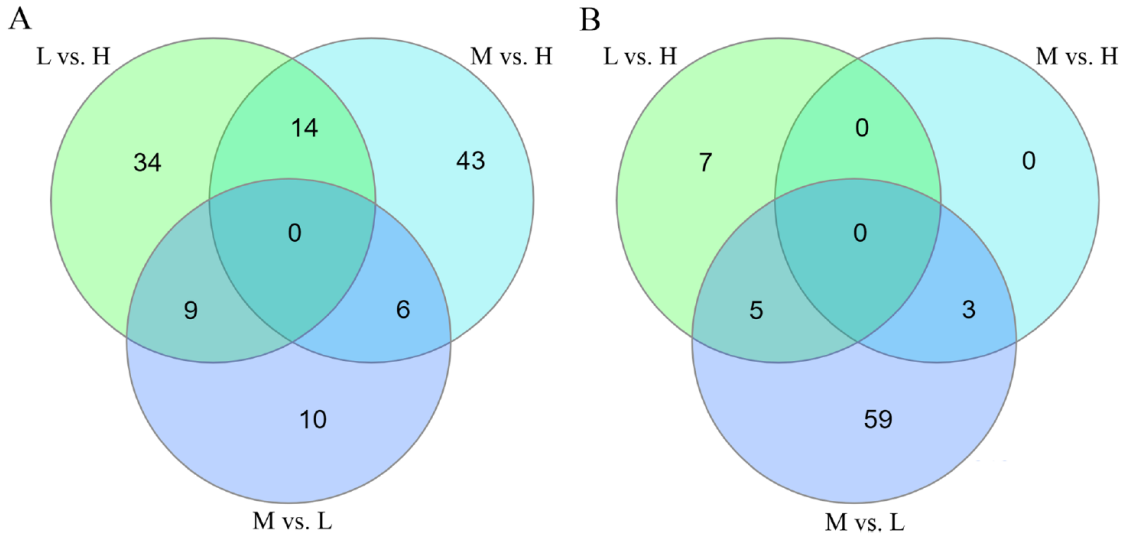

Fig. 3 Venn diagram of DEGs in the kidney (**a**) and the jejunum (**b**) of sows fed diets containing low (L), medium (M) and high (H) dietary P contents throughout gestation and lactation. L vs. H: comparison between group L and H; M vs. H: comparison between group M and H; M vs. L: comparison between group M and L

Effects of dietary P supply on the expression of renal genes involved in P homeostasis and lipid metabolism

The KEGG pathway analysis of DEGs in the kidney revealed 16 significantly affected pathways. A total of 18 genes including the *CYP27B1 (cytochrome P450 family 27 subfamily B member 1)* were enriched in the "metabolic pathways" (Table 3). *CYP27B1* is significantly up-regulated in L compared to H (FC: 2.4, L > H). Renal *CYP27B1* is regulated by hormones like parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), and is induced by a reduced availability of P [9, 27]. In fact, *CYP27B1* encodes an enzyme called 1-alpha-hydroxylase, which is responsible for the conversion of the storage form of vitamin D_3 (calcidiol, 25(OH)VitD_3) to the active form calcitriol ($1,25(OH)_2$VitD_3). Consequently, serum measurements showed significantly higher levels of calcitriol in the blood of L animals compared to M and H at 28 days of lactation. Calcitriol is important for maintaining the proper balance of Ca and P in the body by affecting intestinal absorption, renal excretion and bone remodeling [28, 29]. Despite the increased mineral requirements during pregnancy and lactation, serum analyses in this study suggest that L sows are capable to maintain P homeostasis via endogenous mechanisms and to control serum levels of Ca and P and the respective Ca:P ratio within a narrow range [30].

Interestingly, the *STC1 (Stanniocalcin 1)* gene, a glycoprotein hormone, was down-regulated in group H compared to M (FC: 1.83). It has been demonstrated that the expression of *STC1* is induced by circulating Ca [31], calcitriol and PTH [32] in the kidney, and that *STC1* stimulates P absorption in the small intestine and reabsorption in the proximal tubules of the kidney [33]. This suggests that a high intake of dietary P induces molecular mechanisms that counteract an oversupply with Ca and P of the organism. Due to the absence of regulations at the level of transcellular P transporters, this might mainly involve actions on paracellular transport processes in kidney and intestine. At the physiological level, a recent study on growing piglets also showed that an increased dietary P intake has no further beneficial effects on bone tissue synthesis [34]. Overall, the feeding recommendations for sows seem to exceed the current requirements.

In kidney, a group of genes associated to "metabolic pathways", including *ACOX2 (branched chain acyl-CoA oxidase), ACSL1 (Long-chain Acyl-CoA synthetase-1), ACADVL (Long chain acyl-CoA dehydrogenase), ACAA1 (acetyl CoA acyltransferase 1)* and *CPT2 (carnitine palmitoyltransferase 2)* is also associated with "PPAR signaling pathway", "peroxisome" and "fatty acid metabolism/degradation". Indeed, all genes mentioned above are involved in the β-oxidation of fatty acids, which is the major pathway for fatty acid degradation.

Previous studies in rodents suggested that a high P diet can affect tissue-specific energy metabolism by hampering lipid synthesis, while increasing the expression of genes associated with lipid oxidation [35, 36]. However, in our study on pregnant and lactating sows, all genes related to β-oxidation mentioned above were more abundant in the kidney of L compared to H animals.

Table 3 Significantly enriched canonical pathways in kidney and jejunum of sows fed diets containing low, medium, and high dietary P contents throughout gestation and lactation

Tissue	KEGG pathway	Number of annotated genes in the pathways	FDR-adjusted *P*-value	Involved genes
Kidney	Metabolic pathways	1388	1.38E-04	GALT,ENTPD5,ACADVL,MAT2A, ACSL1,ACOX2,CBR1,MGLL, DGAT2,G6PC,GSTA1,ACSM4, ACAA1,ALDH3A1,CYP27B1, HAO1,FADS2,BDH1
	PPAR signaling pathway	75	2.68E-06	CPT2,ACOX2,ACSL1,ACAA1, FABP3,FADS2,SLC27A2
	Protein processing in endoplasmic reticulum	161	6.72E-03	HSPA8,DNAJB1,DNAJA1,HSPH1, HSPA5
	Peroxisome	84	5.78E-04	ACOX2,HAO1,ACAA1,ACSL1, SLC27A2
	Fatty acid metabolism	57	1.38E-04	ACADVL,ACSL1,ACAA1,FADS2, CPT2
	Protein digestion and absorption	86	6.72E-03	COL3A1,SLC7A7,COL1A2,COL1A1
	Fatty acid degradation	41	5.78E-04	ACSL1,ACAA1,ACADVL,CPT2
	Metabolism of xenobiotics by cytochrome P450	49	1.20E-02	ALDH3A1,CBR1,GSTA1
	Complement and coagulation cascades	80	3.10E-02	C1S,PLG,C7
	ECM-receptor interaction	84	3.16E-02	COL1A2,FN1,COL1A1
Jejunum	Metabolic pathways	1388	3.58E-03	GALK1,B3GALT2,HMOX1,DHRS4,NT5E, XDH,IDUA,FUT2,PHOSPHO1,ADA,CHPF, SPR,PLA2G2D
	Viral protein interaction with cytokine and cytokine receptor	84	4.38E-02	CXCL9,CCL20,CXCL10
	IL-17 signaling pathway	88	4.38E-02	CCL20,MAPK6,CXCL10

Consequently, it can be deduced that the renal up-regulation of these genes under reduced dietary P supply could reflect the fact that energy is locally provided by increased β-oxidation for the function of the kidney cells i.e. to retain P in the organism. However, the NCBI gene databases [37] showed that all these genes related to fatty acid metabolism are highly expressed in many organs and tissues including kidney. Considering the fact that lactating sows can mobilize their nutrient reserves (primarily fat) for milk production, the observed differences between L and H groups might indicate a metabolic shift towards energy supply from lipids via β-oxidation in L animals. In fact, other studies clearly showed that fatty acid metabolism changes significantly as gestation progresses [38], and sows gain excessive fat during gestation to be mobilized in lactation [39]. This metabolic change could also increase the rate of mortality at birth [40], but there is no evidence for this in the current study.

In addition, a group of DEGs including the heat shock proteins (HSPs) *HSPA5, HSPA8, HSPH1, DNAJA1* and *DNAJB1* was higher abundant in L group compared to H animals, and is enriched in the KEGG pathway "protein processing in endoplasmic reticulum (ER)". The HSPs act as molecular chaperones that exert a critical role in protein homeostasis by preventing the aggregation of un−/misfolded proteins and cell death in stress conditions [41, 42]. The ER is also the place of phospholipid synthesis and storage of Ca, and many other protein-folding chaperones in the ER require a high level of Ca for their work [43]. Obviously, the maintenance of mineral homeostasis for L sows at the cellular level might be considered as a stressor, which effects should be assessed across tissues in further studies. In addition, several genes associated with the extracellular matrix formation including the collagen type *COL1A1 (type I collagen alpha 1), COL1A2 (type I collagen alpha 2)* and *COL3A1 (type III collagen alpha 1)*, which are known to be involved in the "Protein digestion and absorption" pathway were higher abundant in H compared to L. The *COL1A1* and *COL1A2* genes jointly produce a large molecule called type I collagen, which is known as a key protein involved in bone density, mineralization and development [44]. Interestingly, it has been demonstrated that high P diet induced collagen fibril organization and caused fibrosis in rat kidney [45]. Furthermore, *FMOD (fibromodulin)* and *FN1 (fibronectin 1)* were significantly down-regulated in L compared to the other groups.

Fibromodulin plays a role in bone mineralization, and its deficiency causes osteoporosis [46]. Fibronectin, an extracellular matrix protein, plays an essential role in the initiation and progression of fibrillogenesis through interaction with other fibronectin molecules and extracellular matrix components such as collagens [47]. Thus, this might indicate that a reduced dietary P level could prevent the excessive accumulation of extracellular matrix proteins (e.g. collagen, fibronectin) and fibrogenesis in the kidney.

Moreover, one member of the transmembrane water and small solute channels, *AQP11* (*Aquaporin 11*), was significantly down-regulated in the high P group (H < M, H < L). The human orthologue (h*AQP11)* is found to be localized in the adipocytes and function as both water and glycerol channel [48]. However, the exact role of *AQP11* in the kidney, whether it transports only water or also other molecules like glycerol, is unclear [49]. Further, several members from the solute carrier (SLC) family were significantly altered between the two extreme dietary groups. The *SLC44A4 (Thiamine Pyrophosphate Transporter)*, *SLC5A9 (Sodium/Glucose Cotransporter)*, *SLC26A6 (Anion Transporter)* and *SLC4A1 (Anion Exchanger)* were higher abundant in the H group, while the *SLC16A13 (Monocarboxylic Acid Transporters)* and *SLC25A45 (acyl carnitine transporter)* were higher abundant in L.

Effects of dietary P supply on gene expression in jejunum

Differentially expressed genes in jejunum revealed three canonical pathways (Table 3). A group of genes including the *PHOSPHO1* and *NT5E* genes was shown to be associated with the "metabolic pathways". *PHOSPHO1* is a phosphatase and was down-regulated in group L compared to M. It has been reported that the gene product of *PHOSPHO1* is involved in providing inorganic P for cartilage and matrix mineralization and in the initiation of calcification processes [50, 51]. Thus, a reduced P diet might prompt adaptations at peripheral tissue sites to preserve the extracellular mineral pool. The *NT5E* (5′-Nucleotidase Ecto) gene is down-regulated in the M group compared to L and H (M vs. H, FC: − 4.92; M vs. L, FC: − 3.97). This gene encodes CD73, which hydrolyzes extracellular adenosine monophosphate into adenosine and inorganic phosphate, and plays a role in the inhibition of ectopic tissue calcification [52] and cellular immune response [53]. The *CXCL9, CCL20 and CXCL10* genes were shown to be associated with the canonical pathway of "Viral protein interaction with cytokine and cytokine receptor". Moreover, *CCL20, MAPK6* and *CXCL10* were shown to be associated with the canonical pathway of "IL-7 signaling pathways". Considering the differential abundance of these genes, the observations might indicate that a high P diet has an inhibitory effect on the immune response in the jejunum. In previous studies, similar immune changes were also observed in the jejunum [11, 54, 55].

The *SLC10A2 (Sodium/Bile Acid Cotransporter)* and *CPT1A (carnitine palmitoyl transferase 1a)* genes had a significantly higher mRNA abundance in group H compared to L. It has been reported that the *SLC10A2* encoded transporter is essential for intestinal reabsorption of bile acid, which is associated with cholesterol/lipid metabolism [56, 57]. The *CPT1A* gene is also suggested as a key player in lipid metabolism due to its role in β-oxidation of long-chain fatty acids [58]. Comparing the M and the H group, the *PON3 (paraoxonase 3)* gene was significantly up-regulated in H. *PON3* was demonstrated to be associated with high-density lipoproteins [59], which play a role in the modulation of cholesterol levels in the body [60].

Deviating dietary P seems to have no significant effects on the sodium dependent P absorption in the jejunum of sows. Actually, it has been demonstrated that intestinal P absorption is mediated by trans- and paracellular transport systems and P transport is affected by factors like intestinal pH and phosphonoformic acid levels [61]. Therefore, in this study, effects of divergent levels of dietary P on the gene expression of major intestinal P transporters could be masked by other cofactors in the jejunum.

Conclusions

We investigated the transcriptional responses in kidney and jejunum to different dietary P supplies and its correlation with the phenotypic characteristics of sows. The differential P supply of the sows throughout pregnancy and lactation showed no influence on the performance characteristics, but triggered endocrine adaptation. The transcriptional responses of kidney and jejunum showed the regulation of pathways related to protein processing in the endoplasmic reticulum, fatty acid metabolism and innate immune characteristics. Interestingly, DEGs are evident within signaling pathways that have been functionally characterized for their role in pathological processes of mineral homeostasis such as ectopic calcification, osteoporosis or fibrillogenesis. This study shows that the regulation of these genes is involved in the physiological response to slight nutritional imbalances. Taken together, intestinal and renal responses to a continuous dietary P reduction can trigger rather complicated molecular mechanisms, whereby a distinction must be made between the local provision of energy and structures and the organismic maintenance of mineral homeostasis.

Methods

Animals, experimental design and sample collection

The study was approved by the Scientific Committee of the Leibniz Institute for Farm Animal Biology (FBN).

The experiment was generally licensed and authorized by the ethics committee of the federal state of Mecklenburg-Western Pomerania, Germany (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei; LALLF M-V/TSD/7221.3–1–053-15).

In this study, 14 nulliparous German Landrace sows from the same herd owned by the Leibniz Institute for Farm Animal Biology (FBN) with an age of 11 months were randomly assigned to three dietary groups prior to synchronization (Fig. 4). Sows were fed soy/barley standard diets with medium (M, $n = 4$), reduced (L, $n = 5$) and higher (H, $n = 5$) amounts of dietary P throughout an adaptation period (10 days until insemination), gestation (115 days) and lactation (29 days). Iso-energetic and iso-nitrous dietary formulations for adaptation and gestation were mixed in two batches and contained total dietary P contents of about 0.46% (M), 0.37% (L) and 0.56% (H). Lactating sows received total P contents of 0.61% (M), 0.48% (L) and 0.72% (H). Dietary P contents in diet M corresponded to the current standard feeding recommendations [20]. No phytase was added to the diets. All animals were fed on the same phase feeding regimen (early gestation, late gestation, lactation) and received restricted rations of 2.8 kg up to 6.4 kg to meet the stage-specific requirements. Sows had ad libitum access to water. The sows were kept in two batches, each representing individuals of all three feeding groups. Pregnant sows were kept in group pens supplied with concrete floor. At 110 days of gestation, sows were moved to individual farrowing pens where they remained with their litter throughout the lactation period. No cross-fostering was applied.

Individual body weights were documented prior to synchronization until slaughter (0, 30, 56, 84, 105 days of gestation; day 28 of lactation; carcass weight). Blood samples were taken from *V. jugularis* at 110 days of gestation and at 28 days of lactation after overnight fasting for serum preparation. Veterinary inspection of the carcasses and organs after slaughter confirmed the lack of any impairments, disease symptoms and pathological signs. Sows were anaesthetized by electrical stunning, sacrificed by exsanguination and slaughtered at the Institute's experimental slaughter facility at day 29 of lactation (corresponds to day 154 on trial) and individual tissue samples were taken. In brief, the left kidney was cut open and cortex was retrieved from the lateral part. Moreover, a section of 10 cm of jejunum was taken at a distance of 200 cm from the stomach. The intestinal tube was opened, the digesta removed and the surface rinsed with ice-cold NaCl (0.9%) to remove residual contaminants. The intestinal epithelium was scraped off with a scalpel. All samples were stored in liquid nitrogen immediately after preparation and stored at − 80 °C until RNA isolation.

Serum analyses and meat characteristics

Serum minerals including inorganic P (IP), calcium (Ca), magnesium (Mg), and total alkaline phosphatase activity (ALP) were measured using a Fuji DriChem 4000i device (FujiFilm, Minato, Japan). The level of total calcitriol in stored serum samples was measured by an immunoassay using a commercially available kit (AC-62; Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany).

Individual pH of *M. longissimus dorsi* (MLD) and *M. semitendinosus* (MSM) were recorded at 45 min and 24 h post mortem (pH-Star, Matthäus, Pöttmes, Germany). The ash content of MLD samples was determined in triplicate by calcination in a muffle furnace at 600 °C using established protocols [62].

Measurements on zootechnical parameters, serum minerals and post mortem meat characteristics were subjected to a linear model (R language, version 3.6.2, package stats). For the analysis of serum minerals, slaughter batch was included in the model. The post-hoc test of Tukey was used to derive differences between the three experimental groups. The significance level was set at $P < 0.05$.

RNA library preparation and differential gene expression analysis

Total RNAs were isolated from each of the jejunum and kidney tissue samples of 14 sows fed medium ($n = 4$),

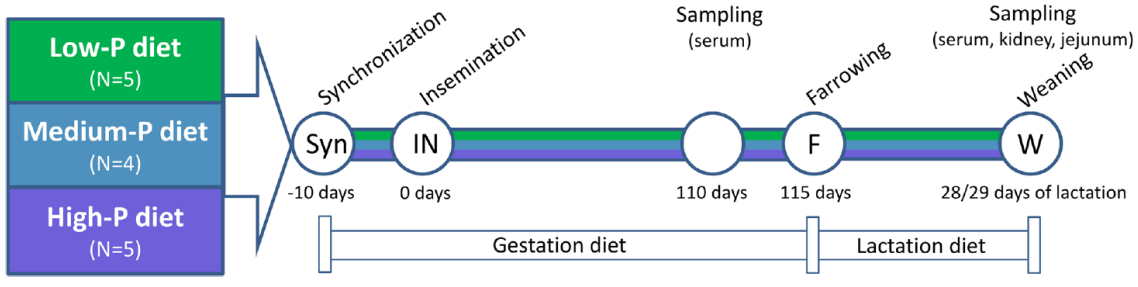

Fig. 4 Experimental design of the trial with divergent P supplies throughout gestation and lactation ($n = 14$). Samples were obtained at 110 days of gestation and day 28/29 of lactation

lower ($n = 5$) and higher ($n = 5$) level of dietary P by TRI Reagent according to user guides (Sigma-Aldrich, Taufkirchen, Germany), followed by Baseline-ZERO DNase treatment (Biozym, Hessisch Oldendorf, Germany) and purification with the column-based NucleoSpin RNA II-Kit (Macherey–Nagel, Düren, Germany). Concentration of purified RNA samples was measured by Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). The quality of RNA extracts were checked on a Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) yielding RNA integrity numbers (RIN) from 6.3 to 9.1 (average of 8.0). RNA libraries were prepared from the final purified total RNAs according to the TruSeq Stranded mRNA protocol (Illumina, San Diego, CA, United States) and the quality was validated using a Agilent DNA-1000 chip kit (Bioanalyzer 2100). Subsequently, paired-end reads with a length of 2×101 bp were generated by RNA sequencing on an Illumina HiSeq2500 instrument. Raw data were quality-checked and pre-processed including the removal of low quality reads (a mean Q-score < 20) and adapters with FastQC v.0.11.7 and Trim Galore v.0.5.0 programs. High quality reads were then mapped to the reference Sscrofa11.1 (Ensembl release 93) and gene features using HISAT2 (2.1.0) [63] and HTSeq 0.8.0 [64] tools. Initial data visualization of gene expression profiles was performed using the mixOmics R package [65]. A sparse Partial Least Squares Discriminant Analysis (sPLS-Da) was used to select the variables (transcripts) with the highest contribution to a component. To account for the experiment including the two tissues and the three experimental groups, in total 4 components were considered in the analysis. Each of these components included 150 variables. Hierarchical clustering of the data was performed using mixOmics with default setting. The differentially expressed gene analysis in the contrast of the three dietary groups (pairwise comparisons) was performed per tissue by the R package DESeq2 v3.4.0 [66]. Very low abundant transcripts having observations in less than four samples were filtered out. The statistical model included information on the mother of the sow to account for the relatedness of pigs. A false discovery rate (FDR) < 0.10 was set as significance threshold to detect differentially expressed genes (DEG) between dietary groups. To assess the differences along the dietary P gradient in the three groups, tissue-specific lists of significant DEGs were combined (132 DEGs for kidney and 79 DEGs for jejunum) and assigned to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [Release 93.0, accessed 2/06/2020, reference organism is pig (*Sus scrofa*)] to investigate significant biological alterations using KOBAS 3.0 web server [67]. Pathways were considered significant at FDR (Benjamini and Hochberg)-adjusted P- value ≤0.05. Pathways containing less than 3 input genes and some disease-related pathways that were considered irrelevant were excluded from the KEGG pathway table. The complete results of KEGG pathway enrichment analysis for each tissue are shown in Supplementary Table S2.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12864-020-07049-0.

Additional file 1: Table S1. Most differentially expressed transcripts in jejunum between diverging phosphorus diet groups.

Additional file 2: Table S2. Results of the KEGG pathway enrichment analysis considering 79 DEGs obtained from analysis of jejunum.

Abbreviations

Calcitriol: 1,25(OH)2VitD3; DEG: Differentially expressed gene; ER: Endoplasmic reticulum; Ca: Calcium; Mg: Magnesium; P: Phosphorus; IP: Inorganic phosphate; ALP: Alkaline phosphatase activity; FDR: False discovery rate; FC: Fold change; H: High P diet group; L: Low P diet group; M: Medium (recommended) P diet group

Acknowledgements

The authors would like to thank the farm stuff for assistance with pig management. We thank Angela Garve, and Marlies Fuchs for their excellent technical help.

Authors' contributions

PW and KW made the conception and design of research; CG, MO, PW and KW supervised the experiment; AW, CG, HR, MO and NT performed experiments; AW, CG, HR, MO and NT analyzed data; AW, HR, MO, PW and KW interpreted results of experiments; EM, SP, PW and KW provided reagents/materials/resources; AW drafted the manuscript; AW and HR prepared figures; AW, CG, HR, MO, EM, NT, SP, PW and KW edited, discussed and revised the manuscript; AW, CG, HR, MO, EM, NT, SP, PW and KW read and approved the manuscript.

Funding

This study has received funding from the European Research Area Network on Sustainable Animal Production (ERA-NET SusAn) as part of the Pegasus Project (2817ERA02D). It was supported by the Leibniz Science Campus Phosphorus Research Rostock and matched funding from the Leibniz Institute for Farm Animal Biology (FBN). The funding had no influence on the design of the study and the collection, analysis and interpretation of data and the preparation of the manuscript. Open Access funding provided by Projekt DEAL.

Availability of data and materials

Raw data are deposited in the ArrayExpress database at The European Bioinformatics Institute (EMBL-EBI, https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9101/,accession number: E-MTAB-9101). RNAseq-reads were mapped to the reference Sscrofa11.1 (Ensembl release 93, https://www.ensembl.org/Sus_scrofa/Info/Index).

Ethics approval and consent to participate

Animals were provided by the Leibniz Institute for Farm Animal Biology (FBN). The experimental protocol was approved by the ethics committee of the federal state of Mecklenburg-Western Pomerania, Germany (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei; LALLF MV 7221.3–1053/15).

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Received: 5 June 2020 Accepted: 3 September 2020
Published online: 11 September 2020

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Publisher's Note

3.4. Own contribution paper III & pdf

Mineral phosphorus supply in piglets impacts on the microbial composition and phytate utilization in the large intestine.

Reyer, Henry, Per J.R. Sjöberg, Michael Oster, Aisanjiang Wubuli, Eduard Murani, Siriluck Ponsuksili, Petra Wolf, and Klaus Wimmers. 2021. Mineral Phosphorus Supply in Piglets Impacts the Microbial Composition and Phytate Utilization in the Large Intestine. *Microorganisms* 9, no. 6: 1197. (https://doi.org/10.3390/microorganisms9061197)

I hereby declare that my contribution in this publication summarized in this dissertation is as follows:

- Tissue sampling and processing
- Carried out DNA extraction and 16S rRNA PCR experiments
- Reviewing and editing the manuscript

Abstract:

A sufficient supply of phosphorus (P) to pigs in livestock farming is based on the optimal use of plant-based phytate and mineral P supplements to ensure proper growth processes and bone stability. However, a high P supplementation might bear the risk of higher environmental burden due to the occurrence of excess P and phytate degradation products in manure. In this context, the intestinal microbiota is of central importance to increase P solubility, to employ non-mineral P by the enzymatic degradation of phytate, and to metabolize residual P. A feeding experiment was conducted in which piglets were fed diets with different P levels, resulting in three groups with low, medium (covering requirements), and high concentrations of available P. Samples from caecum and colon digesta were analyzed for microbial composition and phytate breakdown to estimate the microbial contribution to metabolize P sources. In terms of identified operational taxonomic units (OTU), caecum and colon digesta under the three feeding schemes mainly overlap in their core microbiome. Nevertheless, different microbial families correlate with increased dietary P supply. Specifically, microbes of *Desulfovibrionaceae, Pasteurellaceae, Anaerovoracaceae*, and *Methanobacteriaceae* were found significantly differentially abundant in the large intestine across the dietary treatments. Moreover, members of the families *Veillonellaceae, Selenomonadaceae*, and *Succinivibrionaceae* might contribute to the observed phytate degradation in animals fed a low P diet. In this sense, the targeted manipulation of the intestinal microbiota by feeding measures offers possibilities for the optimization of intestinal phytate and P utilization.

microorganisms

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Article

Mineral Phosphorus Supply in Piglets Impacts the Microbial Composition and Phytate Utilization in the Large Intestine

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Abstract: A sufficient supply of phosphorus (P) to pigs in livestock farming is based on the optimal use of plant-based phytate and mineral P supplements to ensure proper growth processes and bone stability. However, a high P supplementation might bear the risk of higher environmental burden due to the occurrence of excess P and phytate degradation products in manure. In this context, the intestinal microbiota is of central importance to increase P solubility, to employ non-mineral P by the enzymatic degradation of phytate, and to metabolize residual P. A feeding experiment was conducted in which piglets were fed diets with different P levels, resulting in three groups with low, medium (covering requirements), and high concentrations of available P. Samples from caecum and colon digesta were analysed for microbial composition and phytate breakdown to estimate the microbial contribution to metabolize P sources. In terms of identified operational taxonomic units (OTU), caecum and colon digesta under the three feeding schemes mainly overlaP in their core microbiome. Nevertheless, different microbial families correlate with increased dietary P supply. Specifically, microbes of *Desulfovibrionaceae, Pasteurellaceae*, *Anaerovoracaceae*, and *Methanobacteriaceae* were found significantly differentially abundant in the large intestine across the dietary treatments. Moreover, members of the families *Veillonellaceae, Selenomonadaceae*, and *Succinivibrionaceae* might contribute to the observed phytate degradation in animals fed a low P diet. In this sense, the targeted manipulation of the intestinal microbiota by feeding measures offers possibilities for the optimization of intestinal phytate and P utilization.

Keywords: phosphorus; inositolphosphate; large intestine; 16S rRNA; pigs

Citation: Reyer, H.; Sjöberg, P.J.R.; Oster, M.; Wubuli, A.; Murani, E.; Ponsuksili, S.; Wolf, P.; Wimmers, K. Mineral Phosphorus Supply in Piglets Impacts the Microbial Composition and Phytate Utilization in the Large Intestine. *Microorganisms* **2021**, *9*, 1197. https://doi.org/10.3390/microorganisms9061197

Academic Editor: Ryo Inoue

Received: 7 May 2021
Accepted: 28 May 2021
Published: 1 June 2021

1. Introduction

The viability of all organisms depends on a demand-covering supply of phosphorus (P) for the establishment of structural body compartments and the maintenance of other important physiological functions, such as energy supply, cell signalling, and blood buffering. For pigs, the main P sources are phosphorus-containing mineral supplementations (dicalcium-/monocalcium-phosphates) produced from rock phosphates and also phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis; InsP6), which is usually present as phytate and represents the storage form of P in plants [1,2]. The provision of P available to the animal from phytate is essential to meet the needs of the organism while using a sustainable mineral source. However, the phytate utilization requires its gradual hydrolysis, which is catalysed by phytases and other phosphatases, such as the intestinal alkaline phosphatase.

Depending on dietary supply and gastrointestinal site, the cleavage steps result in variable amounts of phosphate, inositolphosphates (InsPx), and *myo*-inositol. However, due to the low impact of endogenous intestinal phosphatases on phytate hydrolysis, pigs have a low capacity to utilise phytic acid, which requires the involvement of phytases of plant or microbial origin [3]. In practice, exogenous phytases of microbial origin are added to pig feed, improving the available P content based on the amount and origin of phytases [2]. However, considering the microbial community of the gastrointestinal tract of pigs and chickens, it is known that some of the microbes present are able to produce phytase and secrete them into the lumen. These include *Bifidobacteria* [4], various isolates of *Lactobacillus* [5,6], and *Pediococcus* [7] as well as *Pseudomonas* spp. [8]. This exemplifies the potentially broad microbial capacity to degrade phytate and provide P in the gastrointestinal tract of monogastric animal species. Besides the benefits of phytate as a P source, antinutritive effects of InsP6 (inositol hexakisphosphate), InsP5 (inositol pentakisphosphate), InsP4 (inositol tetrakisphosphate), and InsP3 (inositol triphosphate) have been described. These include mainly the impairment of the protein-degradation capacity of pepsin and the drastic reduction of the solubility of zinc at pH values relevant for the proximal small intestine in the presence of InsPx [2,9]. Therefore, an efficient breakdown of phytate in the gastrointestinal tract is preferable. Moreover, there is an interaction of dietary mineral P and InsPx such that high P hampers the degradation of phytase [10]. At present, several approaches proved beneficial for the rapid hydrolysis of phytate, e.g., liquid feeding, phytase superdosing, or feed pre-treatment [11,12]. Considering the above-mentioned potential of microbes present in the gastrointestinal tract to provide phytases and improve P efficiency, dietary strategies might further include the establishment of a favourable intestinal microbiome profile. Shifts in the microbial composition of the intestine will facilitate meeting the required P supply by exploiting a higher degree of non-mineral P of plant origin to reduce P excretion and thus to reduce the environmental impact. Therefore, the current study focuses on the investigation of the microbial composition and the profile of inositolphophates in the pig large intestine in response to varying dietary P intakes.

2. Materials and Methods

2.1. Animal Trial and Sample Preparation

The animal experiment to which this study refers was approved by the ethics committee of the federal state of Mecklenburg-Western Pomerania, Germany (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei; LALLF-M-V/TSD/7221.3-1-053-15). A feeding trial was carried out in an experimental pig house with varying levels of mineral P fed to piglets between 28 and 64 days of life, as previously described [13]. In brief, dietary groups were composed as follows: grouP L consisting of 8 piglets fed with a lower mineral P level (P = 0.6% of DM), grouP M consisting of 6 piglets fed recommended P levels (P = 0.9% of DM), and grouP H consisting of 7 piglets fed with a higher mineral P level (P = 1.1% of DM). Except P, all other nutrient contents of the feed were based on current recommendations [14]. The diets had comparable levels of protein (L: 205 g/kg DM; M: 203 g/kg DM; H: 201 g/kg DM), metabolizable energy (L: 12.5 MJ/kg DM; M: 12.5 MJ/kg DM; H: 12.3 MJ/kg DM), and calcium (L: 13.0 g/kg DM; M: 13.0 g/kg DM; H: 13.0 g/kg DM) [13]. Vitamin D3 was provided at the level of 1000 IU for all dietary groups. No microbial phytases were added to the wheat/barley/soybean meal-based diets. The piglets originated from 4 litters and were equally assigned to dietary groups with at least 3 males and females per group. The trial was carried out in March–April at ambient stable temperature and relative humidity. A constant 12-h light-dark cycle was applied. The piglets were kept individually on a flat-deck and had ad libitum access to pelleted feed and water [13]. At the end of the trial period, pigs were killed by exsanguination following electric stunning. From all pigs, digesta samples were collected from the terminal tiP of the caecum and the mid region of the colon. Subsequently, samples were shock frozen in liquid nitrogen and stored at −80 °C until microbiota and inositolphosphate analyses.

2.2. 16S rRNA Profiling

DNA extraction of pig digesta samples followed the instructions of the PowerLyzer PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) with additional heating steps of 10 min at 70 °C and 95 °C prior to beat beating. Amplificates of the 16S rRNA gene were produced in duplicates using primers specific for variable region V4 (515'F and 806R), including adapters and barcodes [15,16]. Polymerase chain reactions were performed with 5PRIME HotMasterMix (5 PRIME, Hamburg, Germany) as follow: 95 °C for 2 min, 30 cycles at 95 °C for 30 s, 55 °C for 60 s, 72 °C for 90 s, and a final extension for 10 min at 72 °C. PCR products were purified using magnetic beads (Agencourt AMPure XP, Beckman Coulter, Krefeld, Germany) and mixed in equal concentrations. Sequencing was performed on a HiSeq2500 (Illumina, San Diego, CA, USA) generating 250 bP paired-end reads. After demultiplexing of the sequencing reads, raw data were analysed with the mothur software (version 1.44.1) [17]. Sequences were globally aligned to the Silva reference database (release 138) with chimeric sequences removed. Considering a sequence identity of 97%, the sequences were combined into operational taxonomic units (OTU), and OTU annotations were retrieved from the Silva database (release 138).

2.3. Inositolphosphate Analysis

For the analysis of InsP6 and InsP5, approximately 200 mg of digesta samples obtained from caecum and colon were lyophilized. The chemicals used were of analytical quality (Sigma-Aldrich, Taufkirchen, Germany). In-house stock solution was prepared as previously described [18,19] from the dipotassium salt of myo-inositol hexakis (dihydrogenphosphate) (P5681, Sigma-Aldrich, Taufkirchen, Germany). A standard series was prepared by diluting the in-house stock solution with 0.1 M NaOH containing 0.01 M etylenediamin tetraacetic acid (EDTA) in the range between 0.1–55 µM for calibration of the ESI-MS system. Freeze-dried samples (approx. 20 mg) were extracted with 1 mL 1.0 M NaOH containing 0.1 M EDTA. Samples were shaken with a Multi Reax for 16 h (Heidolph Instrument, Schwabach, Germany). To remove particles, the samples were centrifuged at 10,000 rpm for 15 min. The supernatant (0.1 mL) was mixed with 0.9 mL of Milli-Q water in a glass vial before placing it in the LC autosampler for analysis. The 1260 Infinity (Agilent Technologies, Waldbronn, Germany) chromatographic system was used in connection with a 3200 Q TraP LC/MS/MS system (AB Sciex, Concord, ON, Canada). The LC-MS method was the same as previously reported [19]. InsP6 and InsP5 were detected by using multiple reaction monitoring mode (MRM) with suitable precursor and product ion transitions with optimal collision energy (CE) and collision cell exit potential (CXP). These values were evaluated in a previous work [19]. Data acquisition and quantification were carried out with Analyst 1.4.2 (AB Sciex). All MRM transitions for respective InsPx were summed before manual peak integration.

2.4. Data Analysis

The OTU abundances determined were adjusted by means of subsampling, taking into account the library with the fewest sequences. After taxonomic annotation of OTU, the relative abundance of phyla and family level were visualized in taxa plots employing the R software. Dietary differences at family level were assessed separately for caecum and colon using DESeq2 (DOI:10.18129/B9.bioc.DESeq2). Therefore, very low abundant families were excluded by considering only taxa with more than ten observations in at least 30% of all samples. A likelihood ratio test was performed against a base model with mother and sex of piglets as effects. Differences were considered significant at a Benjamini–Hochberg adjusted p-value < 0.05. Data referring to the InsPx levels in caecum and colon contents were summarized as mean ± SE. One sample from the L grouP had to be removed from the analyses due to missing values. Data were analysed in a linear model (R package lmerTest, v3.1-2) considering effects of dietary P content, litter, and sex. The significance threshold was set at $p < 0.05$. For correlation analysis between phenotypes and microbiota, Kendall correlation coefficients were calculated. Therefore, animal-individual values of

P intake and serum P levels were obtained from a previous study and considered in the analysis [13].

3. Results and Discussion

The measurement of inositolphosphates in caecum and colon digesta revealed a gradual increase of InsP6 and InsP5 in the two intestinal sections with increasing mineral P supply in the diets (Figure 1). The L grouP showed significantly lower levels of InsP6 and InsP5 in both intestinal segments compared to M and H ($p < 0.05$), although this difference was not considered significant when comparing InsP5 levels between L and M ($p = 0.05$ for colon, $p = 0.08$ for caecum). Despite the absence of a phytase of exogenous origin in the feed, there is a clear response to reduced mineral P supply in the L grouP resulting in an increased hydrolysis of phytate. Supporting this observation, it was found that phytase activity in the ileum increased with decreasing P in the diet [20]. The group-specific patterns and magnitude of effects were similar for caecum and colon digesta, although, a higher amount of inositolphosphates was found for H in the colon compared to the caecum ($p < 0.05$ for InsP6 and InsP5).

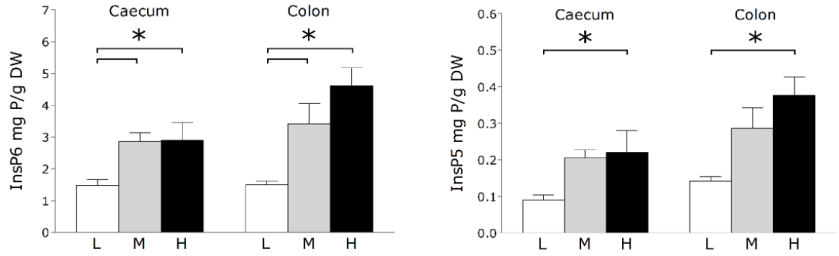

Figure 1. Levels of inositol hexakisphosphate (InsP6) and inositol pentakisphosphate (InsP5) in the caecum and colon of piglets fed a varying supply of mineral P for a period of 5 weeks. Significant differences are indicated by an asterisk (*; $p < 0.05$). L, M, H—dietary groups receiving lower, medium, and higher mineral P levels; DW—dry weight.

Since the experimental design excluded exogenous phytase supplements, the main factor for the observed differences in an inositolphosphate degradation likely refers to the intestinal microbiota composition. The sequencing of the 16S rRNA revealed that, at OTU level, about two-thirds of the taxonomic units overlapped between caecum and colon digesta (Figure 2A). This corresponds to the previous observation that caecum and colon exhibit a high similarity in microbiota profiles [21]. Regarding the specific dietary groups in both tissues, the relations represented in the Venn diagrams revealed a considerable amount of common OTU interpreted as independent of the dietary P supply (Figure 2B,C). However, 39 to 60 OTUs were identified to be specific for a single dietary group, without a particular grouP displaying a considerable increase in microbial richness. Interestingly, the overlaP in OTUs was numerically higher in the extreme P groups (239 and 186 in caecum and colon, respectively, considering L and H) compared to the contrasts with the M group. This might be related to observations that varying dietary P content and phytase addition are associated with the capacity of the cellular adaptive immune system and influence the metabolism in terms of fibre digestion and the concentration of microbial metabolites [22–24].

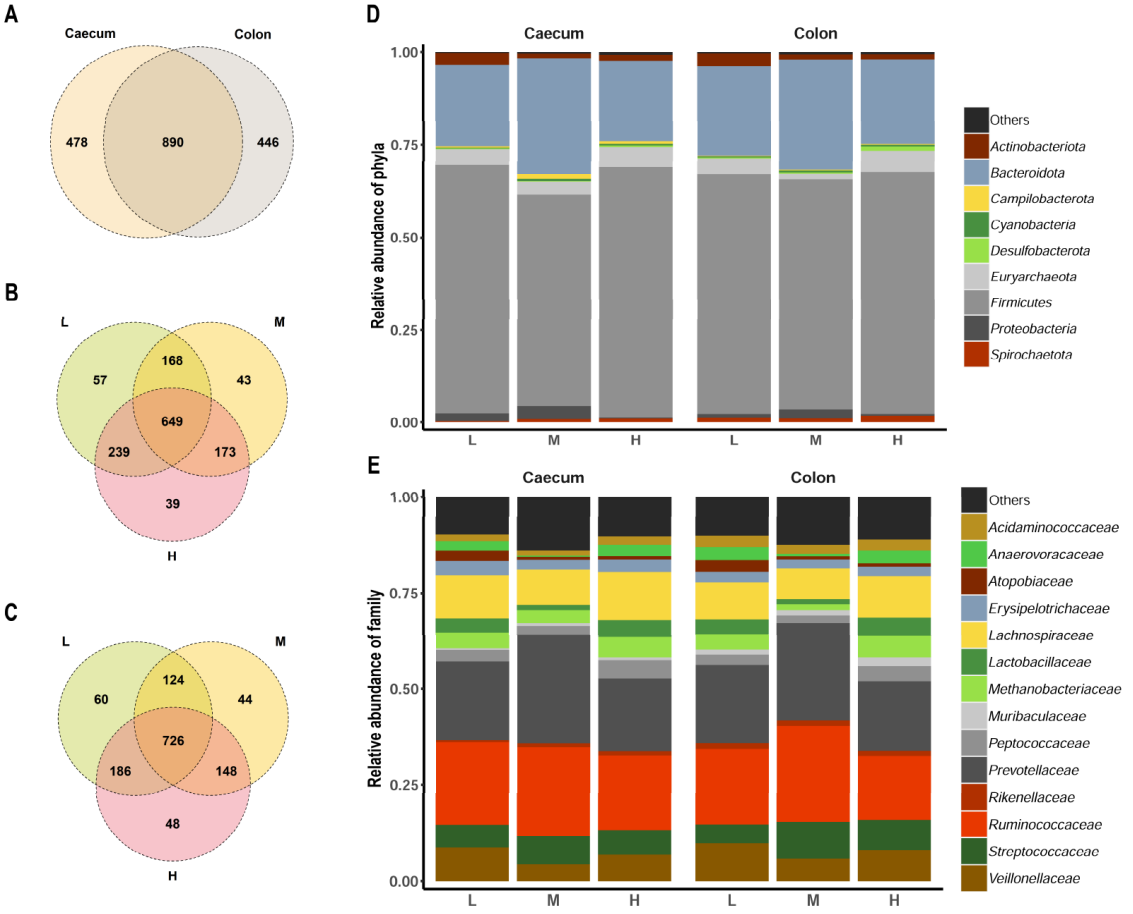

Figure 2. Microbial composition of caecum and colon digesta of pigs fed with varying mineral P levels. Venn diagram representation of specific and overlapping OTUs considering (**A**) caecum and colon digesta samples, (**B**) caecum digesta samples from the three dietary groups, and (**C**) colon digesta samples from the dietary groups. Taxa plots at (**D**) phylum and (**E**) family level with the 9 and 14 toP taxa, respectively. L, M, H—dietary groups receiving lower, medium, and higher mineral P levels.

Diet-specific taxa plots at phylum level revealed a large overlaP in the microbiota composition between the two intestinal segments and the three dietary groups (Figure 2D). The most abundant phyla comprised Firmicutes and Bacteroidota, with an average relative abundance of 0.64 and 0.25, respectively. The abundance of these two predominant phyla reflects the typical microbiota composition in the pig at this age [21]. Group-specific differences in the average abundance were consistent across tissue sites. Considering the toP 14 taxa at the family level, *Prevotellaceae* and *Ruminococcaceae* dominated in caecum and colon (Figure 2E). Statistical analysis at family level revealed that microbes assigned to *Anaerovoracaceae* and *Pasteurellaceae* in the caecal digesta were found to be significantly differentially abundant between dietary groups (adjusted $p < 0.05$, Figure 3, Supplementary Tables S1 and S2). As shown in the animal-individual representation of abundances of *Pasteurellaceae* in Figure 3A, the differences were mainly driven by two samples of the M grouP showing a considerably high proportion of sequences assigned to this taxon. For *Anaerovoracaceae*, samples of L and H groups showed higher abundance compared to M

animals. In the colon digesta, *Anaerovoracaceae* were also differentially abundant between the dietary groups on family classification, showing the same diet-specific pattern likewise in caecum. The family *Anaerovoracaceae* is sparsely characterized. It belongs to the class of *Clostridia*, which are typically involved in the fermentation of plant polysaccharides in the gastrointestinal tract [25]. In colon digesta, also *Methanobacteriaceae* and *Desulfovibrionaceae* differed significantly in their abundance between dietary groups. *Methanobacteriaceae* was lowest abundant in M, whereas microbes of *Desulfovibrionaceae* were found predominantly in the colon digesta of H animals. The predominant process for energy generation in *Methanobacteriaceae* is the reduction of carbon dioxide to form methane [26]. However, it has recently been shown that the abundance of *Archaeae*, which include *Methanobacteriaceae*, is correlated to phytase supplementation in pigs [27]. For *Desulfovibrionaceae*, the genus *Desulfovibrio* was found to have a significantly higher relative abundance in the jejunum and caecum of goats with a high digestibility of P [28]. Therefore, independent of the actual functional involvement in P metabolism, representative species of these two families could drive potential improvements of P availability and phytate utilization in the gastrointestinal tract of pigs.

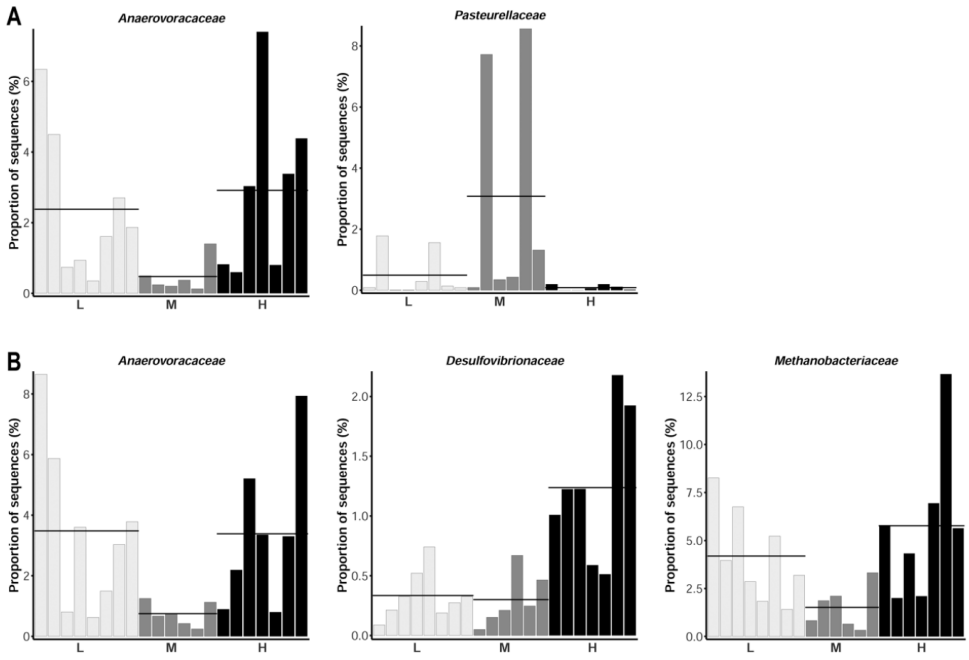

Figure 3. Significant differentially abundant taxa in (**A**) caecum and (**B**) colon digesta at family level (adjusted $p < 0.05$). Individual proportions of sequences assigned to the taxa are represented for each of the three dietary groups. L, M, H—dietary groups receiving lower, medium, and higher mineral P supplements.

The variable dietary P supply provided to the pigs in this study affects various levels of the organism such as the chemical composition and the microstructure of the bones as well as the hormone level and the mineral concentration in the blood to primarily maintain P homeostasis [13]. In order to further explore the interaction between dietary P intake, inositolphosphate degradation, and the abundance of microbial taxa in the caecum and colon, correlation analyses were performed. The majority of the 25 most abundant families included in the analysis originated from *Firmicutes* (Figure 4). With respect to the microbial families with higher relative abundance, significant positive correlation coefficients were identified for *Streptococcaceae* with P intake und serum P levels in colon and with InsP6

values in caecum. This implies favourable conditions for the growth of *Streptococcaceae* under higher available P concentrations in the digesta. It has been described that species of *Streptococcaceae* perform proteolytic processes in the colon and contribute substantially to the turnover of sulfur-containing substrates [29]. Phytase activity has not yet been reported for representatives of this microbial family. *Veillonellaceae* were significantly negatively correlated with InsP6 levels in caecum digesta. This indicates an increased phytate utilization in the presence of microbes of this family. Interestingly, by adding exogenous phytase to the diets of growing pigs, the relative abundance of *Veillonellaceae* significantly decreased [27]. For *Lactobacillaceae*, a positive correlation with InsP6 and InsP5 in colon was retrieved. For low abundant taxa, increasing levels of dietary mineral P intake were suggested to correlate with an increased presence of *Muribaculaceae*, *Rikenellaceae*, and *Desulfovibrionaceae*. For *Muribaculaceae* and *Rikenellaceae*, so far, no information is available about their involvement in P metabolism. Members of the former are known for degradation of carbohydrates and utilization of nitrogen, whereas the latter are mainly hydrogen-producing bacteria [30]. In accordance with the results of the statistical comparison of dietary groups in this study, the identification of *Desulfovibrionaceae* provides clear evidence of a relationshiP between the dietary intake of mineral P and the occurrence of members of this microbial family. The abundance of *Oscillospiraceae* was significantly positively correlated with P intake, InsP6, and InsP5 abundance and serum P levels in both intestinal segments. These conditions of higher dietary P availability and a higher phytate reservoir could lead to conditions with more available P in the large intestine, which represent favourable conditions for certain microbes. Indeed, members of the *Oscillospiraceae* were previously shown to be increased abundantly due to phytase supplementation [27]. Moreover, other representatives of the order *Oscillospirales*, namely Oscillospirales_fa were positively correlated with caecum InsP6 and InsP5 as well as colon InsP5. *Selenomonadaceae* and *Succinivibrionaceae* showed significant negative correlation coefficients with InsP6 and InsP5 levels in different parts of the large intestine. Further, the abundance of *Selenomonadaceae* in the colon was significantly correlated with P intake and serum P levels. Interestingly, a certain phytase activity was detected in several species belonging to the *Selenomonadaceae*, among which, e.g., *Mitsuokella* were considered to have a high activity [31].

Besides the essential capacity for P absorption in the small intestine, the role of P metabolism in the caecum and colon is still largely unclear. It is known for certain that P is released from phytic acid by hydrolysis in the large intestine of pigs, which in turn is available for use by microbial species [32]. However, there are various indications for an uptake of P into the organism also via the epithelium of the large intestine [23]. Specifically, indications are provided by the post-ileal disappearance of P and the expression of P-transporters in the terminal colon [33,34].

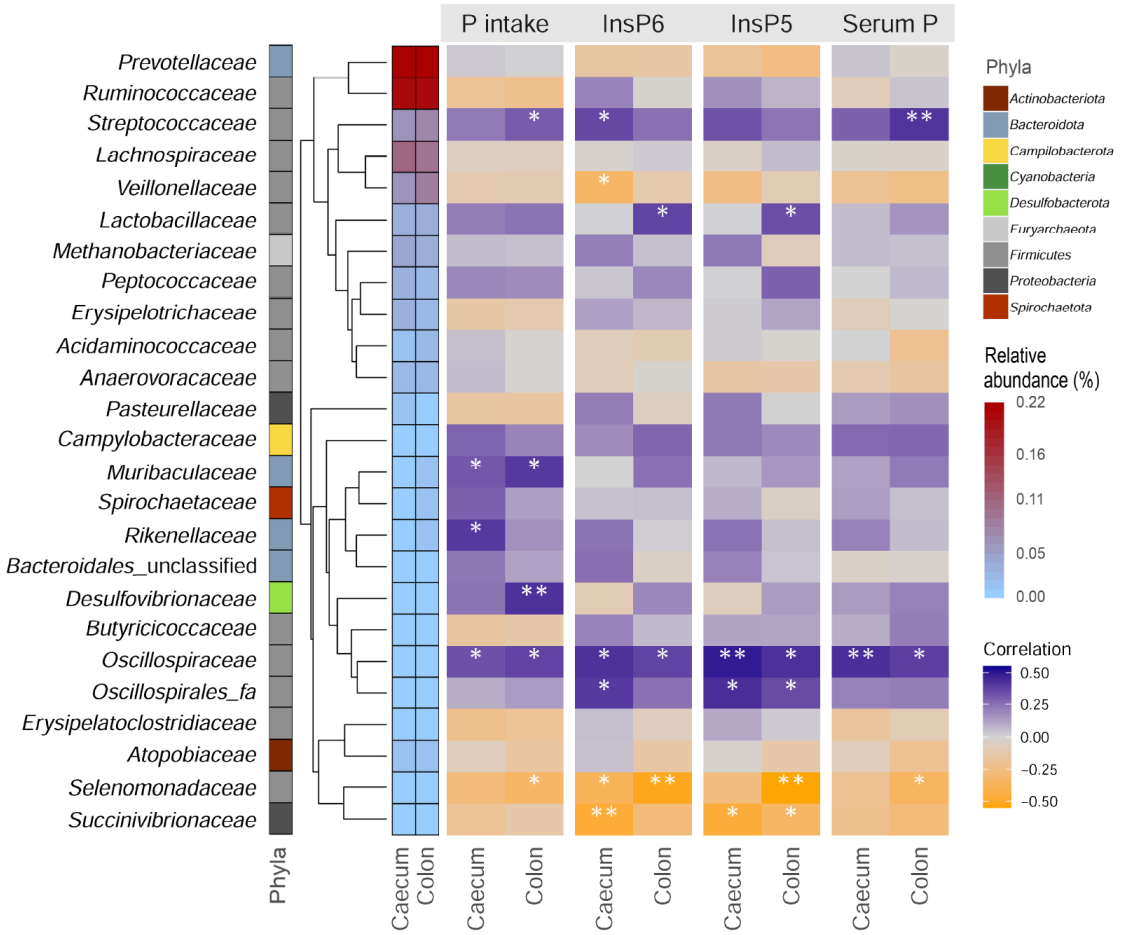

Figure 4. Correlation heatmap. Data of the 25 most abundant microbial families detected in the large intestine were correlated with phosphorus (P) intake, inositol-6 phosphate (InsP6), inositol-5 phosphate (InsP5), and serum P levels. The phyla column indicates the assignment of families to phyla. The cladogram represents the hierarchical clustering of taxa based on their abundance. Correlation coefficients considered significant are indicated by an asterisk (*: $p < 0.05$, **: $p < 0.01$).

4. Conclusions

The current study identifies a number of microbial taxa that either influence the P availability in the large intestine through their phytase activity or are positively or negatively influenced by higher intestinal P levels in terms of growth and replication. Microbial families that might benefit from increasing dietary P supply comprise *Streptococcaceae*, *Muribaculaceae*, *Rikenellaceae*, *Desulfovibrionaceae*, and *Oscillospiraceae*. Potential to provide additional phytase activity and thus induce the hydrolyses of inositolphosphates might be contributed by members of the families *Veillonellaceae*, *Selenomonadaceae*, and *Succinivibrionaceae*. A P-reduced diet for pigs triggers microbial-mediated compensatory actions through phytate cleavage and P release, thereby increasing available P to the host and gut microbiota. In addition to changes in the abundance of certain taxa associated with P digestion and inositol degradation, changes in microbial enzyme expression and

functional shifts of microbes in response to P-variable diets may also be important and can be revealed by metatranscriptome analysis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9061197/s1, Table S1: Group-specific proportions of sequences assigned to taxa at family level in the caecum digesta; Table S2: Group-specific proportions of sequences assigned to taxa at family level in the colon digesta.

Author Contributions: Conceptualization, K.W. and P.W.; methodology, A.W., H.R., M.O., P.J.R.S., and S.P.; formal analysis, H.R., M.O., and K.W.; investigation, M.O., K.W., and P.W.; resources, E.M., P.J.R.S., S.P., and K.W.; data curation, H.R. and M.O.; writing—original draft preparation, H.R.; writing—review and editing, A.W., M.O., P.J.R.S., E.M., S.P., P.W., and K.W.; visualization, H.R.; supervision, K.W.; funding acquisition, P.W. and K.W. All authors have read and agreed to the published version of the manuscript.

Funding: This study has received funding from the European Research Area Network (ERA-NET) co-funds on Sustainable Animal Production (SusAn) as part of the PEGaSus project (2817ERA02D). This work was partly funded by the Leibniz ScienceCampus Phosphorus Research Rostock.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, approved by the Animal Welfare Committee of the FBN, and was licensed by the Ethics Committee of the federal state of Mecklenburg-Western Pomerania, Germany (LALLF-M-V/TSD/7221.3-1-053-15; date of approval: 16 December 2015).

Informed Consent Statement: Not applicable.

Acknowledgments: The authors thank Angela Garve and Frieder Hadlich for their excellent technical help.

Conflicts of Interest: The authors declare no conflict of interest.

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4. General discussion

4.1. Effects of dietary P on gene expression of sodium-dependent P co-transporters

4.1.1. Effects of divergent dietary P intake on tissue-specific expression of sodium-dependent P co-transporters

Some of the sodium-dependent P co-transporter genes including *SLC17A3, SLC34A1, SLC34A3, SLC20A1* and *SLC20A2* were differentially expressed between growing pigs (4 months old) fed high and low P containing diets. The expression level of *SLC17A3* showed to be different in the kidney cortex of pigs fed divergent P-containing diets. Pigs that received lower levels of dietary P showed higher expression levels of the *SLC17A3* gene in the kidney cortex compared with those in the high P group. However, according to studies, *SLC17A3* is actually involved in urate transport rather than P transport (Riches, Wright et al. 2009, Polasek, Jeroncic et al. 2010). The underlying regulatory mechanisms for the differential expression of *SLC17A3* under different dietary P concentrations are still unclear. It is possible that it is indirectly regulated by other genes and factors that could be influenced by the amount of dietary P intake.

The gene expression pattern of sodium-dependent P co-transporters showed that while the *SLC34A1* gene was dominantly expressed at a high level in the kidney (cortex and medulla) of growing pigs, *SLC34A3* had a similar high expression level in both the kidney (cortex and medulla) and intestinal tract (Wubuli, Reyer et al. 2019). These two members of the type II sodium-dependent P co-transporters also showed a significant response to different levels of dietary P intake. While the expression level of *SLC34A1* in the renal cortex was more than three times higher in the low P group than in the high P group, the *SLC34A3* gene also showed significantly higher expression levels in kidney, distal jejunum, and ileum in the low P group compared with the high P group. It has been demonstrated that the levels of dietary P intake has immediate effects on the secretion of PTH in the PTG (Martin, Ritter et al. 2005, Centeno, Herberger et al. 2019). Moreover, an increased dietary P concentration not only increases PTH secretion in PTG (Martin, Ritter et al. 2005, Oster, Gerlinger et al. 2018), but also stimulates the secretion of FGF23 in osteoblasts and osteocytes in bone (see section 1.1.1). Increased serum concentration of PTH and FGF23 then act directly or indirectly on the kidney to downregulate expression of the sodium-dependent P co-transporters *SLC34A1* and *SLC34A3*, which will stimulate P excretion in the kidney. As described previously, the underlying molecular mechanisms of the interaction between dietary P and PTH secretion are associated with the PTH mRNA expression levels and some other miRNA (see section 1.1.2.4). In addition, PTH has been shown to decrease renal expression of SLC34A1 by phosphorylating serine-77 (S77) in the scaffold protein Na+/H+ exchange regulator (NHERF)-1 through activation of PKA (protein kinase A) and PKC (protein kinase C) signaling pathways, leading to degradation of SLC34A1 (Déliot, Hernando et al. 2005, Weinman, Biswas et al. 2007). Furthermore, although it has been debated whether the dietary P acts directly on Ca-sensing receptor or is orchestrated indirectly by downstream signaling cascades to mediate these hormones, a recent study found that dietary P acts directly on the Ca-sensing receptor in the PTG to stimulate PTH secretion. The similar underlying molecular mechanisms were discovered in relation to the phosphaturic action of FGF23 in the kidney. FGF23 was thought to act indirectly on the proximal tubule to decrease apical membrane expression of *SLC34A1* and *SLC34A3* by acting primarily on the distal tubule to generate unknown endocrine or paracrine signals, which in turn act back on the proximal

tubule (White and Econs 2008, Farrow, Summers et al. 2010). However, other studies have found that FGF23 acts directly on the proximal tubule in mice using the co-receptor αKlotho to downregulate renal expression of *SLC34A1* by phosphorylating serine in Na+/H+ exchange regulatory cofactor (NHERF)-1 through activation of ERK1/2 and serum/glucocorticoid-regulated kinase-1 (SGK1) signaling (Andrukhova, Zeitz et al. 2012). This may help explain the threefold low expression of *SLC34A1* in the kidney in the high P diet group than in the low P diet group, in which both the elevated levels of PTH and FGF23 directly suppressed the expression of *SLC34A1* in renal proximal tubules by phosphorylating NHERF-1 via activation of different signaling pathways. Interestingly, a study in parathyroidectomized animals showed that the inhibitory effect of FGF23 on *SLC34A1* is independent of PTH (Shimada, Hasegawa et al. 2004). In addition, a study using transgenic mice has shown that the phosphaturic effects of FGF23 in the kidney are dependent on its critical receptors FGFR1 and FGFR4 (Gattineni, Alphonse et al. 2014). Because *SLC34A3* has a sequence similarity of more than 80% with *SLC34A1*, the low expression of *SLC34A3* in the kidney in the high P diet group could also be caused by the same underlying molecular mechanisms.

In addition, the SLC34A2 is considered the major player of P absorption in the intestinal tract in many species. There is evidence that FGF23 decreases the protein expression level of SLC34A2 and sodium-dependent P transport activity in the small intestine, and this is dependent on the vitamin D receptor (VDR) (Miyamoto, Ito et al. 2005, Goetz, Nakada et al. 2010). However, our data showed that the *SLC34A3* gene, another member of the type II sodium-dependent P transporter family, is predominantly expressed in the small intestine of pigs (Wubuli, Reyer et al. 2019). *SLC34A3* is expressed at lower levels in the intestine of the high P diet group than low P diet group. Because of the high sequence similarity between members of the type II sodium-dependent P transporters (Hilfiker, Hattenhauer et al. 1998, Xu, Bai et al. 1999), it may be that *SLC34A3* represents the role of *SLC34A2* in the intestine of pigs and therefore its expression levels are reduced by the increased level of FGF23 in the high P diet group than in the low P diet group (Perwad, Azam et al. 2005). Results from other studies suggest that the increase in P transport under the low P diet condition may be regulated either directly by higher mTOR activity or indirectly by the suppressive AMPK signaling pathway (Miao, Feng et al. 2017). In addition, calcitriol increases in response to low serum P levels and increases expression of the sodium-dependent P transporter and P transport activity in the small intestine (Lee, Walling et al. 1986, Danisi 1991, Murer, Forster et al. 2004). However, there is also evidence of calcitriol-independent increase in P transport in P deficiency, which occurs in the absence of the calcitriol receptor (Segawa, Kaneko et al. 2004). This suggests that intestinal cells are able to detect changes in intestinal P concentration and increase P absorption via increased synthesis of sodium-dependent P co-transporters. In our study in sows, animals fed a low P diet showed higher levels of calcitriol in blood. These findings indicate that the dietary P regulate intestinal P absorption directly and indirectly via co-regulation of other P regulatory hormones like FGF23 and calcitriol.

The type III sodium-dependent P transporters, *SLC20A1* and *SLC20A2*, also showed differential expression in the kidney and intestine under different dietary P conditions. However, although both *SLC20A1* and *SLC20A2* were shown to be necessary for P-dependent signal transduction, *SLC20A1* and *SLC20A2* contribute only partially to sodium-dependent P transport activity in both organs (Collins, Bai et al. 2004). Both *SLC20A1* and *SLC20A2* showed lower expression in the renal cortex in the high P diet group. Interestingly, in contrast to *SLC20A1* and other P transporters, *SLC20A2* showed high expression in the small intestine, particularly in the jejunum, in the high P diet group. This suggests that the response of *SLC20A2* to increased dietary P concentration may be tissue/cell type specific. A recent study showed that *SLC20A2*, but not *SLC20A1*, is necessary for dietary P dependent regulation of FGF23 synthesis and secretion (Bon, Frangi et al. 2018). In this study, *SLC20A2* knock-out mice lost its ability

to normally regulate their FGF23 level under different dietary P conditions. However, the mechanistic links between *SLC20A2* and FGF23 secretion is unclear.

Overall, our results suggest that differential dietary P intake may directly and indirectly influence gene expression of the major sodium-dependent P co-transporters in the kidney and intestine via a complex regulatory mechanism to maintain P homeostasis in the animal body.

4.1.2. Effect of developmental stages on the activity of sodium-dependent P co-transporters

It should be noted that the mechanisms of increased abundance of SLC34A2 protein and intestinal P absorption do not always clearly correlate with transcriptional activation. However, the results of numerous studies provided evidence for the effects of age differences on the transcriptional activity of sodium-dependent P co-transporters. Our study showed that the major sodium-dependent P transporters in the kidney and intestine of young growing pigs (four months old) responded to high or low levels of dietary P intake (Wubuli, Reyer et al. 2019). However, in adult sows (15 months old), none of these important sodium-dependent P co-transporters were differentially expressed in the kidney and intestine when fed diets with higher or lower than recommended dietary P levels (Wubuli, Gerlinger et al. 2020). For growing and finishing pigs, P requirements are higher in younger pigs than for finishing pigs, and the recommended P levels are sufficient for maximum growth but do not allow for maximum bone mineralization (Council 2012). Generally, additional Ca and P must be included in the diet for maximum bone mineralization. In reproductive sows, Ca and P requirements are influenced by the stages of pregnancy and lactation. A comparative study in rats showed that although calcitriol administration increased intestinal P absorption in both suckling and adult rats, this effect was associated with higher gene expression level of the *SLC34A2* in young but not in adult rats (Xu, Bai et al. 2002). Another study also showed similar results that younger rats had higher P absorption compared to older rats, which corresponded with higher mRNA levels of the *SLC34A2* gene and higher body P balance (Vorland, Lachcik et al. 2018). In that study, the younger animals were found to have lower FGF23 and PTH levels and higher calcitriol levels, implying that hormonal regulation attempts to maintain sufficient P in the body for the increased P requirement for growth by decreasing P excretion in the kidney via decreased FGF23 and PTH and increasing P absorption in the intestine via calcitriol (Marks, Debnam et al. 2010). The latter study also concluded that the regulation of P absorption in the rat small intestine is much closer to that in humans (Walton and Gray 1979, Borowitz and Ghishan 1989). In addition, a recent study reported that αKlotho mRNA and protein levels in the kidney change significantly with age (Yoshikawa, Yamamoto et al. 2018). Some evidence suggests that the FGF23/αKlotho system alters calcitriol and P levels by regulating the expression of *CYP27B1*, *SLC34A1*, and *SLC34A1* in the kidney in response to serum P concentration (Shimada, Hasegawa et al. 2004, Shimada, Kakitani et al. 2004, Segawa, Yamanaka et al. 2007). Yoshikawa et al. also found that differential dietary P intake had significant effects on serum calcitriol levels in young (1 and 2 month old) mice but not in old (13 month old) mice (Yoshikawa, Yamamoto et al. 2018). These results may suggest that age-related changes in the expression of αKlotho in the kidney could affect the sensitivity of P transporters to dietary P at different ages because of altered vitamin D metabolism during aging. Taken together, our observations and data from other studies suggest that the gene expression status of P transporters and their sensitivity to differential dietary P intake may change with age because of changes in hormonal secretion corresponding to the metabolic demand for mineral P at different developmental stages.

4.2. Effects of dietary P on vitamin D metabolism

Vitamin D plays a central role in Ca and P homeostasis and bone mineralization by being activated to form calcitriol, the active form of vitamin D, by hydroxylation in the liver (see section 1.1.2.5) and in the kidney (see section 1.1.2.2). The conversion of vitamin D to calcitriol occurs in the kidney by a mitochondrial enzyme 1a-hydroxylase, which is encoded by the *CYP27B1* gene. Calcitriol plays an important role in the homeostasis of P and other minerals in the body. Our laboratory has previously reported that variable dietary P intake has effects on calcidiol and calcitriol levels in pigs (Oster, Gerlinger et al. 2018, Gerlinger, Oster et al. 2020). According to current knowledge, low dietary P stimulates the synthesis of calcitriol, whereas high dietary P lowers calcitriol levels (Moe 2008, Goretti Penido and Alon 2012). In this study, expression of *CYP27B1* gene is significantly upregulated in the kidney of sows fed low P diet compared to the high P diet group (Wubuli, Gerlinger et al. 2020). In previous studies, the expression of *CYP27B1* is upregulated in the kidney of mice fed a low P diet (Anderson, O'Loughlin et al. 2003, Perwad, Azam et al. 2005, Song and Fleet 2007). Accordingly, in our study, a significantly higher serum calcitriol level was observed in the low P diet group compared to the medium and high P diet group in lactating sows. In a previous study in our laboratory, both the calcitriol and thyroid hormone levels were elevated in pigs on low P diets compared with animals on high P diets (Oster, Gerlinger et al. 2018). Thyroid hormone is essential for skeletal growth and development, controlling bone turnover and maintenance throughout life (Williams 2013, Bassett and Williams 2016). Interestingly, however, a study in mice showed that administration of thyroid hormone decreased plasma calcitriol levels by suppressing renal *CYP27B1* mRNA levels in both the low P and low Ca groups (Kozai, Yamamoto et al. 2013). In this study, thyroid hormones were found to transcriptionally repress the *CYP27B1* gene in the kidney by binding to the heterodimeric junction of thyroid hormone receptors (TRs)/retinoid X receptor α (RXRα) via the involvement of the thyroid hormone negative response element (1a-nTRE). These results suggest that thyroid hormone is also a potent negative regulator of renal *CYP27B1* gene expression. It may be of scientific interest to determine what molecular mechanisms are behind the feedback effects of low dietary P and thyroid hormones on *CYP27B1* gene expression and calcitriol levels. However, it maybe conceivable that endogenous thyroid hormone levels might be influenced by exogenous thyroid hormones or that this was due to the complex feedback regulation mechanism of P homeostasis. In addition, the data from that study provided strong evidence that suppression of *CYP27B1* by thyroid hormones also contributes to the disturbances in mineral and bone homeostasis (Kozai, Yamamoto et al. 2013). Furthermore, a study has shown that thyroid hormone administration upregulates PTH receptor (PTHR) gene expression levels, and PTH also upregulates thyroid hormone receptor (TR) expression in rat osteoblast cells (Gu, Stern et al. 2001). This study provided evidence that these hormones can regulate each other's function in a receptor-mediated manner, which could have significant implications for the regulation of mineral homeostasis and bone remodeling. Thus, the direct and indirect suppression of calcitriol by thyroid hormones and their interaction with the PTH receptor may be physiologically important for vitamin D homeostasis in bone, kidney, and intestine, indicating the physiological role of thyroid hormones in the regulation of vitamin D metabolism by dietary P.

Moreover, it is well known that FGF23 and PTH are important regulators of vitamin D metabolism that directly and indirectly regulate mineral homeostasis in the body. It has been previously reported that, dietary P play an important role in maintaining bone and mineral homeostasis by regulating the expression of the *CYP27B1* gene in the kidney via changes in PTH and FGF23 levels (Anderson, O'Loughlin et al. 2003, Perwad, Azam et al. 2005, Song and Fleet 2007). Increased serum P has been shown to suppress *CYP27B1* primarily via enhancement of FGF23 as well as direct effects on renal

CYP27B1 (Bikle, Murphy et al. 1975, Bikle and Rasmussen 1975). FGF23 has been shown to repress *CYP27B1* transcription in the kidney, at least in part, through transcriptional mechanisms by activating the mitogen-activated protein kinase (MAPK) signaling pathway via MEK/ERK1/2 signaling (Perwad, Zhang et al. 2007, Chanakul, Zhang et al. 2013). Interestingly, another study reported that injection of recombinant FGF23 triggered the phosphaturic effect but did not affect mRNA expression of *CYP27B1* in the kidney or serum calcitriol levels in mice (Kagi, Bettoni et al. 2018). However, injection of recombinant FGF23 significantly decreased the plasma levels of endogenous FGF23. Therefore, this null effect of recombinant FGF23 on *CYP27B1* gene expression could be due to the reduced levels of endogenous FGF23, which are probably counterbalanced by the recombinant FGF23 or by other regulatory factors, and could also depend on different doses or treatment lengths (Kagi, Bettoni et al. 2018). Furthermore, it has been previously demonstrated that high level of dietary P increases secretion of PTH (Martin, Ritter et al. 2005, Oster, Gerlinger et al. 2018). It has been shown that the synthesis of calcitriol is tightly regulated by PTH through regulation of *CYP27B1* activity (Friedman, Coutermarsh et al. 1996, Murayama, Takeyama et al. 1998, Murayama, Takeyama et al. 1999). In vitro studies also showed that PTH increase gene expression of *CYP27B1* in renal proximal tubular cells (Bajwa, Forster et al. 2008). In addition to that, a low P diet downregulated the expression of key 25-hydroxylases in the liver such as *CYP27A1* and *CYP2R1* in mice (Kagi, Bettoni et al. 2018). Moreover, a low P diet decreased mRNA abundances of *VDR* in the kidney of pigs (Oster, Gerlinger et al. 2018). These observations suggest that divergent dietary P intake may affect vitamin D metabolism via regulating the expression of hepatic and renal vitamin D hydroxylases by altering PTH and FGF23 levels. However, further studies are needed to clarify whether this is a direct effect of dietary P intake or whether it requires FGF23 or PTH, as both hormones have been shown to be elevated under the condition of a high P diet.

Taken together, the dietary p appears to affect vitamin D metabolism by regulating renal expression of the *CYP27B1* gene via direct and indirect effects on several other regulatory factors and hormones such as thyroid hormone, PTH, FGF23 and VDR within a rather complex regulatory network of P homeostasis. The mutual regulation via positive and negative feedback loops between these hormones and factors makes it difficult to distinguish the direct and indirect effects of individual regulators of P homeostasis.

4.3. Tissue-specific contributions to the maintenance of P homeostasis

In our study, sodium-dependent P co-transporters, the major P transporters of the body, were found in a variety of tissues in pigs. P homeostasis is maintained by various organs and tissues, including key players such as the intestinal tract, kidney, and bone, by responding in a tissue-specific manner to variable or challenging dietary P intake, as each tissue/organ plays a specific role in maintaining P homeostasis. For example, dietary P deprivation increased the abundance of sodium-dependent P transporters and P uptake in the small intestine (Hattenhauer, Traebert et al. 1999, Katai, Miyamoto et al. 1999). In our study, the intestine and kidney responded simultaneously to low P intake by increasing the abundance of sodium-dependent P transporters in both tissues to maintain sufficient P in the body by increasing both intestinal absorption and renal retention of P in growing pigs. This observation is confirmed by similar results from another study, which was accompanied with lower urinary P levels in pigs fed a low P diet (Pokharel, Regassa et al. 2017). However, the tissue/organ hierarchy in maintaining P balance under challenging P supply conditions is poorly understood.

It has previously been shown that the cultured renal cells are capable to respond directly to changes in environmental P concentrations (Markovich, Verri et al. 1995). Several other studies have also suggested that intestinal cells also respond directly to changes in medium P concentration independently of vitamin D by altering the efficiency of P transport (Lee, Walling et al. 1986, Segawa, Kaneko et al. 2004). Others have demonstrated that there is a P sensor in the intestine that is capable of inducing phosphaturia in the kidney within a short time after the intestinal mucosa is exposed to elevated P concentrations, and that the response that occurs in the kidney is independent of any of the known phosphaturic factors such as PTH and FGF23 that regulate P excretion in the kidney (Martin, Ritter et al. 2005, Berndt, Thomas et al. 2007). Other studies also suggest that early and rapid changes in renal P excretion occur after ingestion of a high P diet independently of FGF23 (Berndt and Kumar 2009). In addition, Berndt et al. also demonstrated the presence of a factor or factors in the intestinal mucosa that directly modulate renal P reabsorption after release and entry into the portal circulation (Berndt, Thomas et al. 2007). These data may suggest that the intestine senses elevated intestinal luminal P concentrations and releases an intestinal mediator/intestinal phosphatonin into the circulation that inhibits renal P reabsorption and increases renal P excretion (Berndt and Kumar 2009). Therefore, it is likely that the intestine, with its high sensitivity to intestinal luminal P concentrations, is at the center of the regulation of P homeostasis and attempts to keep P levels in the body in balance by releasing specific signaling molecules into the circulation when there is a nutritional deficiency in the body.

In addition, skeletal muscle is the main store for most of the P in the body. While the P concentration in the bone depends mainly on the amounts of P in the diet, the P concentration in soft tissues (e.g. muscles and viscera) can be assumed to be fixed and to takes precedence over P deposition in bone (Feaster, Shirley et al. 1953, Brautbar, Lee et al. 1979). It has been reported that when absorbed P resources are limited due to P deficiency nutrition, P is released from bone and mobilized into soft tissue until the depletion of bone P reserves reaches dramatic levels (Day and McCollum 1939, Köhler, Grünberg et al. 2021), suggesting that bone P reserves are mobilized to sustain protein and lipid growth when dietary P intake cannot meet the requirements for soft tissue growth. This observation was recently further demonstrated that P required for soft tissue growth takes precedence over P required for bone maintenance, suggesting that the animal will attempt to maintain its maximum lean tissue retention because soft tissue has essential P requirements to maintain vital functions and bone may tolerate reduced P levels (Létourneau-Montminy, Narcy et al. 2015, Misiura, Filipe et al. 2020). This fact of prioritization of P retention in soft tissue is consistent with the NRC recommendation (Council 2012) that animal has a significantly lower P requirement to maintain the maximum rate and efficiency of weight gain than the P requirement for maximum bone strength and density.

4.4. Effects of dietary P on P utilization in relation to the intestinal microbiota

There is sufficient evidence to support the beneficial role of the intestinal microbiota in the digestibility and utilization of P in the body via its phytase activity (see section 1.1.2.7). Although intestinal P absorption occurs mainly in the small intestine, however, endogenous phytase produced by microbial phytase activity is present mainly in the large intestine (Selle and Ravindran 2008), and there are evidence and indications for phytate hydrolysis by microbial phytases in the large intestine of monogastric animals (Seynaeve, Janssens et al. 2000, Leytem, Turner et al. 2004, Schlemmer, Frølich et al. 2009). In our study, we found the expression of all major sodium-dependent P transporters of

the type II family in the terminal colon of pigs (Wubuli, Reyer et al. 2019). In addition, there is further evidence of P uptake into the organism via the epithelium of the colon (Seynaeve, Janssens et al. 2000, Heyer, Weiss et al. 2015). Further investigation of the effects of dietary P on phytate hydrolysis and the release of phytate-bound P produced by microbial phytase in the large intestine of pigs may provide new insights for improved P utilization. Therefore, we investigated the effects of varying dietary P intake on the microbial composition and profile of inositolphophates in the large intestine of pigs. Measurement of inositol phosphates in the caecum and colon digesta showed that the high P diet increased the concentration of InsP6 and InsP5 in the two intestinal segments. The low P diet group showed significantly lower concentrations of InsP6 and InsP5 in the caecum and colon compared with the high P groups. This result suggests that reduced mineral P intake increases the hydrolysis of phytate in the large intestine of pigs, although no exogenous phytases were added to the diet. Similarly, it was previously reported that lower dietary P content resulted in increased phytase activity in the ileum (Seynaeve, Janssens et al. 2000). It was also observed that a high concentration of mineral P suppressed the degradation of phytase by interacting with inositol phosphates (Shastak, Zeller et al. 2014). Since no exogenous phytases were added to the diets, it is likely that the composition of the intestinal microbiota is the main factor responsible for the observed differences in inositol phosphate degradation.

In addition, investigation of the microbial composition revealed that microbes dedicated to the families *Anaerovoracaceae* and *Pasteurellaceae* were significantly different in abundance between dietary groups. This could mean that some members of these two families have the potential to increase phytate utilization and P availability in the gastrointestinal tract of pigs. In addition, correlation analyses showed a negative correlation for abundance of *Veillonellaceae* with InsP6 content in Caecum digesta, indicating that microbes of this family increase phytate utilization. Interestingly, the addition of exogenous phytase to the diet significantly decreased the abundance of *Veillonellaceae* in growing pigs (Klinsoda, Vötterl et al. 2020). Moreover, the abundances of *Selenomonadaceae* and *Succinivibrionaceae* also showed a significant negative correlation with InsP6 and InsP5 levels in the large intestine. In fact, several species of *Selenomonadaceae*, e.g. *Mitsuokella* with high activity, showed some phytase activity (Yanke, Bae et al. 1998). Shifts in gut microbial composition affect the utilization of P via phytase activity by utilizing higher levels of non-mineral P of plant origin to reduce P excretion and environmental impact. The aforementioned effects of reduced dietary P on the gut microbial community to trigger microbial mediated compensatory actions through phytate cleavage and P release, thereby improving P efficiency, may provide insights into dietary strategies to establish a favorable intestinal microbiome profile.

4.5. Conclusion & outlook

Based on the tissue-specific expression profile of the P transporters, the SLC34A3 appears to be predominant in porcine intestines and could represent the role of SLC34A2 in other species. However, protein expression of the corresponding P-transporters needs further investigation to confirm these observations. Low dietary P intake resulted in higher gene expression of some P transporters, including SLC34A1 and SLC34A3, two major P transporters, compared with the high P group in kidney and intestinal tissues. These results suggest that gene expression of transcellular P transporters is tissue-specific and responds to varying amounts of dietary P to maintain P homeostasis in animals. Regarding the underlying mechanisms, data from the literature search suggest that the intestine is likely at the center of regulating P homeostasis and contributes to maintaining P balance in the body by regulating the expression of P transporters in the intestine and kidney via releasing specific signaling molecules into the bloodstream when P is deficient, and that P requirements for soft tissue growth take

precedence over P requirements for bone maintenance, suggesting that the animal will attempt to maintain its maximum growth rate. In addition, analysis of serum parameters and transcriptional responses in kidney showed that low P diet resulted in an increase in serum calcitriol level and CYP27B1 expression in the kidney of sows compared to the high P group, while serum parameters showed maintained P levels in animals in the low and high P groups. These results suggest that variable P-containing diets may influence vitamin D metabolism in animals to maintain bone and mineral homeostasis by regulating CYP27B1 gene expression in the kidney along with other regulatory factors such as calcitriol, PTH and FGF23. Moreover, analysis of the microbial composition and profile of inositolphophates show that low P diets for pigs trigger microbial mediated compensation through phytate cleavage and P release, thereby increasing the availability of P to the host. These results suggest that targeting the intestinal microbiota through feeding interventions offers opportunities to optimize intestinal phytate and P utilization. In addition, changes in microbial enzyme expression and functional movements of microbes in response to variable dietary P intake may also be critical and detected by metatranscriptome analysis.

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Acknowledgement

I would like to express my great appreciation to all those who contributed to the completion of my PhD thesis.
First and foremost, I would like to thank my PhD supervisor, Prof. Dr. Klaus Wimmers, for giving me the opportunity to do my PhD at the Institute of Genome Biology. This work would not have been possible without the financial support of the PEGaSus project headed by Prof. Klaus Wimmers.
I would like to thank Dr. Henry Reyer very much for his comprehensive support and help with all questions and problems that arose during the course of my PhD. I greatly appreciate the valuable contributions of Henry, who was always willing to help solve difficult analytical questions and has contributed to many aspects that have shaped this project.
I would also like to thank Dr. Michael Oster for his constant advice, detailed discussions, and valuable suggestions that contributed greatly to improving my research skills.
I would like to thank both of them for providing me with the interesting topic and giving me the opportunity to learn and apply a wide range of methods and knowledge. I also thank them for their encouragement and constant guidance in writing my papers and for their comments on the numerous revisions of the published manuscripts and dissertation. Without their valuable support, it would not have been possible to conduct this research.
I would like to thank Prof. Dr. Ulrike Gimsa for her kind supervision of the dissertation at FBN and for taking on the role of reviewer.
I would like to thank Prof. Dr. Volker Stefanski from the University of Hohenheim for taking on the role of reviewer.
My heartfelt thanks go to the technical staff in the laboratory, Angela Garve, Angelika Deike and Janine Wetzel, who always ensured the timely supply of biological samples and access to laboratory equipment, and for the friendly atmosphere in the laboratory.
A big thank you goes to my family and friends for their support during my PhD.
Finally, I would like to thank all colleagues at FBN Dummerstorf who accompanied me during my PhD for the good cooperation and the positive working atmosphere.

Declaration of Independence:

I hereby certify that I have written this thesis independently. For this purpose, I have not used any aids and sources other than those indicated by me, and I have marked as such the passages taken verbatim and in terms of content from the works used.

Rostock, 25. August 2021 ----------

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