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Behavioral Physiology of Livestock

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IMPACT OF DIETARY PHOSPHORUS AND FERMENTABLE SUBSTRATES ON THE IMMUNE SYSTEM AND THE INTESTINAL MICROBIOTA OF THE PIG

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LIST OF ABBREVIATIONS

with the exception of abbreviations only used in Chapter 2-5

BW	Body weight
CaP	Calcium-phosphorus
GIT	Gastrointestinal tract
InsP	Inositol phosphate
$InsP_1$	*Myo*-inositol monophosphate
$InsP_2$	*Myo*-inositol biphosphate
$InsP_3$	*Myo*-inositol triphosphate
$InsP_4$	*Myo*-inositol tetrakisphosphate
$InsP_5$	*Myo*-inositol pentakisphosphate
$InsP_6$	*Myo*-inositol 1,2,3,4,5,6-hexakisphosphate
Itpk	*Myo*-inositol 1,4,5-triphosphate 3-kinase
Ipmk	Inositol polyphosphate multikinase
KLH	Keyhole limpet hemocyanin
NaP_i	Sodium-dependent phosphate
N:L	neutrophile:lymphocyte ratio
P	Phosphorus
PtdIns	Phosphatidylinositol
RANKL	Receptor activator of nuclear factor-κB ligand
SCFA	Short-chain fatty acid
Th	T helper cell
Treg	Regulatory T cell
TRF	Terminal restriction fragment

CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 INTRODUCTION

Phosphorus (P) represents a crucial input for global food systems used for soil fertility, farmer livelihoods and agricultural productivity (Cordell & White, 2015). In the current systems, P is primarily derived from limited mined phosphate rock resources, but only about 20% of P in phosphate rock is part of human food (Cordell et al., 2009). In addition to the limited resources of high-quality mineral P, phosphate rock reserves are located in a few countries, in particular Morocco that controls approximately 75% of the world's remaining phosphate (Neset & Cordell, 2012). The critical challenge of global P shortage is therefore directly linked to future food security and sustainable resource management, especially in the European Union, which is dependent on the supply of raw P from outside Europe. Over the last years, an increasing awareness of possibilities to save P by reducing P pollution from manure and slurries has arisen. The accumulation of P in soil, leaching and P runoff has a tremendous impact on the aquatic ecosystem leading to eutrophication and formation of oceanic dead zones (Singh, 2008). Apart from alternative activities in animal nutrition such as the adjustment of the stocking density on a farm and regional level, and the sewage handling, new dietary formulations of livestock diets emerged as a potential approach to increase the digestibility of plant P, to reduce the supplementation with phosphate (Rodehutscord, 2008) and to minimize losses at the farm level. Particularly in non-ruminant animals such as the pig, the hydrolysis of phytate (any salt of *myo*-inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$)), which is the main storage form of P in plants, is incomplete due to the lack of sufficient enzymes such as endogenous mucosal phytase and phosphatase in the small intestine (Maenz & Classen, 1998; Onyango & Adeola, 2009; Selle et al., 2012). Therefore, there is rising scientific interest to improve the understanding of $InsP_6$ degradation in the digestive tract, as influenced by dietary factors such as the fermentation of carbohydrates and proteins.

P is an essential macronutrient for all life on earth, and it is the second most abundant mineral in the body of human beings, mostly found in bones and teeth in the form of hydroxyapatite. In addition, P is involved in various biochemical reactions including genetical material (DNA, RNA), energy transfer through ATP, and as structural membrane component (phospholipids) of cells (Westheimer, 1987; Desmidt et al., 2015). About 2-4% of dry matter in most cells consists of P (Karl, 2000). Evidence exists that the P supply has a modulating impact on the porcine immune system (Kegley et al., 2001) and the porcine microbiota of the gastrointestinal tract (GIT) (Metzler-Zebeli et al., 2011; Metzler-Zebeli et al., 2013).

Although studies on the interaction between variations in dietary P supply and the immune system are rare, the overall picture from current studies indicates that dietary P has a positive impact on the adaptive immune response due to modulations of lymphocyte proliferation and antibody response (Heyer et al., 2015). In addition, P considerably contributes to bacterial structure and metabolic processes (Durand & Komisarczuk, 1988; Lengeler et al., 1999). Several *in vivo* studies (Metzler et al., 2009; Varley et al., 2010; Metzler-Zebeli et al., 2011; Metzler-Zebeli et al., 2013) described the impact of variations in dietary P supply and carbohydrate sources on the intestinal microbiota composition and activity in pigs. However, only one study by Metzler-Zebeli et al. (2012) unfolds the impact of dietary β-glucan and calcium-phosphorus (CaP) level on interactions between the intestinal microbiota, including their metabolic activities, the integrity of the epithelial barrier function, and the immune system.

1.2 OVERVIEW AND OBJECTIVES OF THE INCLUDED MANUSCRIPTS

Based on a comprehensive literature review presented in Chapter 2, the aim of the present work was to investigate the impact of dietary P, $InsP_6$ and $InsP_6$ hydrolysis products in combination with different fermentable substrates on the porcine immune system and the porcine intestinal microbiota. Therefore, a study with growing pigs was conducted to evaluate modulating effects of varying mineral CaP levels and fermentable substrates on intestinal CaP concentration, and the impact of $InsP_6$ hydrolysis on local and systemic immune parameters, haematological parameters and the intestinal microbiota composition and activity (Chapter 3, 4 and 5). In the following, the objectives of the manuscripts included in this work are given:

MANUSCRIPT 1 (literature review): The impact of phosphorus on the immune system and the intestinal microbiota with special focus on the pig

There is rising scientific evidence concerning possible interactions between the diet composition, the intestinal microbial equilibrium and the host's defence mechanisms, including a potential impact on host health. Dietary supplementation of P has been suggested as a promising strategy to maintain health and performance. Therefore, variations in P availability and the formation of individual inositol phosphate (InsP) isomers due to differences in $InsP_6$-P and in the activity of phytases also have to be taken into account. Special attention was given on the role of dietary P with regard to the immune system and the intestinal microbiota with specific focus on intestinal pathogenic microorganisms. In particular, the impact of individual InsPs on immune functions and bacterial metabolism is of great interest. Thus, the review's objective was to present the

current state of the art on the impact of dietary P, $InsP_6$ and $InsP_6$ hydrolysis products on the immune system and the microbiota along the GIT with special focus on the pig.

The manuscript was published in *Nutrition Research Reviews*.

MANUSCRIPT 2: The impact of dietary phosphorus and calcium on the intestinal microbiota and mitogen-induced proliferation of mesenteric lymph node lymphocytes in pigs

Evidence is emerging that dietary CaP may modulate the porcine intestinal microbiota and immune parameters, although results are not always consistent. In addition, a potential impact of P on the intestinal microbiota is not only restricted to members of the indigenous microbiota, but may also apply to potentially pathogenic bacteria. Any change in the intestinal microbial ecosystem could therefore shift the balance between protective microbiota and pathogens in favour of the pathogens. An *in vivo* study using growing pigs was conducted to evaluate the effect of diets differing in mineral CaP level (low vs. high) and protein source (soybean meal vs. peas). The aim of Manuscript 2 was to describe the effects of differences in mineral CaP content and fermentable substrates on the composition of jejunal, caecal, and colonic bacterial communities, caecal ammonia concentration, and certain aspects of the intestinal immune system in pigs.

The manuscript was published in the *Journal of Animal Science*.

MANUSCRIPT 3: Dietary calcium-phosphorus content and different fermentable substrates modulate distribution and activity of immune cells and the intestinal microbiota in growing pigs

Based on the same experiment as Manuscript 2 and 4, this work is directed on the impact of two dietary CaP levels and different contents of fermentable substrates on the adaptive immune response and the intestinal microbiota in growing pigs. Moreover, in Manuscript 3 the significance of these dietary treatments on animal growth was discussed, and their effects on the gut-associated, but also the peripheral parameters were investigated to determine effects on animal's health.

The manuscript has been submitted to the *Journal of Nutrition*.

MANUSCRIPT 4 (draft): Effect of supplemented mineral calcium-phosphorus and fermentable substrates on phytate hydrolysis, innate immune cell numbers and hematological parameters of growing pigs

Based on the literature review (Manuscript 1), differences in P availability and the formation of individual InsPs due to variations of phytase activity have to be taken into account. In particular, studies on the formation of various InsPs in pigs are rare, and there is no *in vivo* study with pigs describing effects of individual InsP on the immune system. Thus in Manuscript 4, the impact of varying mineral CaP levels and fermentable substrates on intestinal CaP concentration, $InsP_6$ hydrolysis, innate immune cell numbers, and hematological parameters in growing pigs have been assessed.

Manuscript in preparation for publication.

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CHAPTER 2

THE IMPACT OF PHOSPHORUS ON THE IMMUNE SYSTEM AND THE INTESTINAL MICROBIOTA WITH SPECIAL FOCUS ON THE PIG

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Abstract

There is increasing interest in dietary ingredients to support digestive and immune functions, but also maintain a stable microbial ecosystem in the gastrointestinal tract (GIT), especially in weaned pigs. Phosphorus (P) is a non-renewable resource and an essential nutrient both for the gastrointestinal microbial ecosystem and the host, as it is involved e.g. in bone formation, energy metabolism, cellular signalling and stabilisation of cell membranes. There is little information on the impact of dietary P on the immune system of pigs, whereas several studies have shown interactions between dietary calcium-phosphorus (CaP) supply, fermentation activity and microbial composition of the GIT in pigs. In non-ruminant animals, the hydrolysis of phytate, the main storage form of P in plant seeds, is incomplete, as the small intestine lacks sufficient enzymes such as endogenous mucosal phytase and phosphatase, resulting in the formation of variable phosphorylated inositol phosphates (InsPs). The present review focuses on interactions between variations in dietary P supply, the immune system of the host, and the microbiota along the GIT. Though results on the interaction between P and the immune system are inconsistent, several studies in different species have shown a promoting impact of dietary P and phytase supplementation on the adaptive immune response. Current studies with pigs indicate that dietary P may influence the intestinal microbial composition and activity. Individual InsPs or phosphate may also have a modulating impact on pathogenic microorganisms, such as metabolism or virulence. It can be concluded that P may be considered as part of an integrated approach to support immune functions and maintain a stable intestinal microbiota, thereby providing a barrier against potential pathogenic microorganisms. Within this regard, variations in phytate-P content and intrinsic phytase activity of plant feedstuffs, as well as the formation of individual InsPs, have to be considered when formulating diets.

CHAPTER 3

THE IMPACT OF DIETARY PHOSPHORUS AND CALCIUM ON THE INTESTINAL MICROBIOTA AND MITOGEN-INDUCED PROLIFERATION OF MESENTERIC LYMPH NODE LYMPHOCYTES IN PIGS

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Abstract

The objective of the study was to assess the effect of 2 dietary protein sources and 2 calcium-phosphorus (CaP) levels on the microbial ecosystem of the gastrointestinal tract (GIT) and on the intestinal immune system in growing pigs. Thirty-one pigs were fed 4 different diets, corn-soybean meal (SBM) vs. corn-pea meal (PM)-based diets supplemented with 2 different CaP contents each (CaP 20% below (CaP-) or above (CaP+) the animals' Ca and P requirement). The abundance of total bacteria, *Enterobacteriaceae*, and *Bacteroides-Prevotella-Porphyromonas* in the jejunum, cecum, and colon and cecal ammonia concentration were examined. Jejunal and ileal mesenteric lymph node (MLN) lymphocytes (LC) were analysed for mitogen-induced proliferative response against concanavalin A (ConA). The CaP- diets showed higher jejunal gene copy numbers of total bacteria ($P < 0.01$), and tended to have higher jejunal, cecal and colonic gene copy numbers of *Bacteroides-Prevotella-Porphyromonas* ($P < 0.10$) compared to CaP+ diets. The abundance of intestinal *Enterobacteriaceae* were higher ($P < 0.05$), whereas cecal ammonia concentration was lower ($P < 0.01$) for the PM diets compared to the SBM diets. The proliferation after ConA stimulation tended ($P < 0.10$) to be higher for CaP- diets in MLN LC compared to CaP+ diets. A higher abundance of *Enterobacteriaceae* for the PM diet in combination with CaP-content might increase the risk for intestinal disturbances and the lower cecal ammonia concentration for the PM diets may be beneficial for gut health.

CHAPTER 4

DIETARY CALCIUM-PHOSPHORUS CONTENT AND DIFFERENT FERMENTABLE SUBSTRATES MODULATE DISTRIBUTION AND ACTIVITY OF IMMUNE CELLS AND THE INTESTINAL MICROBIOTA IN GROWING PIGS

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Running head: CaP affects gut-health in pigs

Dietary calcium-phosphorus content and different fermentable substrates modulate distribution and activity of immune cells and the intestinal microbiota in growing pigs[1]

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ABSTRACT: This study was conducted to evaluate the effects of diets with varying calcium-phosphorus (CaP) contents and different protein sources on the peripheral and gut-associated immune system, as well as on the composition and activity of the intestinal microbiota in pigs. There is increasing interest in dietary ingredients that are appropriate to support digestive and immune functions, but also maintain a stable microbial ecosystem in the gastrointestinal tract (GIT). Physiologic effects of dietary CaP in combination with different fermentable substrates on immune cell structures and functions, and the intestinal microbiota have not been investigated in pigs. Growing pigs randomized in 4 groups received either a corn-soybean meal or a corn-pea based diet, each with 2 different CaP contents: low (66% of pigs' CaP requirement) vs. high (120% of pigs' CaP requirement) for a period of 9 wk. Blood and secondary lymphoid tissue were examined for immune cell distribution and activity. Antigen-specific immunoglobulin (Ig) concentration in plasma samples was measured after keyhole limpet hemocyanin (KLH) immunization. Digesta content was analyzed for bacterial composition and short-chain fatty acid (SCFA) concentration. The ratio of naive:antigen-experienced T-helper cells (Th) in the blood ($P < 0.01$), as well as the mitogen-induced proliferation of T cells were higher ($P < 0.05$), but the plasma anti-KLH IgG concentration was lower for the low CaP diets in wk 8 ($P < 0.05$). The caecal bacterial composition was significantly shaped by dietary CaP content. Gene copy numbers of saccharolytic bacteria, such as *Bifidobacterium* spp., *Eubacterium rectale*, and *Roseburia* spp. ($P < 0.05$), and caecal SCFA concentrations ($P < 0.10$) were higher for the high CaP and soybean meal diets. These results demonstrate that both, CaP supply and the amount of fermentable substrates may beneficially affect gut health through modulation of the adaptive immune response and the intestinal microbiota.

Key words: immune system, intestinal microbiota, phosphorus, pig

INTRODUCTION

Evidence that dietary phosphorus (**P**) may modulate the porcine intestinal microbiota (Metzler-Zebeli et al., 2011) and immune parameters (Kegley et al., 2001) is emerging, although results are not always consistent. The possible impact of P supply on the adaptive and the innate arm of the immune system of different farm animals, such as lymphocyte proliferation and antibody response (Kegley et al., 2001), and on the porcine microbiota along the gastrointestinal tract (**GIT**) has recently been reviewed in (Heyer et al., 2015). The intestinal microbiota responds to variations in dietary carbohydrate content and protein sources. Graded levels of soybean meal at the expense of cornstarch seem to have a positive linear effect on the growth of saccharolytic and potentially beneficial groups such as bifidobacteria in ileal and faecal samples (Rist et al., 2014). Possible interactions between CaP and protein/carbohydrate have not been studied yet. Among other methods, the use of terminal restriction fragment length polymorphism (**T-RFLP**) has been proven to characterize structure of porcine gut microbiota (Castillo et al., 2007; Ivarsson et al., 2012; Pedersen et al., 2013; Burbach et al., 2016). In general, a high diversity in gut microbial composition is considered to be beneficial for the host health (Kühn et al., 1993; Konstantinov et al., 2004).

Thus, our objective was to assess the effects of diets differing in their calcium-phosphorus (**CaP**) content as well as protein and carbohydrate sources on the peripheral and gut-associated immune system, the jejunal, caecal, and colonic bacterial communities, and intestinal short-chain fatty acid (**SCFA**) concentrations in pigs. We hypothesized that a minimum requirement of P is needed to ensure normal immune functioning as well as a stable microbial ecosystem, thus it can be suggested that the low CaP diet might negatively affect host health.

MATERIALS AND METHODS

Animals and diets

The study was conducted at the experimental unit of the department of Behavioral Physiology of Livestock of the University of Hohenheim. All Exp. and care of animals were approved by the local authorities (Regierungspräsidium Stuttgart, Germany; permit number: V308/13 TH) in accordance with the German Welfare Legislation. Three wk before the Exp. started, pigs were obtained from the Agricultural Experimental Station of the University of Hohenheim (Germany) to facilitate their handling and adaption to the new housing conditions. Animals were housed in individual pens (each 3.25 m^2) under controlled environmental conditions (temperature about 20°C, light regime 12/12 h). Each pen was equipped with a drinking nipple and a stainless steel feeder. The health status of the animals was monitored daily.

In total, 31 German landrace × Piétrain pigs (initial BW: 54.7 ± 4.1 kg) were used in a 2 × 2 factorial arrangement of dietary treatments with 2 consecutive periods with 15 or 16 pigs. The pigs were fed one of 4 assay diets based on corn-soybean meal or corn-peas, and formulated to meet or exceed pigs' nutrient requirements (NRC, 2012), except for CaP content (Heyer et al., 2016). Diets were supplemented with 2 different CaP contents, referred to as low and high, with CaP contents amounting to 66% and 120% of pigs' actual CaP requirement, respectively, based on animals' BW in the range from 50 to 75 kg (NRC, 2012). Feed was offered twice daily at 0800 and 1500 h, and pigs had free access to water. Pigs were weighed each wk to adjust their daily feed allowances to an amount of 4% of the average BW of all pigs within each period. Feed refusals were measured in wk 4, 6, and 8 for each pig separately. Each sampling period consisted of 4 d per wk. At the end of the Exp., pigs received the last meal 3 to 4 h before being euthanized to ensure that digesta had reached each section of the GIT (Pieper et al., 2008).

Immunization and sample collection

Each period was composed of 9 wk, including an adaptation of 19 d to the diets. In wk 4 and 6, all pigs were immunized with a 2 ml intramuscularly injection of 2.5 mg keyhole limpet hemocyanin (**KLH**; Sigma-Aldrich Corporation, St. Louis, MO) in 1 ml incomplete Freund's adjuvant (Sigma-Aldrich Corporation, St. Louis, MO). Blood samples were collected on Monday between 0900-1030 h from all animals before the start of the experimental dietary treatment (wk 1) and in wk 4, 6, 8 using a nose snare. Approximately 18 ml blood was collected by jugular vein puncture in heparinized tubes (Monovette 9 ml sodium heparin; Sarstedt AG & Co, Nümbrecht, Germany) for immunological measurements. In addition, 5 ml blood in EDTA KE tubes (Sarstedt AG & Co, Nümbrecht, Germany) were collected for determination of white blood cells by an automated hematology system (pocH 100-iV Diff, Sysmex Deutschland GmbH, Norderstedt, Deutschland). Two ml of the heparinized blood were centrifuged (1000 × g for 10 min at 10°C) and plasma samples were stored at -20°C until further analysis of anti-KLH IgG and anti-KLH IgM.

At the end of the Exp., general anesthesia was induced in pigs with an injection of ketamin (20 mg/kg BW, Serumwerk Bernburg AG, Bernburg, Germany) and azaperon (2 mg/kg BW, Lilly Deutschland GmbH, Bad Homburg, Germany). Pigs were euthanized by intravenous injection via the ear vein with pentobarbital (about 70 mg/kg BW, CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany). Immediately thereafter, the abdominal cavity was opened and the entire GIT was removed. The gut sections (jejunum, ileum, caecum, colon) were separated using clamps to prevent mixing of digesta. First, the spleen, the ileal and the

jejunal mesenteric lymph nodes (**MLN**) were taken and transferred into ice cold phosphate-buffered saline (**PBS**) without Ca^{2+}/Mg^{2+} and with 50 µg/ml gentamycin (Biochrom GmbH, Berlin, Germany). Then, digesta from the jejunum (80 cm from the *Plica ileocaecalis*), ileum, caecum and colon were aseptically collected. Digesta of each gut section were immediately stored at -20°C and transferred to -80°C after sampling procedure. In addition, other subsamples of digesta for measurements of SCFA were stored at -20°C.

Tissue processing and separation of Peripheral Blood Mononuclear Cells

About 4 g of the mid-spleen (cross section), randomized ileal and jejunal MLN were separated into small pieces and processed by gentleMACS Dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were isolated by 100 µm sterile Cell Strainer (Greiner Bio-One GmbH, Frickenhausen, Germany) and stored in PBS without Ca^{2+}/Mg^{2+} (Biochrom GmbH, Berlin, Germany) and with 50 µg/ml gentamycin (Biochrom GmbH, Berlin, Germany) at 4°C. After all samples were processed, the immune cells were centrifuged (300 × g for 5 min at 4°C). Then, the single-cell suspension was resuspended in RPMI-10 (RPMI 1640 (Biochrom GmbH, Berlin, Germany)) supplemented by 10% fetal calf serum (Seromed Biochrom KG, Berlin, Germany) and 50 µg/ml gentamycin (Biochrom GmbH, Berlin, Germany). The cell concentration was determined by a Z2 Coulter Counter (Beckman Coulter GmbH, Krefeld, Germany).

Peripheral Blood Mononuclear Cells (**PBMC**) were isolated of the processed spleen and blood samples according to (Grün et al., 2013) with few modifications for the splenic single-cell suspension, as follows. Tubes were filled with 15 ml of Biocoll separating solution (Biochrom GmbH, Berlin, Germany) and were coated with 10 ml splenetic single-cell suspension and 15 ml PBS without Ca^{2+}/Mg^{2+} (Biochrom GmbH, Berlin, Germany). After centrifugation (500 × g for 35 min at 20°C), the PBMC layer was transferred into a new tube, washed and the cell concentration was determined by a Z2 Coulter Counter (Beckman Coulter GmbH, Krefeld, Germany).

Leukocyte distribution and mitogen-response

Blood samples and single-cell suspension of the spleen and the MLN were stained as described previously (Grün et al., 2013), except for regulatory T cells (**Tregs**). The number of white blood cells was determined by an automated hematology system (pocH 100-iV Diff, Sysmex Deutschland GmbH, Norderstedt, Deutschland). The following fluorochrome-labeled

antibodies were used: CD3 (clone PPT3), CD4 (clone 74-12-4), CD8α (clone 76-2-11), and CD 172α (clone 74-22-15) (Biozol Diagnostica Vertrieb GmbH, Echingen, Germany).

The percentage of Tregs in blood and tissue samples were determined according to (Käser et al., 2011) with few modifications, as follows. Blood PBMC, the splenic PBMC, as well as cells of jejunal and ileal MLN were stained with the fluorochrome-labeled antibody CD4 (clone 74-12-4), fixed, permeabilized using the Foxp3 Staining Buffer Set (eBioscience Inc., San Diego, CA) according to manufactures' instructions, and then stained with an anti-Foxp3 antibody (clone FJK-16s) for 30 min at room temperature in the dark. After washing, cells were analysed by FACSCanto^{TM} flow cytometer (BD Biosciences, San Jose, CA) using the software BD FacsDiva^{TM}.

In vitro-immune cell activity of blood PBMC, the splenic PBMC, as well as cells of jejunal and ileal MLN were determined by the mitogen-induced lymphocyte proliferation assay according to (Grün et al., 2013) with few modifications. Briefly, 1.5×10^5 immune cells were pipetted per well of a U-bottom 96-well cell culture plate (neoLab Migge GmbH, Heidelberg, Germany) in triplicates per treatment and stimulated subsequently or left without stimulation. For stimulation of the blood and spleen immune cells, either 5 µg/ml of the mitogen concanavalin A (**ConA**; Biochrom GmbH, Berlin, Germany), 5 µg/ml of the mitogen pokeweed mitogen (**PWM**; Sigma-Aldrich Corporation, St. Louis, MO) or 10.0 µg/ml KLH (Sigma-Aldrich Corporation, St. Louis, MO) were used. The jejunal and ileal MLN samples were only stimulated with PWM (Sigma-Aldrich Corporation, St. Louis, MO). After 48 h of incubation (37°C, 5% CO_2), 0.25 µCi titrated thymidine ([6-3H], PerkinElmer Inc., Waltham, MA) were given to each well. One d later, cells were harvested on glass fiber filters and radioactivity was measured by liquid scintillation analyzer (PerkinElmer Inc., Waltham, MA). The Δ counts per minute (**cpm**) for ConA, PWM, and KLH were determined for each individual (Δ cpm = stimulated (cpm) - unstimulated cells (cpm)).

Anti-KLH IgG and anti-KLH IgM ELISA measurements

Concentrations of plasma anti-KLH IgG were measured by ELISA as described previously (Grün et al., 2014). Concentrations of anti-KLH IgM were measured by ELISA as described by (Schrama et al., 1997; Bolhuis et al., 2003; Grün et al., 2014). ELISA plates were coated with KLH (Sigma-Aldrich Corporation, St. Louis, MO) diluted in coating buffer (15 mM $NaHCO_3$ and 35 mM Na_2CO_3, pH 9.6) and incubated overnight at 4°C. Diluted plasma samples were then added to the plates, and the anti-KLH antibodies were detected with HRP-labeled goat anti-pig

IgM (GeneTex Inc., Irvine, CA). Plasma samples were quantified by reference to standard curves constructed with a pooled plasma control and calculated as arbitrary units.

T-RFLP analysis, cloning and sequence analysis

Profiles of bacterial communities in digesta samples were obtained by analysis of T-RFLP as described by (Burbach et al., 2016) with few modifications. Bacterial 16S rRNA gene fragments were amplified from the genomic DNA using primer pair 27F, 5'labeled with 6-carboxyfluorescein, and 1492R (Lane, 1991). The PCR mixture included 3% DMSO and the PCR was performed with 33 cycles. 200 ng amplicon DNA were digested with 5 U MspI and analyzed on an ABI 3130xI Genetic Analyzer (Applied Biosystems; Foster City, CA).

To assign single terminal restriction fragments (**TRF**) clone libraries from ileal digesta and faecal samples of one pig fed with soybean meal, low CaP diet were constructed. 16S rRNA gene fragments were amplified with primer 27F and 1492R. Amplicons were gel-purified using Double Pure Kit (Bio&Sell GmbH, Feucht bei Nürnberg, Germany) and cloned into pGEM®-T Easy Vector System (Promega, Madison, WI) according to manufactures instructions. Randomly selected clones were amplified using vector primers M13F and M13R. PCR products of positive clones were screened by amplified rDNA restriction analysis for variable phylogenetic clusters. M13 amplicons were digested with 5 U RsaI (New England BioLabs, Ipswich, MA) at 37°C for 1 h and separated on a 2% agarose gel (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) with 50 V for 2.5 h. Representatives of the different amplified rDNA restriction analysis patterns were amplified with vector primers T7 and SP6 and send to Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). Sequence assembly and manual editing was done in Codon-Code Aligner (http://www.codoncode.com/aligner/) and BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Sequences were taxonomically assigned using RDP Sequence Match and submitted to NCBI GenBank under the accession number KU705874-KU705900.

Quantitative real-time PCR analysis of 16S rRNA abundance, and SCFA concentration

Genomic DNA extraction and quantification of jejunal, caecal, and colonic digesta samples was determined according to (Weiss et al., 2016) using previously described primer sets (**Table 1**). Results were reported as $\log_{10}$ 16S rRNA gene copies/g fresh matter. Concentration of SCFAs in jejunal and caecal samples was analyzed by GC according to (Wischer et al., 2013).

Statistics

All data are expressed as mean ± SEM of the raw data without transformation. Statistical analyses were performed with use of the statistical package R version 3.1.0 (Development Core Team R, 2014). Shapiro-Wilk test was used to evaluate normal distribution, as well as homogeneity of variance by a plot of the fitted values against the residuals. Logarithmic or square route transformations were used for those variables that were not normally distributed or where homogeneity of variance could not be assumed. Treatment effects (protein source and CaP content) and their interactions, BW, as well as for the immunological parameters sampling duration were analyzed using linear mixed-effect models with the function "lmer" of the R package lme4 (Bates et al., 2014). Every model was corrected for dam, period, as well as for the digesta and tissue parameters sampling dates by including those parameters as random effects. The covariate BW and sampling duration was excluded from the model, if no significant effect ($P \geq 0.05$) could be observed. From each pig, blood samples were collected before starting the experimental dietary treatment (wk 1) to determine their individual baseline and were statistical analyzed, as described above. No treatment effect has been determined, except for cytotoxic T cells that were lower ($P < 0.05$) for pigs fed the low CaP diets (**Table 2**). Changes from this baseline were calculated for each individual for wk 4, 6, 8 to determine a treatment effect. For all values, differences were considered significant at $\alpha < 0.05$ and $0.05 < P < 0.10$ as a tendency.

Statistical analysis of multivariate T-RFLP data sets were carried out using PRIMER v6 (Clarke and Warwick, 2001). Abundance data of TRF in the range of 70 bp to 1400 bp were standardized by total and Bray-Curtis similarity matrix. Bacterial community structures based on T-RFLP data were explored by principal coordinate analysis. Group-average cluster were superimposed onto principal coordinate analysis plot to show similarity within groups of samples. TRF contributing to dissimilarity between sample groups were determined by similarity percentage analysis and bubbles representing relative abundance of mostly contributing TRF were superimposed onto principal coordinate analysis plots. Significant differences ($P \leq 0.05$) in bacterial communities were evaluated by analysis of similarity (**ANOSIM**). The R statistic value expresses the separation between groups in range from -1 to 1, with the higher R value the more distinct are the groups.

Table 1. Oligonucleotide primers used for quantitative real-time PCR

Target bacterial group	Primer sequences (5′–3′)	Amplicon length, bp	Annealing temp, °C	Reference
Total eubacteria	**F:** GTG STG CAY GGY YGT CGT CA	147	52	Fuller et al., 2007
	R: ACG TCR TCC MCN CCT TCC TC			
Lactobacillus spp.	**F:** AGA GGT AGT AAC TGG CCT TTA	391	59	Malinen et al., 2003
	R: GCG GAA ACC TCC CAA CA			
Bifidobacterium spp.	**F:** TCG CGT CYG GTG TGA AAG	243	59	Rinttilä et al., 2004
	R: CCA CAT CCA GCR TCC AC			
Roseburia spp.	**F:** AGG CGG TAC GGC AAG TCT	353	59	Rinttilä et al., 2004;
	R: AGT TTY ATT CTT GCG AAC G			Veiga et al., 2010
Clostridium cluster IV	**F:** GGC GGC YTR CTG GGC TTT	147	65	Ramirez-Farias et
	R: CCA GGT GGA TWA CTT ATT GTG TTA A			al., 2009; Lay et al.,
				2005
Clostridium cluster	**F:** CGG TAC CTG ACT AAG AAG C	429	63	Rinttilä et al., 2004
XIVa	**R:** AGT TTY ATT CTT GCG AAC G			
Eubacterium rectale	**F:** AAG GGA AGC AAA GCT GTG AA	200	65	Balamurugan et al.,
	R: TCG GTT AGG TCA CTG GCT TC			2008

Table 2. T cell populations[1] and T cell proliferation to ConA[2] in the blood before starting the experimental dietary treatment (baseline, wk 1) in growing pigs

	Soybean meal		Peas		*P* value			
Item	Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
T cells	7401 ± 651	7307 ± 236	6815 ± 391	7053 ± 392	0.12	0.48		
Naive Th[3]	1626 ± 164	1603 ± 104	1418 ± 168	1634 ± 96	0.45	0.42		
Antigen-experienced Th	1192 ± 94	1564 ± 202	1325 ± 143	1359 ± 135	0.05	0.22		
Ratio of naive:antigen-experienced Th	1.40 ± 0.15	1.16 ± 0.19	1.11 ± 0.12	1.28 ± 0.14	0.66	0.97		0.96
Cytotoxic T cells	941 ± 106	1348 ± 117	1107 ± 57	1177 ± 86	0.85	0.01		0.19
Treg[4]	115 ± 15	125 ± 7	120 ± 14	139 ± 9	0.53	0.67		0.04
B cells	1528 ± 202	1615 ± 162	1732 ± 169	1705 ± 143	0.38	0.20		
Lymphocyte proliferation	11542 ± 1695	16249 ± 2343	11743 ± 2024	12090 ± 1722	0.17	0.19		

[1]Expressed as number/µl blood. Values are means ± SEM, *n* = 7-8.

[2]Expressed as Δcpm calculated for each triplicate and determined for each individual as follows: Δcpm = stimulated cells (cpm) - unstimulated cells (cpm). ConA = Concanavalin A; counts per minute = cpm.

[3]Th = T-helper cell.

[4]Treg = regulatory T cell.

RESULTS

Leukocyte distribution and activity

Several interactions between CaP content and protein source could be determined for the change from baseline of various immune cell subtypes in the blood at different sampling points. In detail, the number of total T cells, antigen-experienced T-helper cells (**Th**), cytotoxic T cells in wk 6 ($P < 0.10$), and naive Th in wk 8 ($P < 0.10$) were lower for the soybean meal diets in combination with the low CaP content (**Table 3**). The number of blood B cells was lower compared to the baseline in wk 4 ($P < 0.05$) for the low CaP diets. The number of antigen-experienced Th was lower ($P < 0.10$) for pigs fed the low CaP diets in wk 8, resulting in a higher naive:antigen-experienced Th ratio ($P < 0.10$) for the low CaP diets. For the secondary lymphoid tissue, several interactions between CaP content and protein source could be observed for splenic naive Th ($P < 0.10$), as well as the naive:antigen-experienced Th ratio ($P < 0.10$) for all analyzed tissues (**Table 4**). In the ileal MLN, there was a tendency for a lower number of T cells ($P < 0.10$), naive Th ($P < 0.10$), cytotoxic T cells ($P < 0.10$), and Treg ($P < 0.10$) for the low CaP contents. Jejunal antigen-experienced Th were lower ($P < 0.10$) for the pea diets.

In blood, proliferative responses of lymphocytes induced by the mitogen ConA in wk 6 were higher ($P < 0.05$) in pigs fed the pea diets (**Fig. 1**). However, the low CaP concentrations resulted in a higher cell proliferation in wk 8 ($P < 0.05$). No difference between the experimental groups were found for the lymphocyte proliferation in blood samples to PWM and KLH for the different wk, in the spleen samples to ConA, PWM, and KLH, and for the MLN to PWM (data not shown).

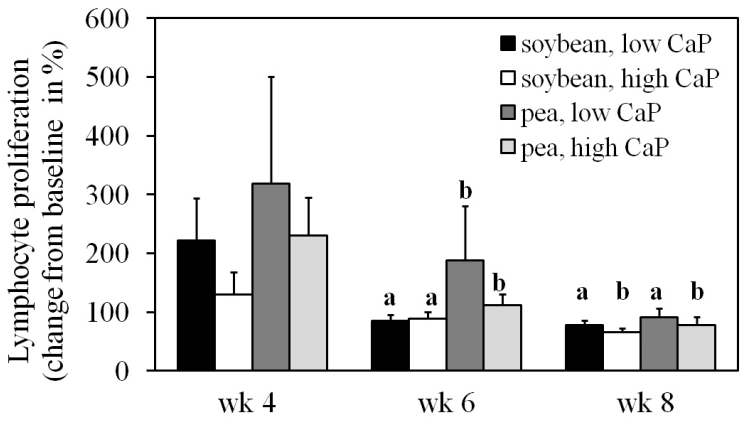

Figure 1. Effect of CaP content (low/high) and protein source (soybean meal/pea) on lymphocyte proliferation after stimulation with Concanavalin A in wk 4, 6, 8 in growing pigs. Data are expressed as percentage of the respective proliferation determined before starting the experimental dietary treatment (wk 1). Values are means ± SEM, n = 4-8 pigs. For each wk, labeled means without a common letter differ, $P < 0.05$.

Anti-KLH plasma IgM and anti-KLH plasma IgG

One main objective was to assess the effects of different CaP and fermentable substrates on the primary humoral immune response to the apathogenic neo-antigen KLH. Thus, the plasma concentration of anti-KLH IgM (main immunoglobulin (**Ig**) of the first immune response) and anti-KLH IgG (main Ig of the second immune response) upon KLH immunization were determined. After the first immunization (wk 6), no dietary treatment effect could be observed for plasma anti-KLH IgM concentrations (**Table 3**). Plasma anti-KLH IgG concentrations were lower ($P < 0.05$) after the second immunization in wk 8 for pigs fed the low CaP diets. Though plasma anti-KLH IgG concentration appears higher for the pea diets, this effect was not significant.

Table 3. Blood T and B cell populations as well as plasma anti-KLH Ig concentrations of pigs fed diets with different protein sources and CaP contents[1]

	Soybean meal		Peas		*P* value			
Item	Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
T cells								
wk 4	119 ± 7.2	117 ± 5.1	116 ± 6.9	123 ± 7.8	0.92	0.26		0.18
wk 6	120 ± 4.8	120 ± 13.3	132 ± 5.5	125 ± 6.1	0.36	0.40	0.08	0.26
wk 8	124 ± 8.7	126 ± 7.6	135 ± 8.4	128 ± 4.5	0.88	0.22		0.17
Naive Th[2]								
wk 4	98 ± 2.6	100 ± 9.1	97 ± 2.7	97 ± 7.6	0.53	0.39		0.14
wk 6	99 ± 6.9	93 ± 9.3	111 ± 6.1	96 ± 4.7	0.61	0.24		
wk 8	100 ± 10.6	95 ± 3.5	115 ± 6.3	94 ± 2.8	0.72	0.79	0.05	0.43
Antigen-experienced Th								
wk 4	113 ± 7.9	112 ± 9.1	102 ± 11.0	123 ± 12.4	0.52	0.12		0.19
wk 6	108 ± 8.9	137 ± 30.3	115 ± 10.0	116 ± 10.8	0.61	0.03	0.06	0.07
wk 8	113 ± 17.4	130 ± 14.5	113 ± 10.4	122 ± 10.2	0.50	0.09		0.20
Ratio of naive:antigen-experienced Th								
wk 4	89 ± 5.6	90 ± 7.9	109 ± 17.3	81 ± 5.5	0.61	0.19		
wk 6	93 ± 6.1	79 ± 7.4	105 ± 15.5	87 ± 7.7	0.11	0.003		0.42
wk 8	92 ± 7.4	79 ± 7.3	108 ± 12.4	81 ± 6.6	0.57	0.07		
Cytotoxic T cells								
wk 4	135 ± 11.7	118 ± 15.8	122 ± 16.7	138 ± 16.2	0.96	0.93		0.82
wk 6	133 ± 9.8	135 ± 25.3	158 ± 14.5	145 ± 16.1	0.14	0.54	<0.10	0.18
wk 8	149 ± 18.9	143 ± 24.8	163 ± 16.3	141 ± 12.9	0.64	0.31		0.10
Treg[3]								
wk 4	112 ± 8.5	126 ± 8.4	119 ± 9.6	113 ± 6.3	0.51	0.24		0.17
wk 6	86 ± 19.0	69 ± 16.1	84 ± 19.5	86 ± 21.2	0.41	0.61		0.08
wk 8	77 ± 26.0	81 ± 16.8	69 ± 21.0	65 ± 15.9	0.27	0.92		0.77
B cells								
wk 4	88 ± 9.3	100 ± 4.6	79 ± 11.3	100 ± 5.3	0.53	0.02		0.57
wk 6	85 ± 6.7	91 ± 8.9	82 ± 5.4	99 ± 4.6	0.66	0.32		0.21
wk 8	86 ± 7.3	95 ± 4.1	83 ± 6.5	100 ± 5.2	0.41	0.96		0.09
anti-KLH[4] IgM								
wk 6	2819 ± 311	3528 ± 731	3415 ± 773	3558 ± 913	0.71	0.15		0.12
anti-KLH IgG								
wk 8	2937 ± 1217	2929 ± 432	4612 ± 1359	5613 ± 989	0.18	0.03		0.12

[1]T and B cell populations expressed as changes from baseline (wk 1) in %. Values are means ± SEMs, n = 6-8. [2]Th = T-helper cell. [3]Treg = regulatory T cell. [4]KLH = keyhole limpet hemocyanin.

Table 4. T cell populations in the spleen, and the jejunal and ileal MLN[2] of pigs fed diets with different protein sources and CaP contents[1]

	Soybean meal		Peas		*P* value			
Item	Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
T cells								
Spleen	5886 ± 823	5802 ± 773	5415 ± 965	5644 ± 505	0.63	0.72		0.55
Jejunal MLN	7082 ± 993	6744 ± 1462	10331 ± 3744	6884 ± 1506	0.84	1.00		0.24
Ileal MLN	12616 ± 3688	9624 ± 1412	7685 ± 1115	9689 ± 1854	0.22	0.07		0.04
naive Th[3]								
Spleen	490 ± 104	371 ± 70	332 ± 63	421 ± 63	0.25	0.56	0.09	0.45
Jejunal MLN	2039 ± 274	1735 ± 437	2709 ± 843	2017 ± 366	0.70	0.94		0.27
Ileal MLN	4562 ± 1853	2619 ± 498	1880 ± 214	2709 ± 529	0.18	0.09		0.04
Antigen-experienced Th								
Spleen	1045 ± 223	960 ± 124	797 ± 133	856 ± 123	0.12	0.44		0.33
Jejunal MLN	3315 ± 539	4923 ± 1156	4029 ± 553	2874 ± 689	0.06	0.80		0.39
Ileal MLN	4334 ± 788	6117 ± 829	4877 ± 716	4634 ± 905	0.16	0.10		0.15
Ratio of naive:antigen-experienced Th								
Spleen	0.5 ± 0.05	0.4 ± 0.04	0.4 ± 0.05	0.5 ± 0.09	0.58	0.09	0.08	0.045
Jejunal MLN	0.6 ± 0.06	0.4 ± 0.09	0.6 ± 0.12	0.8 ± 0.15	0.21	0.96	0.06	0.45
Ileal MLN	1.0 ± 0.31	0.5 ± 0.14	0.4 ± 0.03	0.6 ± 0.09	0.29	0.94	<0.01	0.38
Cytotoxic T cells								
Spleen	839 ± 166	858 ± 129	782 ± 148	749 ± 127	0.19	0.72		
Jejunal MLN	1721 ± 355	1628 ± 555	3541 ± 2114	1515 ± 318	0.97	0.79		0.16
Ileal MLN	3294 ± 935	2354 ± 521	1970 ± 387	2552 ± 613	0.19	0.05		0.02
Treg[4]								
Spleen	38 ± 8	35 ± 5	26 ± 5	42 ± 9	0.81	0.26		0.52
Jejunal MLN	204 ± 45	214 ± 72	283 ± 59	237 ± 38	0.64	0.97		0.27
Ileal MLN	442 ± 97	442 ± 80	344 ± 61	607 ± 180	0.72	0.06		0.11

[1]T cell populations expressed as number/g tissue × 10^4. Values are means ± SEM, n = 6-8.
[2]MLN = mesenteric lymph node.
[3]Th = T-helper cell
[4]Treg = regulatory T cell.

Analysis of intestinal bacterial communities

T-RFLP profiles from jejunal, ileal, caecal and colonic digesta samples were analyzed and a total number of 156 TRF were detected with a core community of 11 TRF found in all gut sections. ANOSIM test revealed significant differences between all four analyzed gut section (R = 0.598, $P = 0.001$). In a principal coordinate scaling plot (**Fig. 2**) the bacterial communities from digesta samples are grouped by small (jejunum and ileum) or large intestine (caecum and colon) origin. T-RFLP profiles from jejunal and ileal digesta share 77% average similarity and caecal-colonic profiles 41% average similarities, among each other these two intestine sections showed in average 79% dissimilarity. This dissimilarity was mainly caused by TRF 521, which was predominated in all gut sections, with a fourfold higher average abundance in small intestine than large intestine.

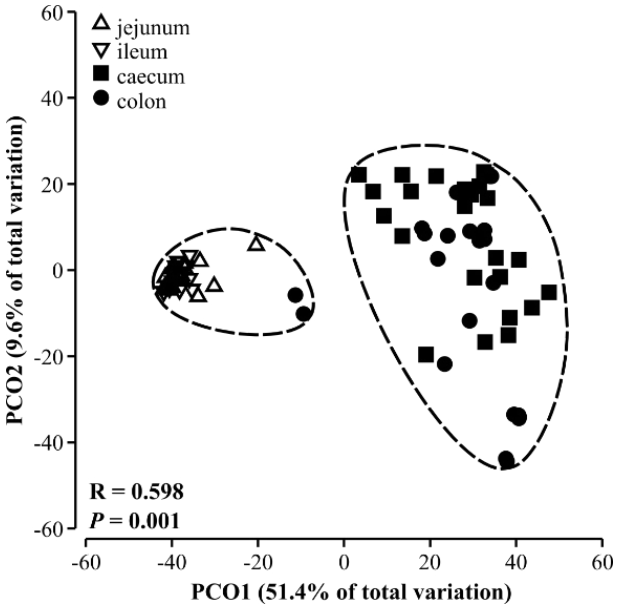

Figure 2. Principal coordinate analysis plot of bacterial community structure by T-RFLP data based on Bray-Curtis similarity matrix. Community profiles are displayed according to gut section and clusters of 30% similarity are overlaid as determined by group-average clustering. T-RFLP = terminal restriction fragment length polymorphism.

The bacterial community structures in each gut section were further investigated in respect to diet-associated effects. The T-RFLP patterns of the caecal digesta samples showed separations of communities based on the dietary treatment (R = 0.387, $P = 0.001$) (**Fig. 3**). These differences were not associated with the protein source ($P > 0.05$) but significantly associated with CaP level ($P = 0.001$). Within the other gut sections, no significant effects related to dietary treatments were found. Caecal TRF profiles from pigs fed the low CaP content diets and those fed the high

CaP content diets showed 55% dissimilarity between the groups. We identified discriminating TRF that contributed to more than 5% to these dissimilarities: TRF 92, TRF 219 and TRF 521. By comparing T-RFLP profiles with clone sequences we could assign TRF 521 to an uncultured bacterium related to unclassified *Porphyromonadacea*e (clone HomPi_148) in the order of *Bacteroidales* (**Table 5**). TRF 92 bp can probably be assigned to a member of the *Clostridiales* as several clones sequences (clone HomPi_107 and HomPi_127) share relevant sequence similarity and showed an experimental TRF of 91 bp, which can be considered to be equivalent, by accepting technical bias of +/-1 bp. The discriminating TRF 219 was not assigned to a known sequence. As shown in Fig 4, TRF 521, unclassified *Porphyromonadacea*e, was detected in higher abundance in samples of higher dietary CaP content (23.5%) than in samples of lower CaP content (23.5% *vs.* 13.0%). For the abundance of TRF 92, *Clostridiale*s spp., the opposite ratio was observed with a higher abundance in low dietary CaP (12.6% *vs.* 5.5%).

Quantification of certain bacterial groups and SCFA concentration

In the jejunum, the low CaP content enhanced gene copies of *Clostridium* cluster IV ($P < 0.05$) and *Bifidobacterium* spp. ($P < 0.01$) (**Table 6**). Jejunal gene copy numbers of *Lactobacillus* spp. were lower ($P < 0.05$) for the pea based diets. In the caecum, gene copy numbers of *Eubacterium rectale* ($P < 0.01$) and *Roseburia* spp. ($P < 0.05$) were lower for the low CaP diets. Furthermore, caecal numbers of *Eubacterium rectal* ($P < 0.01$), *Roseburia* spp. ($P < 0.10$) and *Bifidobacterium* spp. ($P < 0.05$) were lower for the pea diets. In the colonic digesta, interactions between protein sources and CaP contents were found for *Clostridium* cluster IV and XIVa ($P < 0.05$). Colonic gene copy numbers of *Eubacterium rectale* were lower ($P < 0.10$) and *Bifidobacterium* spp. ($P < 0.05$) were higher for the low CaP diets. Colonic gene copy numbers of *Roseburia* spp. were lower ($P < 0.05$) for the pea diets.

In jejunal and caecal digesta, acetate was the predominant fermentation end-product (**Table 7**). In the jejunum, there were CaP-protein source interactions for total SCFA ($P < 0.05$) and acetate ($P < 0.05$), indicating that the low CaP content increased their concentration when combined with the soybean meal diets. The concentration of isovalerate was lower ($P < 0.01$) for the low CaP diets. Pigs fed the pea diets had a lower concentration of propionate ($P < 0.10$) and isovalerate ($P < 0.10$). In caecal digesta, the concentration of total SCFA ($P < 0.01$), acetate ($P < 0.01$), propionate ($P < 0.01$), and valerate ($P < 0.10$) were lower for the low CaP diets. Total SCFA ($P < 0.10$), acetate ($P < 0.01$), butyrate ($P < 0.05$), and isovalerate ($P < 0.10$) were lower for the pea diets.

Immunological and microbiological values were associated with body weight

The BW gain of the pigs averaged from 585 g/d to 965 g/d and was lower for the pigs fed the low CaP diets ($P < 0.01$) and the pea diets ($P < 0.05$) (**Table 8**). Although, the feed intake was lower ($P < 0.01$) for pigs fed the low CaP diets, the feed:gain ratio was higher ($P < 0.05$). In the blood, the number of antigen-experienced Th, and Treg were lower in wk 6 ($P < 0.10$), whereas the number of B cells was higher ($P < 0.10$) with an increased BW in wk 8 (**Table 3**). For the immunological tissues, the number of T cells, naive Th, and cytotoxic T cells in ileal MLN, as well as the ratio of naive:antigen-experienced Th in the spleen were lower ($P < 0.05$) with an increased BW (**Table 4**). The lymphocyte proliferation to the mitogen ConA in wk 6 ($P < 0.05$) and wk 8 ($P < 0.10$) was higher with an increased BW. The gene copy numbers of jejunal *Roseburia* spp. ($P < 0.05$), jejunal and colonic *Bifidobacterium* spp. ($P < 0.10$), and caecal *Lactobacillus* spp. ($P < 0.05$) were higher with an increased BW, whereas, the gene copy numbers of caecal and colonic *Eubacterium rectal* ($P < 0.10$), as well as jejunal isovalerate ($P < 0.01$) and caecal acetate concentration ($P < 0.10$) were lower with an increased BW (**Table 6 and 7**).

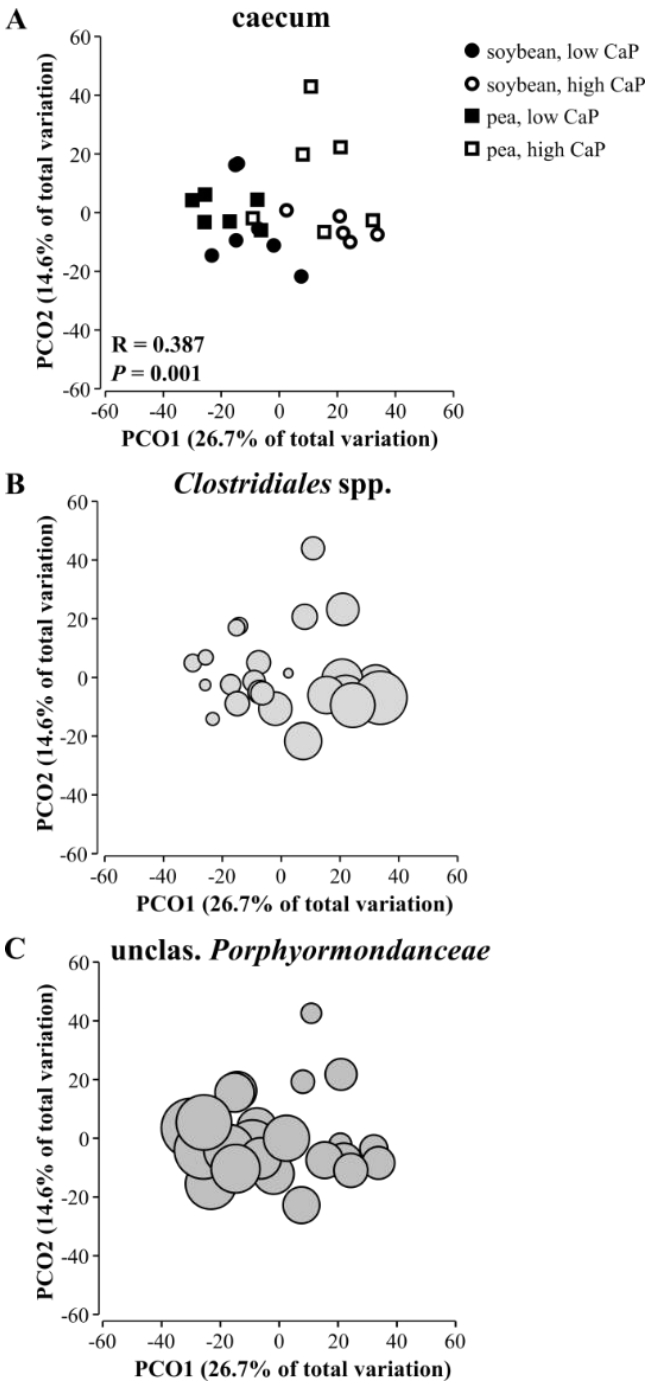

Figure 3. Effect of different dietary CaP levels on bacterial community in caecal digesta. (A) Bubbles representing the relative abundance of single TRFs with contribution > 5% to pattern of the caecal community are superimposed onto principal coordinate analysis plot. The bubble area of TRF 92, *Clostridiales* spp., (B) depicts higher abundance in samples of low dietary CaP content, whereas TRF 521, unclassified *Porphyromonadaceae*, (C) shows higher abundances in samples from high CaP groups. TRF = terminal restriction fragment.

TABLE 5. 16S rRNA gene clone library analyzed with T-RFLP and putative identity aligned to closest relative in RDP database

genebank accession number	TRF	clone name	putative identity	similarity score	order	family
KU705900	91	HomPi_107	*Clostridium sensu stricto*	0.92	*Clostridiales*	*Clostridiaceae* 1
KU705883	91	HomPi_127	*Clostridium* cluster XI	0.94	*Clostridiales*	*Peptostreptococcaceae*
KU705885	95	HomPi_129	*Prevotella*	0.98	*Bacteroidales*	*Prevotellaceae*
KU705878	96	HomPi_92	*Clostridium sensu stricto*	0.99	*Clostridiales*	*Clostridiaceae* 1
KU705897	97	HomPi_162	*Clostridium sensu stricto*	1.00	*Clostridiales*	*Clostridiaceae* 1
KU705887	131	HomPi_135	unclassified_*Ruminococcaceae*	0.90	*Clostridiales*	*Ruminococcaceae*
KU705875	155	HomPi_84	*Clostridium* cluster XI	0.98	*Clostridiales*	*Peptostreptococcaceae*
KU705894	192	HomPi_157	*Clostridium sensu stricto*	0.93	*Clostridiales*	*Clostridiaceae* 1
KU705876	193	HomPi_86	*Clostridium* cluster XI	0.99	*Clostridiales*	*Peptostreptococcaceae*
KU705881	194	HomPi_105	*Clostridium sensu stricto*	0.98	*Clostridiales*	*Clostridiaceae* 1
KU705884	212	HomPi_128	unclassified_*Clostridiales*	0.98	*Clostridiales*	unclassified *Clostridiales*
KU705877	281	HomPi_88	*Clostridium* cluster XI	0.94	*Clostridiales*	*Peptostreptococcaceae*
KU705879	283	HomPi_96	*Clostridium sensu stricto*	0.92	*Clostridiales*	*Clostridiaceae* 1
KU705896	284	HomPi_160	unclassified_*Ruminococcaceae*	0.97	*Clostridiales*	*Ruminococcaceae*
KU705893	295	HomPi_153	*Clostridium sensu stricto*	0.94	*Clostridiales*	*Clostridiaceae* 1
KU705890	297	HomPi_147	unclassified "*Porphyromonadaceae*"	0.98	*Bacteroidales*	*Porphyromonadaceae*
KU705882	308	HomPi_125	unclassified *Erysipelotrichaceae*	0.96	*Erysipelotrichales*	*Erysipelotrichaceae*
KU705874	455	HomPi_74	unclassified *Clostridiales*	0.95	*Clostridiales*	unclassified *Clostridiales*
KU705899	458	HomPi_190	*Clostridium* cluster XI	0.99	*Clostridiales*	*Peptostreptococcaceae*
KU705889	466	HomPi_143	*Clostridium sensu stricto*	0.94	*Clostridiales*	*Clostridiaceae* 1
KU705880	469	HomPi_97	*Clostridium sensu stricto*	0.94	*Clostridiales*	*Clostridiaceae* 1
KU705886	472	HomPi_130	*Clostridium sensu stricto*	0.97	*Clostridiales*	*Clostridiaceae* 1
KU705895	519	HomPi_158	*Clostridium* cluster XI	0.91	*Clostridiales*	*Peptostreptococcaceae*
KU705892	520	HomPi_152	*Clostridium sensu stricto*	0.99	*Clostridiales*	*Clostridiaceae* 1
KU705891	521	HomPi_148	unclassified "*Porphyromonadaceae*"	0.98	*Bacteroidales*	*Porphyromonadaceae*
KU705888	522	HomPi_136	*Oscillibacter*	0.99	*Clostridiales*	*Ruminococcaceae*
KU705898	523	HomPi_181	*Clostridium sensu stricto*	1.00	*Clostridiales*	*Clostridiaceae* 1

TABLE 6. Bacterial numbers in the jejunum, caecum, and colon of growing pigs fed diets with different protein sources and CaP contents[1]

	Soybean meal		Peas		*P* value			
Site and bacteria	Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
Jejunum								
Lactobacillus spp.	7.3 ± 0.25	6.7 ± 0.40	6.8 ± 0.39	6.9 ± 0.20	<0.05	0.88		
Clostridium cluster IV	4.7 ± 0.15	4.5 ± 0.09	4.9 ± 0.21	4.5 ± 0.12	0.41	0.02		
Clostridium cluster XIVa	ND	ND	ND	ND				
Eubacterium rectale	ND	ND	ND	ND				
Roseburia spp.	4.7 ± 0.15	4.7 ± 0.11	4.7 ± 0.15	4.7 ± 0.12	0.03	0.08	0.07	0.02
Bifidobacterium spp.	7.2 ± 0.24	6.1 ± 0.48	6.5 ± 0.53	5.2 ± 0.48	0.29	<0.01		0.09
Caecum								
Lactobacillus spp.	7.5 ± 0.31	8.0 ± 0.21	7.8 ± 0.11	8.0 ± 0.05	0.42	0.58		0.04
Clostridium cluster IV	7.8 ± 0.07	8.1 ± 0.06	8.0 ± 0.15	8.2 ± 0.15	0.18	0.83		
Clostridium cluster XIVa	9.9 ± 0.08	9.8 ± 0.12	9.8 ± 0.12	9.9 ± 0.12	0.92	0.57		
Eubacterium rectale	5.9 ± 0.27	6.5 ± 0.36	5.2 ± 0.22	6.2 ± 0.12	<0.01	<0.01		0.02
Roseburia spp.	8.5 ± 0.14	8.7 ± 0.17	8.1 ± 0.11	8.6 ± 0.14	0.09	0.01		
Bifidobacterium spp.	7.3 ± 0.21	7.0 ± 0.24	6.8 ± 0.31	6.4 ± 0.39	0.01	0.12		
Colon								
Lactobacillus spp.	7.1 ± 0.38	8.3 ± 0.27	7.4 ± 0.23	8.2 ± 0.13	0.47	0.29		
Clostridium cluster IV	8.2 ± 0.06	8.6 ± 0.05	8.5 ± 0.07	8.6 ± 0.08	<0.05	0.10	0.03	
Clostridium cluster XIVa	10.1 ± 0.08	10.0 ± 0.10	10.3 ± 0.10	9.9 ± 0.03	0.90	0.26	0.04	
Eubacterium rectale	6.1 ± 0.34	6.5 ± 0.30	6.2 ± 0.40	6.1 ± 0.20	0.14	0.06		0.07
Roseburia spp.	8.7 ± 0.12	8.7 ± 0.09	8.4 ± 0.11	8.5 ± 0.11	0.03	0.12		
Bifidobacterium spp.	7.1 ± 0.30	6.9 ± 0.23	7.1 ± 0.31	6.6 ± 0.38	0.83	0.01		0.06

[1]Expressed as $\log_{10}$ 16S rRNA gene copy numbers g^{-1} fresh matter. Values are means ± SEMs, n = 4-6.

[2]ND = not detected.

TABLE 7. Concentrations of fermentation metabolites in the jejunum and caecum of growing pigs fed diets with different protein sources and CaP contents[1]

	Soybean meal		Peas		*P* value			
Site and metabolite	Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
Jejunum								
Total SCFA	308 ± 12.6	287 ± 36.0	131 ± 25.6	263 ± 27.2	<0.01	0.14	<0.05	
Acetate	273 ± 10.0	259 ± 29.1	111 ± 22.6	230 ± 19.8	<0.01	<0.10	0.03	
Propionate	1 ± 0.2	1 ± 0.2	1 ± 0.1	2 ± 1.4	0.05	0.67		
Isobutyrate	ND	ND	ND	ND				
Butyrate	31 ± 3.2	23 ± 8.0	16± 2.5	27 ± 6.0	0.38	0.66		
Isovalerate	2 ± 0.4	3 ± 0.8	1 ± 0.5	2 ± 0.1	0.09	<0.01		<0.01
Valerate	ND	ND	ND	ND				
Caecum								
Total SCFA	1005 ± 52.0	1160 ± 83.6	812 ± 79.2	1079 ± 54.1	0.05	<0.01		
Acetate	672 ± 32.1	793 ± 52.8	518 ± 51.5	742 ± 34.2	<0.01	<0.001		0.08
Propionate	176 ± 11.8	230 ± 16.6	180 ± 15.8	215 ± 7.2	0.32	<0.01		
Isobutyrate	5 ± 0.7	5 ± 0.7	4 ± 0.6	5 ± 0.4	0.57	0.56		
Butyrate	141 ± 11.1	118 ± 13.7	100 ± 13.1	107 ± 7.5	0.03	0.93		
Isovalerate	5 ± 0.8	5 ± 0.7	4 ± 0.7	4 ± 0.3	0.07	0.49		
Valerate	6 ± 1.9	10 ± 1.2	6 ± 0.8	7 ± 0.7	0.24	0.05		

[1]Expressed as mmol/kg digesta. Values are means ± SEM, *n* = 4-6.
[2]ND = not detected.

TABLE 8. Body weight gain, feed consumption and feed:gain ratio of growing pigs fed diets with different protein sources and CaP contents[1]

Item	Soybean meal		Peas		*P* value	
	Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP
BW gain (g/d)	656 ± 25	965 ± 36	585 ± 33	848 ± 70	0.04	<0.001
FC[2] (g/d)	2419 ± 158	3174 ± 67	2490 ± 147	2910 ± 262	0.58	<0.01
F:G[3] ratio (g/g)	3.7 ± 0.24	3.3 ± 0.08	4.4 ± 0.44	3.4 ± 0.18	0.15	0.02

[1]Data were recorded in wk 4, 6, and 8 of the Expt. Values are means ± SEMs, $n = 6$. [2]FC = feed consumption.

[3]F:G = feed:gain.

DISCUSSION

In the current study, results demonstrated that the low CaP diets resulted in a higher ratio of naive:antigen-experienced Th caused by a lower number of antigen-experienced Th in the blood. This might be indicative for an impaired immunological memory functioning and probably a limited protection against pathogens. These findings are consistent with the tissue data showing a higher ratio of naive:antigen-experienced Th for pigs fed the soybean meal diets in combination with the low CaP content. Thus, the dietary treatment had local and systemic modulatory impact. Accordingly, plasma concentration of anti-KLH IgG was lower for pigs fed the low CaP diets, suggesting an inhibition of the adaptive, humoral arm of the immune system. In contrast to our results, Kegley et al. (2001) found in weaned pigs after immunization with either sheep red blood cells or ovalbumin a decreased antigen-specific IgG antibody response. The authors also found no effect on PWM-induced proliferation with increasing dietary P supply (monocalcium-dicalcium phosphate). Similarly, in the present study the *in vitro*-proliferative response of lymphocytes to PWM was not influenced by the dietary treatment and the one to ConA stimulation was even higher in pigs fed the low CaP diets. Thus, it can be assumed that the lower number of antigen-experienced Th in blood and the lower *in vivo* response to KLH found in pigs fed the low CaP diets most probably did not result from an impaired reactivity of lymphocytes per se in these pigs. Further studies are needed to elucidate, whether the innate immune system, such as migration patterns as well as cytokine secretion profile of antigen-presenting cells, are affected by the dietary treatment and thus causing or mediating the observed modulations in the adaptive immune response.

The interaction between the intestinal microbiota including their metabolic products such as butyrate, the integrity of the epithelial barrier function, and the local and systemic immunity describes the close relationship between these structures (Grenham et al., 2011). There is increasing evidence that P has to be considered as part of an integrated approach to support digestive and immune function, with special focus on maintaining the intestinal eubiosis of pigs (Heyer et al., 2015; Mann et al., 2014). One TRF assigned to bacterial order *Clostridiales* was identified as discriminator in bacterial communities of low CaP content. By clone library matches, this TRF is assumed to be affiliated to *Clostridium sensu stricto* and *Clostridium* cluster XI. With the two main representatives *C. perfringens* and *C. difficile*, the potential identity of TRF 92 is conditionally associated with a less healthy microbiota (Songer and Uzal, 2005). By qPCR, *Clostridium* cluster IV and *Clostridium* cluster XIVa were examined, but here no significant CaP effect was found. These groups of *Clostridiales* contain many butyrate producers and therefore are associated with a healthy microbiota (Louis and Flint, 2009; Levine et al., 2013). In general, *Clostridiales* dominate porcine microbiota in large intestine (Zhao et al., 2015) and low numbers of pathogenic species can be found in healthy subjects (Ozaki et al., 2004). In contrast, numbers of caecal saccharolytic bacteria, such as *Bifidobacterium* spp., *Eubacterium rectale*, and *Roseburia* spp., as well as the SCFA concentration were lower for the low CaP and pea diets. Short chain fatty acids, especially acetate, propionate and butyrate, are the major end-products of carbohydrate fermentation, contributing to lower the pH in the colon and thereby preventing the growth and activity of pathogenic bacteria (Conlon and Bird, 2015). In particular, butyrate, plays an important role in cell growth, differentiation, intestinal barrier function, and also immune regulation (Macfarlane and Macfarlane, 2012). These finding suggest that the intestinal microbiota, in particular specific bacteria, affects the host immune system in a different manner, which is in agreement with studies showing the presence of a gut-brain-axis (Grenham et al., 2011). In the present work, the butyrate concentration was modulated by the protein source, thus may have influenced immune parameters determined in the present study. Future research should focus on the role of active microbial fractions and their functions, possibly causing activation of the immune system due to e.g. LPS present on the outer membrane of gram-negative bacteria.

Evidence exists that the CaP availability and the formation of individual inositol phosphates affects the intestinal microbiota in pigs (Heyer et al., 2015) due to insolubility of mineral Ca-phytate complexes at pH levels usually prevalent in the small intestine (Selle et al., 2009). In consequence, the P digestibility might be reduced due to the formation of insoluble Ca-phytate complexes and the entailed limited efficiency of mucosal phytase, in particular, in the

proximal gut (Steiner et al., 2007). The decrease in CaP availability for the intestinal microbiota can be associated with a lowered fermentation activity, similar to studies with rumen microbes (Komisarczuk et al., 1987). Komisarczuk et al. (1987) examined the effects of variations in P supply on rumen microorganisms in sheep and determined that the available P in the surrounding medium affects the activity of bacterial fibrolytic enzymes. The authors concluded that a P deficiency may cause a reduced SCFA synthesis due to a decreased fermentation of cellulose by the microorganisms. According to a study in pigs (Metzler-Zebeli et al., 2010), an increased concentration of Ca ions may reduce the adhesion potential of strains such as *Lactobacillus*, resulting in decreased colonization of mucosal areas due to competition for the same adhesion sites with other bacterial species such as *E.coli* (Larsen et al., 2007).

The impact of type and amount of fermentable carbohydrates in the diet on the intestinal microbiota composition and activity has been reported in several studies (Aumiller et al., 2015). Compared to soybeans, peas are rich in slowly digestible or resistant starch (Aumiller et al., 2015; Jezierny et al., 2011), thereby possibly affecting selectively specific bacterial groups (Ryan et al., 2006). In an *in vitro* study with colonic bacteria isolated from pigs (Jha et al., 2011) using the gas technique to determine fermentation characteristics of different feed ingredients differing in carbohydrate and protein composition, peas and pea fibers increased bacterial protein synthesis. It needs to be taken into account that nitrogen incorporated into bacterial mass would not be immediately available for metabolite production such as ammonia. This suggests that increased bacterial nitrogen assimilation might be beneficial for host health (Mosenthin et al., 1992). Moreover, the experimental diets contained different amounts of corn, resulting in different amounts of carbohydrates, mainly resistant starch, available for microbial fermentation. In a study of Rist et al. (2014), an increased supply of cornstarch may have supported starch escaping digestion in the small intestine to be subsequently utilized by the microbiota in the large intestine. Therefore, a shift from protein-fermenting to carbohydrate-fermenting bacterial groups occurs. These results are in agreement with our present study with higher caecal gene copy numbers of *Enterobacteriaceae* for pigs fed the pea based diets compared to the soybean meal diets (data presented in (Heyer et al., 2016)). In contrast, caecal numbers of *Eubacterium rectale*, *Roseburia* spp. and *Bifidobacterium* spp. were higher for the soybean meal diets. The bacterial shift might have modulated the bacterial metabolite production, resulting in a lower total SCFA, acetate, and butyrate concentration in caecal digesta for pigs fed the pea diets compared to the soybean meal diets. It can be concluded that regardless of CaP content variations in fermentable substrates might have a modulating impact on the intestinal microbiota, thereby influencing the host immune system (Grenham et al., 2011).

The high dietary CaP content increased abundance of one TRF that was assigned to an uncultured bacterium related to *Porphyromonadaceae*. This certain clone sequence was assigned by its similarity to a 16S rRNA sequence isolated from feces of pigs with high levels of antibiotic resistance (Kalmokoff et al., 2011). Members of *Porphyromonadaceae* are common members of porcine intestine (Leser et al., 2002) and found in increased abundance in human intestine of Crohn's disease patients (Manichanh et al., 2006) as well as in malnourished children (Gupta et al., 2011). A mice study revealed negative correlation between gain in body weight and *Porphyromonadaceae* abundance (Ryan et al., 2014). This is in contrast to our findings, where the abundance of the related T-RF showed an increase along with an increased BW gain in dietary groups of high CaP content. In addition, there is rising evidence that growth hormones affect immunological parameter (Murphy et al., 1995), such as stimulatory effects on human and murine T cells *in vitro* (Snow et al., 1981; Mercola et al., 1981). Although, no consistent effect of BW gain on immunological and microbiological values could be observed and no determination of growth hormones has been performed, further research is needed to elucidate the impact of animal growth and growth-related hormones with e.g. immune-modulatory properties such as somatotropin or cortisol (Webster Marketon and Glaser, 2008; Welniak et al., 2002).

In conclusion, the present study demonstrated that CaP and fermentable substrates have a distinct effect on the peripheral and gut-associated immune system, as well as microbial composition and activity in pigs. In particular, the CaP content has a modulating effect on the outcome of the adaptive immune response, on the composition of the caecal microbiota and in particular on saccharolytic bacteria that might be beneficial for animal’s health.

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CHAPTER 5

EFFECT OF SUPPLEMENTED MINERAL CALCIUM-PHOSPHORUS AND FERMENTABLE SUBSTRATES ON PHYTATE HYDROLYSIS, INNATE IMMUNE CELL DISTRIBUTION AND HEMATOLOGICAL PARAMETERS OF GROWING PIGS

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Running head: CaP and phytate affects health in pigs

Effect of supplemented mineral calcium-phosphorus and fermentable substrates on phytate hydrolysis, innate immune cell numbers and hematological parameters of growing pigs

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ABSTRACT: To evaluate the impact of varying mineral calcium-phosphorus (CaP) levels and fermentable substrates on intestinal CaP net absorption, phytate (*myo*-inositol hexakisphosphate, $InsP_6$) hydrolysis, innate immune cell numbers, and hematological parameters in pigs, a 2×2 factorial arrangement was conducted using 31 crossbred pigs with an initial average BW of 54.7 kg. Authors hypothesized that CaP deficiency caused by lower dietary mineral CaP content and modulations in $InsP_6$ hydrolysis lead to an inhibitory effect on the innate, cellular arm of the immune system and values associated with oxygen delivery that might affect animals' health. Pigs were fed either a corn-soybean meal or a corn-pea based diet, each with 2 different CaP levels supplemented with monocalcium phosphate and calcium carbonate (low CaP, 66% of the CaP requirement; high CaP, 120 % of the CaP requirement). After 3 wk of adaptation to the diets, blood samples were collected for analyses of immune cell distribution and hematological parameters. Fecal and digesta samples were examined for P, Ca, inositol phosphate (InsP) isomers and the marker Titanium dioxide (Ti). Jejunal, cecal, and fecal P net absorption was lower ($P < 0.010$) for the low CaP diets. In addition, the $InsP_6$ hydrolysis in digesta samples was not affected by the dietary treatment, nevertheless the $InsP_6$ concentration in the jejunum was lower for low CaP diets ($P < 0.050$). Jejunal and cecal concentration of $Ins(1,2,4,5,6)P_5$ was higher ($P < 0.100$) for the soybean meal diets. Dietary CaP level mainly affected hematological parameters, resulting in a higher number of red blood cells (RBC), and concentration of hemoglobin (HGB) and hematokrit (HCT) ($P < 0.001$) for the low CaP diets in wk 4. On the other hand, numbers of granulocytes, neutrophils and dendritic cells were lower ($P < 0.100$) for pigs fed the low CaP diets. However, spleen immune cell numbers and spleen hematological parameters were rarely affected by dietary treatment, spleen weight was lower ($P < 0.001$) for pigs fed the low CaP diets. In conclusion, the present study suggested that sufficiently high amounts of CaP may be required to support the innate immune response, whereas hematological parameters were higher for the low CaP diets. It needs to be further elucidated whether variations in CaP digestion and/or absorption might explain the complex relationship between P and animals' health.

Key words: immune system, phosphorus, phytate hydrolysis, pig

INTRODUCTION

In pig diets, phosphorus (P) is one of the most expensive ingredients caused by low digestibility of plant dietary P resulting in the need of supplementation of expensive, non-renewable inorganic P to meet animals' requirements. In particular in non-ruminant animals, the hydrolysis of phytate (any salt of *myo*-inositol hexakisphosphate (**$InsP_6$**)), the main storage form of P in plants, is incomplete, as the small intestine lacks sufficient enzymes such as endogenous mucosal phytase and phosphatase (Maenz and Classen, 1998; Onyango and Adeola, 2009; Selle et al., 2012). Therefore, there is rising scientific interest in the understanding of $InsP_6$ degradation in the digestive tract, as well as the effects on nutritional factors and finally support of animals' health. Evidence from a recent study of Heyer et al. (2016) suggests that a minimum requirement of P is needed to ensure normal immune functioning. However, studies on the impact of individual inositol phosphate (**InsP**) isomers and the immune system or haematological parameters are rare. Nakao et al. (1962), for example, observed modulations in the ATP concentration in P-depleted human erythrocytes that resulted in a reduced osmotic resistance of erythrocytes. Thus, haematological measures need to be taken into account when formulating diets in support of potentially improving animals' performance due to modulations of delivering oxygen. Furthermore, it is generally accepted that diet composition is a major factor being responsible for modulations of the intestinal microbiota (Mosenthin et al., 1999). Heyer et al. (2016) determined that pigs fed a corn-soybean meal diet showed higher jejunal gene copy numbers of *Lactobacillus* spp. compared to corn-pea based diets that might lead to variations in microbial phytase production and differences in $InsP_6$ degradation. The aim of the study was to evaluate the impact of varying mineral calcium-phosphorus (**CaP**) levels and fermentable substrates on intestinal CaP concentration, $InsP_6$ hydrolysis, innate immune cell numbers, and hematological parameters in growing pigs.

MATERIALS AND METHODS

The procedures and the use of animals in this experiment were approved by the local authorities (Regierungspräsidium Stuttgart, Germany; permit number: V308/13 TH) in accordance with the German Welfare Legislation.

Table 1. Ingredients and analyzed chemical compositions of the assay diets (as-fed basis).

	Soybean meal		Peas	
	Low CaP	High CaP	Low CaP	High CaP
Ingredients, %				
Corn	66.89	64.15	35.62	32.84
Soybean meal	20.00	20.00	0	0
Peas	0	0	51.00	51.00
Oil[1]	4.32	5.40	4.46	5.56
Potato starch[2]	5.00	5.00	5.00	5.00
Vitamin-mineral premix[3]	2.05	2.05	2.05	2.05
Monocalcium phosphate	0.24	1.59	0.19	1.55
Calcium carbonate	0.54	0.82	0.61	0.89
Sodium chloride	0.17	0.17	0.22	0.22
Lys	0.18	0.18	0.02	0.03
L-Thr	0.03	0.04	0	0
DL-Met	0.01	0.02	0.15	0.16
Potassium chloride	0	0	0.11	0.13
Vitamin E[4]	0.08	0.08	0.08	0.08
Choline chloride	0.03	0.03	0.03	0.03
Copper sulphate	0.001	0.001	0.001	0.001
Titanium dioxide	0.50	0.50	0.50	0.50
Analyzed chemical composition of the diets, g/kg DM				
DM, g/kg	905.1	901.0	900.7	897.3
GE, MJ/kg DM	21.7	21.1	21.1	21.0
Crude ash	54.5	67.5	56.0	70.3
Crude protein	168.0	166.9	152.7	148.0
Crude fat	70.6	78.9	79.7	88.9
Ca	4.3	8.3	4.5	8.3
Total P	4.1	7.5	4.2	7.4
Phytate-P	2.1	2.0	2.0	2.0
Ins(1,2,4,5,6)P_5,nmol/g DM[5, 6]	650	700	600	650
Phytate, nmol/g DM	11 350	10 800	10 900	10 600

[1]50% soybean oil + 50% linseed oil.

[2]50% Spezialstärke 6007 + 50% Kartoffelstärke Superior: Südstärke, Schrobenhausen, Germany.

[3]Vitamin-mineral premix (BASU-Mineralfutter GmbH, Bad Sulza, Germany) provided per kg of diet: Ca, 556 mg; Na, 8 mg; Mg, 8 mg; S, 16 mg; Fe (as $FeSO_4$), 25 mg; Mn, 3 µg; Zn (as ZnO), 46 mg; I (as $Ca(IO_3)_2$), 0,1 mg; Se (as Na_2SeO_3), 0,1 mg; vitamin A, 433 IU; vitamin D, 154 IU; vitamin E, 5 mg; vitamin K, 0,5 mg; vitamin B2, 2 mg; vitamin B12, 5 µg; niacin, 23 mg; panthothenic acid, 5 mg; folic acid, 0,3 mg; biotin, 31 µg.

[4]BASF, Ludwigshafen, Germany; DL-*α*-tocopheryl acetate, >50% purity.

[5]All other inositol phosphate isomers were not quantitative determinable.

[6]$InsP_5$ = *myo*-inositol pentakisphosphate.

Animals and Dietary Treatment

In a 2 × 2 factorial arrangement with 4 dietary treatment groups, 31 growing pigs (German landrace × Piétrain, initial BW 54.7 ± 4.1 kg) were used in 2 consecutive periods with 15 or 16 pigs. Corn-soybean meal diets or corn-pea diets were formulated to meet pigs' nutrient requirements (NRC, 2012), except for CaP content. Two different CaP contents amounting to 66% (low CaP) and 120% (high CaP) of pigs' actual CaP requirement based on animals' BW in the range from 50 to 75 kg (NRC, 2012) were adjusted by supplementation of inorganic CaP sources (monocalcium phosphate, calcium carbonate). Ingredients were selected to meet similar CaP contents, $InsP_6$ concentrations and intrinsic phytase activity. Common used protein sources were chosen for different contents of fermentable substrates, in particular carbohydrate fractions such as starch. Ingredients and analyzed chemical compositions of the assay diets are provided in **Table 1**. Pigs were housed individually in pens (each 3.25 m^2) under controlled environmental conditions (room temperature ± 21°C, light regime 12/12 h) and had free access to water. Animals were fed twice daily (0800-1500 h) with a feeding amount adjusted to 4% of the average BW of all pigs/period determined weekly.

Sampling Procedure, Processing and Analyses

Blood samples were taken by jugular vein puncture (0900-1030 h) before the start of the experimental dietary treatment (wk 1), after adaptation to the diet (wk 4), in wk 6, and 8 using a nose snare. Blood was collected in heparinized tubes (Monovette 9 ml sodium heparin; Sarstedt AG & Co, Nümbrecht, Germany) for immunological measurements and in 5 ml EDTA KE tubes (Sarstedt AG & Co, Nümbrecht, Germany) for determination of white blood cells (**WBC**), thrombocytes and erythrocyte values by an automated hematology system (pocH 100-iV Diff, Sysmex Deutschland GmbH, Norderstedt, Deutschland). Fecal spot samples were collected from Tuesday to Friday between 0800-1800 h in wk 4, 6 and 8. Feces were immediately stored at -20°C after sampling procedure. In wk 9, all pigs were slaughtered after anesthesia with ketamin (20 mg/kg BW, Serumwerk Bernburg AG, Bernburg, Germany), azaperon (2 mg/kg BW, Lilly Deutschland GmbH, Bad Homburg, Germany) and euthanasia by i.v. injection via the ear vein with pentobarbital (about 70 mg/kg BW, CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany). First, the abdominal cavity was opened and the gastrointestinal tract (**GIT**) and the spleen were removed. The spleen was immediately transferred into ice cold PBS without Ca^{2+}/Mg^{2+} and with 50 µg/ml gentamycin (Biochrom GmbH, Berlin, Germany). Thereafter, jejunal and cecal digesta samples were collected and stored at -20°C and the weight of the total spleen was determined. After a cross section of the mid-spleen (about 4 g) was processed by gentleMACS Dissociator (Miltenyi Biotec GmbH,

Bergisch Gladbach, Germany). Cells were isolated by 100 µm sterile Cell Strainer (Greiner Bio-One GmbH, Frickenhausen, Germany) and stored in PBS without Ca^{2+}/Mg^{2+} (Biochrom GmbH, Berlin, Germany) and with 50 µg/ml gentamycin (Biochrom GmbH, Berlin, Germany) at 4°C. Then, cells were centrifuged (300 × g for 5 min at 4°C) and resuspended in RPMI-10 (RPMI 1640 (Biochrom GmbH, Berlin, Germany) supplemented by 10% fetal calf serum (Seromed Biochrom KG, Berlin, Germany) and 50 µg/ml gentamycin (Biochrom GmbH, Berlin, Germany). The cell concentration was determined by a Z2 Coulter Counter (Beckman Coulter GmbH, Krefeld, Germany). Blood and spleen immune cells were stained as described previously (Grün et al., 2013) using the following fluorochrome-labeled antibodies: CD3 (clone PPT3), CD8*α* (clone 76-2-11), and CD 172*α* (clone 74-22-15) (Biozol Diagnostica Vertrieb GmbH, Echingen, Germany). Concentration of proximate nutrients were determined according to the official methods in Germany (VDLUFA, 2007). Diets were analyzed for DM, crude ash, CP, and crude fat. Content of GE in the diets was measured with a bomb calorimeter (IKA calorimeter, C200, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The concentration of P, Ca, Titanium dioxide (**Ti**), and InsP isomers in diets, feces and digesta samples were determined as described by Zeller et al. (2015). A mean for an InsP isomer was calculated only if the isomer was detected in at least 3 out of the 6 samples of one treatment group. If the detected value was below the limit of quantification (**LOQ**) in more than 1/3 or more samples, this was noted as less than the LOQ in the tables, and means were not calculated.

Calculations and Statistical Analyses

P, Ca net absorption and $InsP_6$ hydrolysis in digesta and feces (*y*) were calculated for each treatment on the ratio of P, Ca, or $InsP_6$ and Ti according to the generally accepted equation:

$$y\ (\%) = 100 - 100 \times [\text{Ti in the diet (g/kg DM)/Ti in digesta or feces (g/kg DM)}] \times [\text{P, Ca or InsP}_6\text{ in digesta or feces (g/kg DM)/P, Ca or InsP}_6\text{ in the diet (g/kg DM)}]$$

Data were analyzed by statistical package R version 3.1.0 (Development Core Team R, 2014) using linear mixed-effect models with the function "lmer" of the R package lme4 (Bates et al., 2014). Effects of dietary treatment, BW and sampling duration were analyzed. Random effects (dam, period, for digesta and spleen data also sampling date) were used for every model. The covariate BW and sampling duration was excluded from the model, if no significant effect ($P \geq 0.050$) could be observed. Normal distribution (Shapiro-Wilk test) and homogeneity of variance (plot of fitted values against residuals) were checked. Data were transformed by logarithmic or square route transformation for those variables that were not normally distributed

or where homogeneity of variance could not be assumed. From each pig, blood samples were collected before starting the experimental dietary treatment (wk 1) to determine their individual baseline. Data of the baseline were statistically analyzed as described above (**Table 2**). For the immunological and hematological parameters, changes from this baseline were calculated for each individual for wk 4, 6, and 8 to determine a treatment effect. Fecal samples were analyzed separately for each wk, statistical analyses were done for the mean of the values (wk 4, 6, 8) for each individual separately. The significance level was set at $\alpha < 0.050$ and $0.050 < P < 0.100$ as trend. All data are expressed as mean ± SEM of the raw data without transformation.

RESULTS

P, Ca Net Absorption, and InsP$_6$ Hydrolysis

The P net absorption in jejunal and cecal digesta and feces were lower ($P < 0.010$) for pigs fed the low CaP diets (**Table 3**). In addition, the P net absorption in the cecum were lower ($P < 0.010$) for pigs fed the soybean meal diets. In jejunal digesta, the Ca net absorption was higher ($P < 0.100$) for the soybean meal diets in combination with the low CaP content. In contrast, the interaction of CaP × protein source resulted in a lower ($P < 0.050$) Ca net absorption in cecal digesta. The $InsP_6$ hydrolysis determined in digesta and fecal samples was not affected by the dietary treatment. P, Ca net absorption and $InsP_6$ hydrolysis were not significantly affected by the BW. Negative values for P, Ca net absorption and $InsP_6$ hydrolysis can be caused due to differences in velocity of the undigestible marker Ti compared to other constituents of the diet.

Appearance of InsPs

In jejunal, cecal digesta and feces, *myo*-inositol pentakisphosphate (**InsP$_5$**) isomers and $InsP_6$ were detected (**Table 4 and Table 5**). In jejunal samples, the concentration of Ins(1,2,4,5,6)P_5 and $InsP_6$ were lower ($P < 0.050$) for the low CaP diets and higher ($P < 0.100$) for the soybean meal diets. Similar to the results of jejunal digesta, in the cecum and feces the concentration of Ins(1,2,3,4,5)P_5 and Ins(1,2,4,5,6)P_5 were higher ($P < 0.050$) for the soybean meal diets. Furthermore, there was a significant interaction of CaP × protein source ($P < 0.050$) resulting in a higher concentration of $InsP_6$ for pigs fed the soybean meal diets in combination with the low CaP content in the cecum. In feces, the concentration of Ins(1,2,3,4,6)P_5 was lower ($P < 0.100$) for the low CaP diets. Appearance of InsPs was not significantly affected by the BW.

Table 2. Immune cell populations, hematological parameters and thrombocyte numbers in blood samples before dietary treatment (baseline, wk 1) of growing pigs fed diets with different CaP[1] levels and fermentable substrates.
(*n* = 6-8 pigs; mean values with their SEM)

	Soybean meal		Peas		*P* value			
Item	Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
RBC[2], $\times 10^6$/µl	7 ± 0.2	7 ± 0.2	7 ± 0.2	7 ± 0.2	0.55	0.95		0.01
HGB[3], g/dl	12 ± 0.2	12 ± 0.2	13 ± 0.2	12 ± 0.2	0.18	0.93		0.19
HCT[4], %	43 ± 0.9	43 ± 0.5	44 ± 0.8	44 ± 0.5	0.16	0.69		0.21
MCV[5], fl	59 ± 0.97	59 ± 0.9	59 ± 1.1	60 ± 0.9	0.68	0.72		0.02
MCH[6], pg	17 ± 0.3	17 ± 0.3	17 ± 0.4	17 ± 0.3	0.69	0.92		0.04
MCHC[7], g/dl	29 ± 0.2	29 ± 0.2	29 ± 0.2	28 ± 0.2	0.93	0.37		
WBC[8], $\times 10^3$/µl	16 ± 1.0	17 ± 0.8	15 ± 0.5	16 ± 0.4	0.14	0.28		0.23
Thrombocytes, $\times 10^3$/µl	490 ± 32.8	502 ± 17.2	484 ± 18.9	486 ± 27.7	0.51	0.88		0.08
Granulocytes, number/µl	5058 ± 367	5650 ± 650	4683 ± 313	4637 ± 271	<0.05	0.99		0.36
Neutrophils, number/µl	4844 ± 314	5347 ± 576	4465 ± 306	4191 ± 245	<0.05	0.82		0.35
Eosinophils, number/µl	276 ± 40	271 ± 42	292 ± 51	295 ± 41	0.60	0.81		0.35
Dendritic cells, number/µl	771 ± 162	947 ± 109	874 ± 107	842 ± 155	0.70	0.25		0.53
Monocytes, number/µl	1056 ± 77	1001 ± 79	913 ± 64	965 ± 52	0.22	0.91		0.81
Ratio of N:L[9]	0.6 ± 0.05	0.6 ± 0.06	0.5 ± 0.04	0.5 ± 0.04	0.09	0.63		0.64

[1]CaP = calcium-phosphorus.
[2]RBC = red blood cell.
[3]HGB = hemoglobin.
[4]HCT = hematocrit.
[5]MCV = mean corpuscular volume.
[6]MCH = mean corpuscular hemoglobin.
[7]MCHC = mean corpuscular hemoglobin concentration.
[8]WBC = white blood cell.
[9]N:L = neutrophil:lymphocyte.

Table 3. Net absorption of Ca and P (%) and hydrolysis of $InsP_6$[1] (%) in jejunal, cecal digesta and fecal samples of pigs fed diets with differentCaP[2] levels and fermentable substrates.
(*n* = 4-6 pigs; mean values with their SEM)

	Soybean meal		Peas		*P* value			
Item	Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
P net absorption, %								
Jejunum	-12 ± 9.4	15 ± 2.7	-7 ± 15.0	23 ± 2.8	0.49	<0.01		
Cecum	-3 ± 2.4	22 ± 2.4	-0.2 ± 1.8	26 ± 0.9	<0.01	<0.001		
Feces	3 ± 1.2	20 ± 1.0	4 ± 0.8	24 ± 0.7	0.17	<0.001		
Ca net absorption, %								
Jejunum	51 ± 5.4	-2 ± 28.3	-29 ± 63.3	42 ± 2.7	0.55	0.33	0.07	0.42
Cecum	18 ± 5.8	26 ± 6.3	21 ± 11.3	-2 ± 11.2	<0.01	0.34	0.03	0.08
Feces	45 ± 1.8	34 ± 2.8	42 ± 2.8	34 ± 1.3	0.20	0.38		0.19
$InsP_6$ hydrolysis, %								
Jejunum	-17 ± 7.6	-4 ± 10.1	4 ± 15.3	-9 ± 7.4	0.55	0.79		
Cecum	-12 ± 2.4	-7 ± 3.2	-12 ± 3.7	-12 ± 6.1	0.49	0.44		
Feces	10 ± 2.5	13 ± 0.6	11 ± 2.9	11 ± 3.7	0.60	0.68		0.21

[1]$InsP_6$ = *myo*-inositol 1,2,3,4,5,6-hexakisphosphate.
[2]CaP = calcium-phosphorus.

Table 4. Concentration of different InsP[1] isomers (nmol/g DM) in jejunal and cecal digesta samples of pigs fed diets with different CaP[2] levels and fermentable substrates.

(*n* = 3-6 pigs; mean values with their SEM)

	Soybean meal		Peas		*P* value			
Item	Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
Jejunum, nmol/g DM								
$Ins(1,2,3,4,6)P_5$	ND[3]	ND	ND	ND				
$Ins(1,2,3,4,5)P_5$	ND	907 ± 250	ND	ND				
$Ins(1,2,4,5,6)P_5$	1259 ± 341	1901 ± 492	ND	1524 ± 235	0.07	0.03		0.06
$Ins(1,3,4,5,6)P_5$	ND	ND	ND	ND				
$InsP_6$	26 115 ± 3128	32 268 ± 4686	16 809 ± 4257	30 572 ± 2863	0.06	0.02		
Cecum, nmol/g DM								
$Ins(1,2,3,4,6)P_5$	LOQ[4]	490 ± 176	ND	ND				
$Ins(1,2,3,4,5)P_5$	977 ± 133	950 ± 223	485 ± 121	574 ± 139	0.02	0.63		0.60
$Ins(1,2,4,5,6)P_5$	2203 ± 146	2148 ± 279	1549 ± 172	1940 ± 96	0.02	0.34		
$Ins(1,3,4,5,6)P_5$	ND	ND	ND	ND				
$InsP_6$	36 188 ± 740	38 303 ± 2526	29 925 ± 2123	38 399 ± 1751	0.06	0.03	0.03	0.66

[1]InsP = inositol phosphate.
[2]CaP = calcium-phosphorus.
[3]ND = not detected (InsP isomers was not detectable).
[4]LOQ = limit of quantification (InsP isomers were not quantitative determinable).

Table 5. Concentration of different InsP[1] isomers (nmol/g DM) in feces samples of pigs fed diets with different CaP[2] levels and fermentable substrates.

(n = 6 pigs; mean values with their SEM)

	Soybean meal		Peas		*P* value			
Item	Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
Ins(1,2,3,4,6)P_5, nmol/g DM	696 ± 95	794 ± 29	LOQ[3]	LOQ		0.07		0.36
Ins(1,2,3,4,5)P_5, nmol/g DM	1589 ± 29	1650 ± 72	1122 ± 105	1 011 ± 112	<0.001	0.214		0.16
Ins(1,2,4,5,6)P_5, nmol/g DM	3244 ± 88	3261 ± 166	2833 ± 144	2850 ± 112	<0.01	0.63		0.41
Ins(1,3,4,5,6)P_5, nmol/g DM	ND[4]	ND	ND	ND				
$InsP_6$, nmol/g DM	55 283 ± 353	55 700 ± 851	54 889 ± 2678	54 972 ± 2210	1.00	1.00		0.78

[1]InsP = inositol phosphate.
[2]CaP = calcium-phosphorus.
[3]LOQ = limit of quantification (InsP isomers were not quantitative determinable).
[4]ND = not detected (InsP isomers was not detectable).

Hematological Parameters

Particularly, the dietary CaP concentration affected hematological values. In wk 4, the number of red blood cells (**RBC**) ($P < 0.001$), the concentration of hemoglobin (**HGB**) ($P < 0.001$), hematocrit (**HCT**) ($P < 0.001$), and mean corpuscular volume (**MCV**) ($P < 0.010$) were higher for pigs fed the low CaP diets (**Table 6**). In addition, the number of RBC ($P < 0.010$), the concentration of HGB ($P < 0.010$), and mean corpuscular hemoglobin concentration (**MCHC**) ($P < 0.050$) were higher for the low CaP diets in wk 8. In wk 4, the concentration of MCHC was lower ($P < 0.050$) for the low CaP diets. In wk 8, the soybean meal diets resulted in a lower concentration of MCHC ($P < 0.050$), and a higher concentration of HCT ($P < 0.100$). In the spleen, no dietary treatment effects could be determined for the number of RBC (**Table 7**). The spleen weight was significantly ($P < 0.001$) lower for the low CaP diets (**Figure 1**). Moreover, the spleen weight was lower with an increased BW ($P < 0.010$).

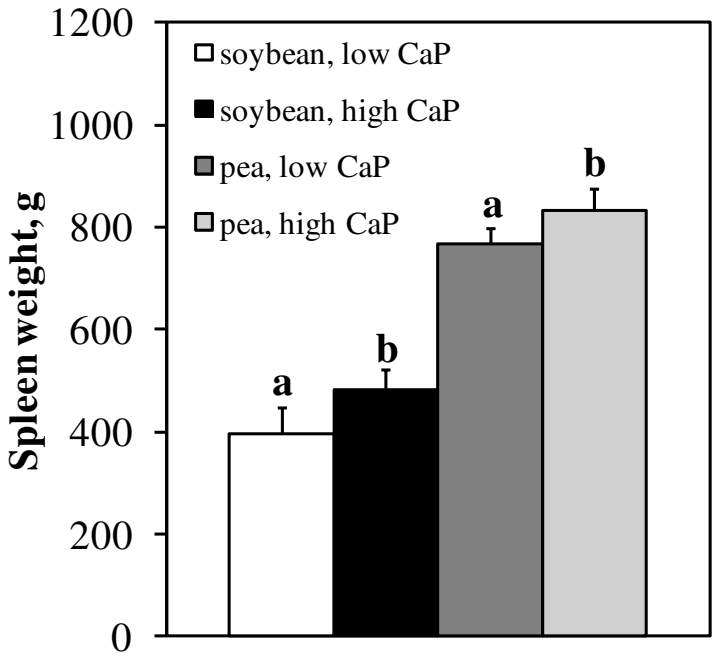

Figure 1. Effect of CaP content (low/high) and fermentable substrates on spleen weight (g). Different letters indicate significant differences between the low and high CaP contents regardless of used protein source (soybean meal, peas): $P < 0.010$ (n = 7-8 pigs; mean values with their SEM).
CaP, calcium-phosphorus.

White blood cells, Thrombocytes, and Immune Cell Numbers

In the blood, the number of WBC in wk 4 ($P < 0.100$) and 6 ($P < 0.050$) and thrombocytes in wk 8 ($P < 0.010$) was lower for the soybean meal diets in combination with the low CaP content (**Table 6**). In the spleen, no dietary treatment effects could be determined for the number of WBC, and thrombocytes (**Table 7**). In the blood, there were CaP × protein source interactions for the number of granulocytes (wk 4; $P < 0.050$), neutrophils (wk 4; $P < 0.050$), monocytes (wk 4, 6, 8; $P < 0.050$), and the ratio of neutrophil:lymphocytes (**N:L**) (wk 4; $P < 0.050$) resulting in lower numbers for pigs fed the soybean meal diet in combination with the low CaP content (**Table 8**). Furthermore, the number of granulocytes (wk 8; $P < 0.050$), neutrophils (wk 6, 8; $P < 0.050$), dendritic cells (wk 8; $P < 0.100$), and the ratio of **N:L** (wk 6, 8; $P < 0.100$) were lower for the low CaP diets. The number of eosinophils was higher (wk 6; $P < 0.050$) for pigs fed the low CaP diets in wk 6. In the blood, the number of neutrophils and monocytes ($P < 0.050$) was higher, whereas the ratio of N:L was lower ($P < 0.010$) with an increased BW in wk 4. For the spleen, there was an interaction of CaP content and protein source for the N:L ratio ($P < 0.100$) and a higher ($P < 0.100$) number of antigen-presenting cells (monocytes and dendritic cells) for the soybean meal diets (**Table 7**).

Table 6. Changes from baseline of hematological parameters (%), white blood cells (%) and thrombocytes (%) in blood of pigs fed of pigs fed diets with different CaP[1] levels and fermentable substrates.
(*n* = 6-8 pigs; mean values with their SEM)

	wk	Soybean meal		Peas		*P* value			
Item		Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
RBC[2], %	4	106 ± 1.6	100 ± 1.7	104 ± 1.4	100 ± 1.9	0.26	<0.001		
	6	108 ± 2.5	102 ± 3.2	108 ± 4.1	103 ± 2.7	0.67	0.57		0.53
	8	109 ± 1.6	106 ± 3.0	108 ± 2.1	103 ± 3.3	0.15	<0.01		0.12
HGB[3], %	[9]4	107 ± 1.6	101 ± 1.6	106 ± 1.2	101 ± 1.6	0.28	<0.001		
	6	111 ± 2.3	104 ± 2.9	111 ± 3.7	105 ± 2.4	0.67	0.48		0.45
	[9]8	114 ± 1.8	109 ± 3.2	112 ± 1.9	106 ± 3.4	0.60	<0.01		
HCT[4], %	4	108 ± 2.0	100 ± 1.8	108 ± 2.3	100 ± 2.2	0.42	<0.001		
	6	110 ± 2.4	103 ± 3.2	110 ± 4.4	104 ± 2.8	0.66	0.49		0.61
	8	111 ± 2.0	108 ± 2.9	109 ± 2.9	104 ± 3.6	0.08	0.59		0.32
MCV[5], %	4	102 ± 0.7	100 ± 0.4	103 ± 1.1	100 ± 0.7	0.84	<0.01		0.48
	6	101 ± 0.8	101 ± 0.6	102 ± 1.0	101 ± 0.9	0.73	0.47		0.46
	8	101 ± 0.8	102 ± 0.8	101 ± 1.1	101 ± 0.7	0.58	0.51		
MCH[6], %	4	102 ± 0.3	101 ± 0.5	102 ± 0.6	101 ± 0.5	0.69	0.21		0.42
	6	102 ± 0.4	102 ± 0.7	103 ± 1.2	102 ± 1.0	0.80	0.82		0.82
	[9]8	104 ± 0.5	103 ± 1.0	104 ± 1.0	104 ± 0.7	0.17	0.13		
MCHC[7], %	4	100 ± 0.6	100 ± 0.5	98 ± 1.2	101 ± 1.0	0.86	0.03		
	6	101 ± 0.6	101 ± 0.6	102 ± 1.1	101 ± 1.2	0.92	0.81		0.70
	8	103 ± 0.6	101 ± 0.5	103 ± 1.3	103 ± 0.6	0.02	0.04		
WBC[8], %	4	109 ± 4.8	123 ± 6.3	127 ± 7.4	120 ± 4.9	0.34	0.98	0.08	0.47
	6	102 ± 5.7	119 ± 5.7	112 ± 4.8	111 ± 4.5	0.42	0.11	0.03	0.27
	8	104 ± 6.0	115 ± 4.2	117 ± 5.3	113 ± 4.3	0.85	0.13		0.22
Thrombocytes, %	4	94 ± 5.5	100 ± 3.9	86 ± 8.2	85 ± 6.5	0.16	0.63		
	[9]6	101 ± 5.5	97 ± 9.8	101 ± 6.5	93 ± 4.2	0.33	0.18		
	[9]8	92 ± 10.6	111 ± 2.3	109 ± 2.1	99 ± 2.7	0.72	0.14	<0.01	0.28

[1]CaP = calcium-phosphorus.
[2]RBC = red blood cell.
[3]HGB = hemoglobin.
[4]HCT = hematocrit.
[5]MCV = mean corpuscular volume.
[6]MCH = mean corpuscular hemoglobin.
[7]MCHC = mean corpuscular hemoglobin concentration.
[8]WBC = white blood cell.
[9]significant effect of sampling duration, $P < 0.05$.

Table 7. Spleen immune cell populations (number/g tissue × 10^4) of pigs fed diets with different CaP[1] levels and fermentable substrates. (*n* = 6-8 pigs; mean values with their SEM)

	Soybean meal		Peas		*P* value			
Item	Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
RBC[2], number/g × 10^7	482 ± 69.1	474 ± 66.2	506 ± 90.6	467 ± 68.0	0.74	0.31		0.08
WBC[3], number/g × 10^7	10 ± 1.5	10 ± 1.0	9 ± 1.4	9 ± 0.9	0.33	0.65		0.50
Thrombocytes, number/g × 10^7	48 ± 7.1	51 ± 13.0	41 ± 8.1	49 ± 10.8	0.76	0.66		0.35
Granulocytes, number/g × 10^4	664 ± 100	650 ± 74	516 ± 87	592 ± 84	0.17	0.74		
Neutrophils, number/g × 10^4	311 ± 49	379 ± 30	322 ± 59	340 ± 57	0.62	0.30		0.60
Eosinophils, number/g × 10^4	437 ± 121	343 ± 45	329 ± 74	328 ± 35	0.41	0.78		
APC[4], number/g × 10^4	2518 ± 519	2168 ± 325	1659 ± 278	1694 ± 303	0.06	0.74		
Ratio of N:L[5]	0.05 ± 0.004	0.07 ± 0.012	0.06 ± 0.005	0.05 ± 0.006	0.54	0.17	0.06	0.20

[1]CaP = calcium-phosphorus.
[2]RBC = red blood cell.
[3]WBC = white blood cell.
[4]APC = antigen presenting cells
[5]N:L = neutrophil:lymphocyte.

Table 8. Changes from baseline of immune cell populations (%) in blood of pigs fed diets with different CaP[1] levels and fermentable substrates.

(*n* = 6-8 pigs; mean values with their SEM)

	wk	Soybean meal		Peas		*P* value			
Item		Low CaP	High CaP	Low CaP	High CaP	Protein source	CaP	Protein source × CaP	BW
Granulocytes, %	4	99 ± 7.2	144 ± 16.2	167 ± 26.0	130 ± 9.8	0.11	0.43	0.02	0.06
	6	82 ± 6.7	97 ± 10.2	96 ± 5.9	100 ± 6.1	0.39	0.12		0.67
	8	80 ± 6.2	101 ± 7.3	88 ± 5.2	101 ± 10.1	0.96	0.02		0.12
Neutrophils, %	4	96 ± 7.3	143 ± 16.3	165 ± 25.3	134 ± 10.8	0.04	0.54	0.03	0.02
	6	77 ± 6.4	94 ± 10.3	89 ± 6.5	102 ± 4.9	0.39	<0.01		
	8	76 ± 6.0	96 ± 6.7	83 ± 3.3	97 ± 7.1	0.63	0.03		0.49
Eosinophils, %	4	165 ± 30.8	155 ± 20.8	173 ± 27.8	176 ± 18.8	0.56	0.51		0.37
	6	194 ± 23.2	168 ± 22.5	207 ± 19.0	165 ± 16.2	0.53	0.03		0.27
	8	206 ± 40.1	212 ± 33.2	214 ± 33.7	209 ± 25.7	0.78	0.36		0.27
Dendritic cells, %	4	131 ± 13.4	145 ± 17.1	132 ± 16.9	159 ± 21.0	0.97	0.13		0.48
	6	131 ± 17.5	134 ± 18.5	126 ± 10.1	147 ± 17.8	0.95	0.23		0.71
	8	140 ± 19.0	146 ± 19.9	131 ± 19.2	155 ± 20.5	0.67	0.09		0.28
Monocytes, %	4	89 ± 5.8	120 ± 9.5	119 ± 7.5	111 ± 5.7	0.13	0.92	<0.01	<0.05
	6	84 ± 5.5	101 ± 11.6	97 ± 8.3	94 ± 5.8	0.74	0.08	0.01	0.24
	8	80 ± 7.2	102 ± 5.6	98 ± 4.1	95 ± 4.5	0.16	0.64	0.04	0.82
Ratio of N:L[2]	4	87 ± 9.9	129 ± 14.4	164 ± 33.7	117 ± 12.1	<0.05	0.09	0.01	<0.01
	6	68 ± 5.3	83 ± 6.8	72 ± 3.2	87 ± 7.0	0.50	0.02		
	8	65 ± 5.2	82 ± 8.0	68 ± 4.3	82 ± 7.8	0.70	0.09		

[1]CaP = calcium-phosphorus.
[2]N:L = neutrophil:lymphocyte.

DISCUSSION

In mammal species such as the pig, body P homeostasis is modulated by absorption from the intestine, bone turnover, and urinary excretion via the kidney (Rodehutscord et al., 1999). In the present study, the lower P net absorption in jejunum, cecum, and feces for pigs fed the low CaP diets might indicate some compensatory mechanisms for P homeostasis of the organism, such as mobilization of P of the bone. These processes are mediated by parathyroid hormone and calcitriol (1,25-dihydroxycholecalciferol), whereby calcitriol is also known to have immune-modulatory function. According to Tsoukas et al. (Tsoukas et al., 1984) and Manolagas et al. (Manolagas et al., 1985), an increased plasma level of calcitriol might stimulate monocytes, thereby enhancing antigen presentation, but inhibit lymphocyte functions. Moreover, it needs to be taken into account that bone resorption is affected by the immune system due to receptor activator of nuclear factor (NF)-κB ligand (**RANKL**) that is a member of the tumour-necrosis factor family essential for osteoclastogenesis. Interaction between RANKL and IFN-γ results in a negative feedback between T-cell activation and bone resorption (Takayanagi et al., 2000). This suggests that apart from the direct impact of P, components of P metabolism, such as calcitriol and RANKL, have a direct impact on immune cell function.

In the cecum, a high content of $InsP_6$ and lower InsPs such as Ins(1,2,3,4,5)P_5 and Ins(1,2,4,5,6)P_5 have been measured. However, in the jejunum mainly $InsP_6$ and Ins(1,2,4,5,6)P_5 were analyzed indicating almost no $InsP_6$ degradation. These results are in agreement with results of Schlemmer et al. (2001) examining almost no $InsP_6$ degradation for a phytase-unsupplemented barley-wheat-rye-soybean meal diet in the small intestine of pigs. Furthermore, higher $InsP_6$ concentrations and higher $InsP_5$ concentrations for the high CaP diets indicate that the mineral CaP supplementation reduced both the initial step of P release from $InsP_6$ and the further breakdown of $InsP_5$ isomers.

The soybean meal diets resulted in a higher fecal, jejunal and cecal concentration of $InsP_6$ and Ins(1,2,4,5,6)P_5, and in the cecum and feces in higher concentration of Ins(1,2,3,4,5)P_5. In addition, the soybean meal diets showed a lower P net absorption in the cecum and feces. The Ins(1,2,4,5,6)P_5 might be the hydrolysis product of a residual intrinsic soybean 3-phytase withstanding the heat treatment in the desolventizer-toaster or formed by microbial phytases of fungi, yeasts and bacteria mainly in the cecum (Zeller et al., 2015). However, several studies (Greiner et al., 1998; Phillippy, 1999; Greiner et al., 2000; Greiner et al., 2001) suggested that intrinsic plant phytases may be rapidly inactivated at low pH values and due to pepsin and

pancreatin. On the other hand, Angel et al. (2005) determined in broilers a higher P retention due to the supplementation of different *Lactobacillus* species to the diet indicating the production of $InsP_6$-degrading enzymes. These results are in agreement with our present study with higher jejunal gene copy numbers of *Lactobacillus* spp. for pigs fed corn-soybean meal diets compared to the corn-pea based diets (Heyer et al., 2016), probably leading to higher concentrations of $Ins(1,2,4,5,6)P_5$. In general, further studies are needed to differentiate between phytase and non-phytase producing bacteria at different sites of the digestive tract of the pig. Furthermore, mineral Ca is known to form insoluble mineral Ca-phytate complexes at pH levels usually prevalent in the small intestine (Selle et al., 2009) and the entailed limited efficiency of mucosal phytase possibly resulting in a reduced P digestibility (Steiner et al., 2007) that might affect microbial activity, similar to studies with rumen microbes (Komisarczuk et al., 1987). However, the effect of mineral Ca and P on the different sources of phytase in the GIT that cannot be separated for this experiment needs to be further evaluated.

Furthermore, it appears that the source of fermentable carbohydrates affected the disappearance of P in pigs (Partridge, 1978; Jongbloed et al., 1992; Baumgärtel et al., 2008). In the present study, the amount of corn in the experimental diets varied between 32.84% and 66.89% leading to different contents of carbohydrates, mainly resistant starch. Furthermore, peas are rich in slowly digestible or resistant starch compared to soybeans (Jezierny et al., 2011; Aumiller et al., 2015). An *in vivo* study of Baumgärtel et al. (2008) found that cellulose and pectin caused a net secretion of P into the lumen of the large intestine, whereas corn starch resulted in a net absorption of P. Authors suggested that a stimulation of microbial growth as a consequence of a higher supply of fermentable carbohydrates associated with an increased P incorporation into microbial mass promoting the demand for P. Especially the number of cecal saccharolytic bacteria, such as *Bifidobacterium* spp., *Eubacterium rectale*, and *Roseburia* spp., as well as the SCFA concentration were higher for the high CaP and soybean meal diets (Heyer et al., 2016) indicating a higher number and activity of carbohydrate fermenting bacteria that might result in an increased demand for P.

In the present study, the low dietary CaP content of the diets showed several modulating effects on blood hematological parameters, such as a higher concentration of RBC, HGB, and HCT indicating a potential better performance of these animals. Whereas the BW gain of the pigs averaged from 585 g/d to 965 g/d and was lower for the pigs fed the low CaP diets and the pea diets (Heyer et al., 2016). Erythrocytes, a vertebrate organism's principal means of delivering oxygen to the body tissue, are dependent on dietary P levels (Nakao et al., 1962). In a human

study of Nakao et al. (1962) assessing possible interactions between ATP levels and *in vivo* viability of erythrocytes, a modulation in the ATP concentration in P-depleted human erythrocytes resulted in a reduced osmotic resistance of erythrocytes, spherocytosis and intravascular haemolysis. However in the present study the low CaP diets showed a positive impact on oxygen delivering indices, thus might be explained by effects of InsPs on erythrocytes (Irvine and Schell, 2001). Through, mechanisms of $InsP_6$ absorption are rather unclear, human and murine cell studies determined potential $InsP_6$ absorbtive capacity (Grases et al., 2000; Grases et al., 2001). A study of Coates (1975) suggested a decreased affinity of HGB for O_2 in vertebrates species due to $Ins(1,3,4,5,6)P_5$.

Results of the current study demonstrated that the low CaP diets resulted in a lower ratio of N:L caused by a lower number of neutrophils in the blood. This might be indicative for an impaired phagocytic activity and probably a limited protection against pathogens including bacteria and fungi (Kumar and Sharma, 2010). These findings are consistent with the spleen data showing a lower ratio of N:L for pigs fed the soybean meal diets in combination with the low CaP content. Thus, the results demonstrate that the dietary treatment had local and systemic modulatory impact. Accordingly, numbers of granulocytes and dendritic cells were lower for pigs fed the low CaP diets and especially numbers of monocytes were lower for pigs fed the low CaP diets in combination with the soybean meal diet, suggesting an inhibitory effect on the innate, cellular arm of the immune system. In accordance to our results, Kiersztejn et al. (1992) found in rats receiving either a low or a high P diet that due to the low P diet the cytosolic Ca^{2+} content increased and the ATP level of granulocytes decreased causing an impaired phagocytic activity. Similarly, in a study with humans investigating possible interactions between InsP metabolism and specific immunological parameters, a modulation of neutrophil functioning due to $InsP_4$ could be observed (Dillon et al., 1987). These findings suggested that InsPs are essential for several cell biological processes, in particular for the immune system. Heyer et al. (2016) found that the low CaP diets resulted in a higher ratio of naive:antigen-experienced T-helper cells (**Th**) by a lower number of antigen-experienced Th in the blood indicating an impaired immunological memory functioning and protection against pathogens. In addition, the concentration of anti-keyhole limpet hemocyanin IgG was lower for pigs fed the low CaP diets two wks after the second immunization. This might be indicative for a limited adaptive, humoral immune response.

Conclusion

The present study demonstrated that sufficiently high amounts of CaP may be required to support the innate immune response and finally animals' health. However, hematological parameters were higher for the low CaP diets without apparent effect on animals' performance. Further studies should elucidate whether variations in CaP digestion and/or absorption might explain the complex relationship between P supply and animals' health.

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CHAPTER 6

GENERAL DISCUSSION

6 GENERAL DISCUSSION

6.1 INTRODUCTION

In 2008, the commodity price of phosphate rock rose by 800% over a period of 18 months. Market concentration and supply risk, physical phosphorus shortage and euthrophication problems demonstrate the critical challenge of global P vulnerability (Neset & Cordell, 2012). About 90% of the world's mined phosphate rock is used in the agriculture and food sector (Smil, 2000). Due to the limited global P resources, research has been intensified to investigate possibilities to recycle P and to save raw phosphate. In the field of livestock production, new dietary formulations emerged to increase the digestibility of plant P and to reduce the supplementation with phosphate and the use of enzyme supplements without negative effects on the animal (Rodehutscord, 2008). To ensure an adequate supply with P, porcine diets are supplemented with the soluble and easily absorbable phosphate. However, the less soluble $InsP_6$ is slowly digestible and can be poorly absorbed in the GIT especially for non-ruminant animals (Eeckhout & De Paepe, 1994; Steiner et al., 2007; Huber et al., 2015). In addition, $InsP_6$ acts as an antinutritive agent, reducing the absorption of trace elements and minerals (Schlemmer et al., 2001). It also has a modulating impact on the digestion and absorption of proteins and carbohydrates (Ravindran et al., 1999; Rutherfurd et al., 2002; Selle et al., 2012).

6.2 MECHANISMS OF INTESTINAL ABSORPTION OF DIETARY PHOSPHORUS

Dietary mineral imbalance or mineral deficiency caused by mineral content not adequate to the actual requirements might affect the absorption of several minerals and limit animals' productive performance (Case & Carlson, 2002). The small intestine, in particular the jejunum, is supposed to be the main site of P absorption (Breves & Schröder, 1991). However, the role of the large intestine is rather contradictory in view of results demonstrating both absorbtive and secretory mechanisms for P (Den Hartog et al., 1988; Larsen & Sandström, 1993; Liu et al., 2000; Seynaeve et al., 2000). Several studies showed that P homeostasis across the GIT is modulated by various factors, such as dietary P and Ca level (Jongbloed et al., 1992; Li et al., 1999; Ruan et al., 2007), $InsP_6$ content (Jongbloed et al., 1992; Schlemmer et al., 2001), ingredient composition of the diet (Fang et al., 2007), feeding level and supply of phosphate sources (Rodehutscord et al., 1999). In the present work, P homeostasis across the GIT was modulated by different dietary contents of mineral P and Ca. These P and Ca concentrations were chosen based on previous studies with pigs (Metzler-Zebeli et al., 2011; Metzler-Zebeli et al., 2012; Metzler-Zebeli et al., 2013), and were adjusted by supplementation of monocalcium phosphate

and calcium carbonate in addition to the P supply from plant sources. Moreover, the impact of variations in fermentable substrates, originating from protein and carbohydrate sources in the diet were assessed by using ingredients frequently used in pig diets (corn, soybean meal, peas). Although in this study, the net absorption of P and the $InsP_6$ hydrolysis in different segments of the GIT have been determined, mechanisms of intestinal P absorption, such as the expression of phosphate transporter, need to be further evaluated. In the following, a comprehensive literature overview on the intestinal phosphate absorption, $InsP_6$ hydrolysis and $InsP_6$ absorption is given.

6.2.1 PHOSPHATE ABSORPTION

The body phosphate homeostasis is modulated by parathyroid hormone and calcitriol (1,25-dihydroxycholecalciferol) primarily in the kidney and via passive, paracellular and active, transcellular transport in the small intestine (Schröder et al., 1996; Ruan et al., 2007; Sabbagh et al., 2011). The transcellular phosphate transport is divided into three steps: phosphate passes from the gut lumen across the luminal brush-border membrane into the enterocyte, then intracellularily from the luminal to the basolateral site of the cell, and finally via phosphate extrusion across the basolateral membrane into the blood (Murer & Hildmann, 1981, Karsenty et al., 1985; Danisi et al., 1988; Shirazi-Beechey et al., 1988). In the apical membrane of absorbtive epithelia, type II sodium-dependent phosphate (NaP_i) co-transporter represent the transepithelial phosphate transport, such as the type-IIb NaP_i transporter in the brush border membrane of enterocytes (Hilfiker et al., 1998). In mice, rats and chickens it has been shown that P deprivation positively affects the intestinal phosphate absorption (Quamme, 1985; Hilfiker et al., 1998; Hattenhauer et al., 1999). In a study with weanling pigs (Saddoris et al., 2010), the impact of available P and different ratios of Ca:available P on the stimulation of NaP_i transporter and mRNA expression of NaP_i-IIb in the small intestine has been investigated. The results indicate a 46% increase of NaP_i transporter with a decrease in dietary available P (0.40% to 0.23% available P). In addition, the expression of the NaP_i-IIb co-transporter protein of the jejunal brush border membrane vesicle was higher (84%) for pigs fed the low P diets compared to pigs fed a diet based on pigs' actual P requirement. The authors concluded that the available P content of the diet has a modulating impact on the NaP_i transporter and expression of the NaP_i-IIb protein in the brush border membrane vesicle of the small intestine due to post-transcriptional mechanisms. In conclusion, it can be suggested that the low CaP diets of the present work enhanced the expression of NaP_i transporter and increased the absorption of phosphate compared to pigs fed the high CaP diets. Modulating effects on the expression of NaP_i transporter might be an additional indicator for a compensatory mechanism

of the organism to prevent deficiency syndromes, such as an impaired cellular adaptive immune response, and need to be further evaluated.

6.2.2 PHYTATE HYDROLYSIS AND PHYTATE ABSORPTION

In the small intestine of non-ruminants at pH 6 to 7, the small sized $InsP_6$ is highly negatively charged. It has therefore been suspected that $InsP_6$ cannot pass the lipid bilayer of plasma membranes, as no adequate carriers have been found (Schlemmer et al., 2009; Humer et al., 2015). In the pig, Schlemmer et al. (2001) examined the effect of a barley-wheat-rye-soybean meal based diet with different intrinsic feed phytase activities (high and low [inactivated phytase via extrusion]) to evaluate the impact of intrinsic feed phytase and endogenous phytase on InsP hydrolysis. In the stomach, hydrolysis of $InsP_6$ was higher for the control diet compared to the phytase-inactivated diet. The intrinsic plant phytase (6-phytases) hydrolyses $InsP_6$ in the stomach and forms mainly *myo*-inositol pentakisphosphate ($Ins(1,2,3,4,5)P_5$; $InsP_5$). This finding is supported by other studies (Rapp et al., 2001; Kemme et al., 2006) in which a low or almost no $InsP_6$ degradation occurred in diets with a low intrinsic phytase activity in comparison to diets with a high intrinsic plant phytase or supplemented phytase. As a consequence of reduced gastric $InsP_6$ degradation, fewer lower phosphorylated and more soluble InsPs, such as $InsP_5$, and *myo*-inositol tetraphosphate ($InsP_4$), were formed. These lower InsPs have a limited mineral binding capacity that results in an impaired intestinal absorption of essential minerals and trace elements (Sandström & Sandberg, 1992; Schlemmer et al., 2009). In the small intestine, almost no $InsP_6$ degradation was observed for the phytase-unsupplemented diets (Schlemmer et al., 2001; Kemme et al., 2006). However, a high content of $InsP_6$ and low InsPs (*myo*-inositol biphosphate ($InsP_2$) - $InsP_5$), mainly $Ins(1,2,3,4,5)P_5$ and $Ins(1,2,4,5,6)P_5$, were measured in the solid phase of the large intestinal digesta (Schlemmer et al., 2001). Figure 1 gives an overview of the pathways of $InsP_6$ hydrolysis in the porcine gut. The results of Schlemmer et al. (2001) are in agreement with results of the present work in which mainly $InsP_6$ and $Ins(1,2,4,5,6)P_5$ were measured in jejunal digesta, indicating almost no $InsP_6$ degradation. However, in the caecum a high content of $InsP_6$ and low InsPs, mainly $Ins(1,2,3,4,5)P_5$ and $Ins(1,2,4,5,6)P_5$ were determined. The higher $InsP_6$ and $InsP_5$ concentration for the high CaP diets indicates that the supplementation with mineral CaP reduced the initial step of P release from $InsP_6$ and the further breakdown of $InsP_5$ isomers. The insolubility of mineral Ca-$InsP_6$ complexes at pH levels usually prevalent in the small intestine (Selle et al., 2009) and related limitations of mucosal phytases (Steiner et al., 2007) might be a factor. In the jejunum and caecum, the concentration of $InsP_6$ and $Ins(1,2,4,5,6)P_5$ were higher

for soybean meal diets. This may be caused due to soybean 3-phytase withstanding the heat treatment in the desolventiser-toaster or due to microbial phytases of fungi, yeast and bacteria (Zeller et al., 2015). As an example, supplemented *Lactobacillus* species to a diet increased P retention, indicating $InsP_6$-degrading enzyme production in broilers (Angel et al., 2005). However, the present work showed that the InsP hydrolysis is negligible in pigs. An improved understanding of $InsP_6$ degradation in the different sections of the GIT, especially the efficiency of different phytases such as microbiota-associated, endogenous mucosal and intrinsic plant phytases is further needed.

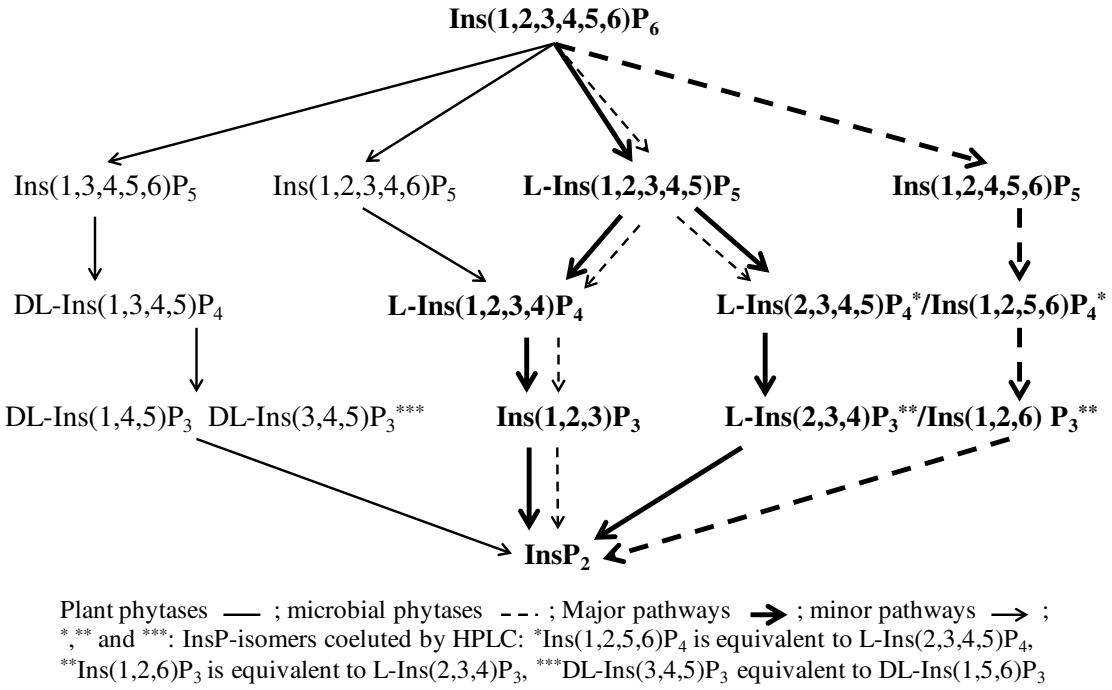

Figure 1. Pathway of phytate hydrolysis in the porcine gut (modified according to Schlemmer et al. (2001) and Humer et al. (2015)).

Mechanisms of $InsP_6$ absorption and their impact on cell metabolism are of crucial importance possibly explaining effects on the gut-associated and peripheral immune system that have been assessed in the present work. Although mechanisms of $InsP_6$ absorption are rather unclear, several studies in cultured cells of humans and rats (Grases et al., 2000, 2001a) have so far examined potential absorbtive capacities of $InsP_6$. In rats, Grases et al. (2000) studied the interaction between oral intake of $InsP_6$, known as an inhibitor of urinary crystallization, and its urinary excretion. Two groups of rats were fed either a control diet or a diet first without $InsP_6$, and then a gradually increasing amount of $InsP_6$. For the control diet, the urinary $InsP_6$ concentration decreased below the detection level. However, the supplementation of $InsP_6$

increased the $InsP_6$ concentration in urinary samples. The authors concluded that the $InsP_6$ urinary concentration is related to the oral intake. Similarly, Grases et al. (2001b) observed elevated InsP concentrations in organs (kidney, brain, bone), urine and plasma of rats fed a diet supplemented with $InsP_6$ in comparison to a diet without $InsP_6$. It appears that the $InsP_6$ concentration is affected by the dietary intake and that the endogenous synthesis of $InsP_6$ is less important (Grases et al., 2001b). In addition, a study of Sakamoto et al. (1993) investigated possible mechanisms of the absorption and distribution of $InsP_6$. The authors determined gastric and small intestinal $InsP_6$ absorption, followed by a fast dephosphorylation in the mucosal cells and a distribution to various organs, such as liver, kidneys, skin and gut, as inositol and *myo*-inositol monophosphate ($InsP_1$). Nevertheless, it needs to be taken into account that other studies, such as Letcher et al. (2008), failed to observe detectable $InsP_6$ concentrations in plasma and urine samples. In conclusion, further studies are needed to show potential mechanisms of dietary InsP absorption to elucidate effects on the whole organism, such as the immune system.

P substantially contributes to animal performance, resulting in a lower body weight (BW) gain for pigs fed diets with a CaP content below pigs' P requirement. In particular, modulating effects of phosphate absorption might be of specific importance with regard to a compensatory regulation of P due to, for example, mobilisation of P of the bone and altered NaP_i transporter expression. Further studies should evaluate parameters associated with P metabolism, such as P concentration in plasma and bone. Although $InsP_6$ hydrolysis is negligible in pigs, research on $InsP_6$ degradation and absorption in different sections of the GIT should be intensified, with special focus to be directed to $InsP_6$ hydrolysis products that might have modulatory function on the intestinal microbiota and the immune system, such as *myo*-inositol triphosphate ($InsP_3$) affecting immune cell signalling processes via receptor. In particular, the impact of different phytases, microbiota-associated, endogenous mucosal and intrinsic plant phytases, needs to be evaluated to describe modulations of P availability from different feed materials. Additionally, the supplementation with exogenous phytases should be taken into account in diet formulation to cover P requirements and avoid excessive use of mineral P. Moreover, several factors, such as fermentable substrates and dietary Ca level that can contribute to the degradation and possible absorbtive mechanisms need to be evaluated. In this regard it is often difficult to differentiate between effects of P and Ca. For a comprehensive overview of calcium digestibility and metabolism in the pig, see González-Vega & Stein (2014).

6.3 DIETARY PHOSPHORUS AND RELEVANCE FOR THE ORGANISM

6.3.1 THE LINK BETWEEN PHOSPHORUS AND THE IMMUNE SYSTEM

Nutrition has an important impact on the organism including the immune system, and represents a crucial factor of health (Reilly, 2002; Saeed et al., 2015). The intestine is supposed to be the largest lymphoid organ that is composed of more immune cells than any other organ (Brandtzaeg et al., 1989). About 10^{12} lymphocytes and a very high antibody concentration compared to other tissues in the body can be detected in the intestinal mucosa (Mayer, 2000). The intestinal immune system is exposed to a wide range of luminal antigens, such as commensal and potentially pathogenic bacteria (Elson, 1985; Mayer, 1997; Mayer, 2000; Nagler-Anderson & Shi, 2001). On the one hand, it is important to achieve an effective immune tolerance towards the intestinal microbiota harbouring the GIT. On the other hand, an efficient immune responsiveness has to be generated to protect the integrity of the intestinal barrier against harmful pathogens and dietary antigens (Artis, 2008; Burkey et al., 2009). Although studies of the impact of P on the immune system are rare, few studies investigated the effect of P on immune cell functions such as lymphocyte proliferation (Kegley et al., 2001; Mullarky et al., 2009), phagocytic activity (Kiersztejn et al., 1992; Jokinen et al., 2003; Mullarky et al., 2009), antibody response (Eya & Lovell, 1998; Kegley et al., 2001; Jokinen et al., 2003; Liu et al., 2008; Ghahri et al., 2012), number of leukocytes (Liu et al., 2008) and further immune parameters (Zyla et al., 2000; Metzler-Zebeli et al., 2012) in blood or other tissues. While the impact of InsPs on mammalian cells has already been reported a quarter century ago, new studies have so far examined effects of InsPs on the immune system, such as T cells, B cells, and neutrophil development and function (Irvine & Schell, 2001; Miller et al., 2008; Sauer & Cooke, 2010). The multifunctional inositides, membrane-anchored inositol lipids and cytosolic InsPs (Cockcroft & De Matteis, 2001, Irvine, 2005; Shi et al., 2006; Michell, 2008), are described as a cellular compound consisting of *myo*-inositol in the chemical structure (Shi et al., 2006; Michell, 2008). In mammalian species, cells produce many higher InsPs, for example membrane-bound lipid phosphatidylinositol (PtdIns) or $InsP_4$ and $InsP_5$ isomers (Michell et al., 2006; York, 2006; Sauer & Cooke, 2010). First, $Ins(1,4,5)P_3$ was identified as important second messenger mediating receptor-induced Ca^{2+} mobilization (Streb et al., 1983). Some recent studies (Hawkins & Stephens, 2007; Rommel et al., 2007; Weichhart & Saemann, 2008; Buitenhuis & Coffer, 2009; Fruman & Bismuth, 2009; Skwarek & Boulianne, 2009) described the impact of other PtdIns ($PtdIns(4,5)P_2$ and $PtdIns(3,4,5)P_3$) in many receptor-mediated cell signalling processes. As an example, $PtdIns(4,5)P_2$ showed a regulating effect on cytoskeletal function and represent a precursor of important cellular signalling

molecules, such as diaclycerol, Ins(1,4,5)P_3 and PtdIns(3,4,5)P_3 (Divecha & Irvine, 1995; Balla, 2001; Schurmans et al., 2011). In the cytosol, Ins(1,4,5)P_3 is phosphorylated to Ins(1,3,4,5)P_4 by Ins(1,4,5)P_3 3-kinases (Itpk) and by the Ca^{2+} sensitive inositol polyphosphate multikinase (Ipmk) (Irvine & Schell, 2001; Pattni & Banting, 2004). To note, both Itpkb and Ins(1,3,4,5)P_4 seems to affect the development of B cells (Schurmans et al., 2011). Although InsP_6 hydrolysis is negligible and the absorption of InsPs is not elucidated yet in pigs, the impact of lower InsP isomers on immune cells needs to be taken into account. Further studies are needed to elucidate interactions between dietary InsPs and immune parameters.

Leukocyte Distribution

The mesenteric lymph nodes represent the largest lymph nodes in the body (Mowat, 2003) and constitute one of the main compartments for priming the adaptive immune cells in the gut that are diffusely distributed throughout the *lamina propria* and the overlying epithelia (Mowat & Agace, 2014). The Peyer's patches are specialized follicle-associated epithelia with areas populated by B and T cells, which are located on the antimesenteric site of the jejunum and ileum (Cornes, 1965; Burkey et al., 2009; Mowat & Agace, 2014). In the Peyer's patches as well as in the mesenteric lymph nodes, the antigens are presented by antigen-presenting cells to naive T cells (Kraehenbuhl & Neutra, 1992). Upon activation, T and B cells differentiate from a mature, but naive status into potent effector cells, such as antigen-specific B cells to predominantly IgA-committed plasma cells (Brandtzaeg et al., 1999; Bauer et al., 2006). Subsequently, lymphocytes pass through the thoracic duct into the blood stream to the mucosal effector sites, such as the *lamina propria* (Kraehenbuhl & Neutra, 1992; Mowat, 2003). The present work showed a tendency for a lower number of T cells, naive T helper cells (Th), cytotoxic T cells, and regulatory T cells (Tregs) for the low CaP contents in the ileal mesenteric lymph nodes. This might be indicative of an impaired adaptive, cellular immune response in pigs fed the low CaP diets. Due to the low CaP diets the resistance against bacteria and parasites and viral, endogenic pathogens might be particularly limited. However, the ratio of naive:antigen-experienced Th in jejunal mesenteric lymph nodes were higher for pigs fed the soybean meal diet in combination with the low CaP content due to a higher number of antigen-experienced Th. Thus, a better immunological memory function and protection against pathogens can be suggested.

The spleen contains various immune cells, such as B cells, T cells, macrophages, dendritic cells and natural killer cells (Kang et al., 2008; Maroof et al., 2008; Bhattacharyya et al., 2011; Bonnefoy et al., 2011; Zhao et al., 2013). In the spleen, migrated antigen-presenting cells, such

as macrophages and dendritic cells, activate T and B cells (Osmond, 1985; Brown, 1992; McGaha et al., 2011; Mitchell et al., 2011; Zhao et al., 2013). In the present work, the ratio of naive:antigen-experienced Th in the spleen was higher due to a higher number of naive Th for pigs fed the soybean meal diet in combination with the low CaP diets, indicating a limited immunological functioning of the adaptive arm of the immune system. The number of antigen-presenting cells (monocytes and dendritic cells) in the spleen was higher for the soybean meal diets, possibly promoting the activation of the highly specialised adaptive immune response.

In the blood, number of cells of the cellular adaptive immune system, such as antigen-experienced Th, cytotoxic T cells and number of cells of the cellular innate immune system, such as granulocytes and monocytes, were lower for the soybean meal diet in combination with the low CaP content. The number of blood B cells, granulocytes, neutrophils, dendritic cells, and the ratio of neutrophile:lymphocyte ratio (N:L) were lower for the low CaP diets. In addition, the number of antigen-experienced Th was lower in pigs fed the low CaP diets, resulting in a higher naive:antigen-experienced Th ratio. Immune cell functioning is dependent on an adequate distribution of lymphocytes and antigen-presenting cells in the organism. The impact of P on immune cell distribution and functioning has not yet been studied in pigs. Thus, the results demonstrate that the dietary treatment affects both cell numbers of the innate and the adaptive arm of the immune system in the blood. Particularly, the low CaP content might cause an impaired first line of defence and limited activation of the cellular adaptive immune response.

Antibody Response

The timely meeting of immune-competent cells such as lymphocytes and antigen-presenting cells in the mesenteric lymph nodes is a key process for the organism to establish an appropriate antibody response. As outlined above, the leukocyte distribution and activity is modulated by CaP level and fermentable substrates. After the first immunization, no dietary treatment effect could be observed for plasma anti-keyhole limpet hemocyanin (KLH) IgM concentrations, but after the second immunization plasma anti-KLH IgG concentrations were lower for pigs fed the low CaP diets. Although there is no other study investigating an inhibitory effect on the humoral, adaptive immune response due to P deficiency in pigs, it can be speculated that due to lower numbers of antigen-presenting cells and T cells in the blood the humoral adaptive immune response is limited for pigs fed the low CaP diets, resulting in an impaired specific antigen recognition and memory functioning against pathogens.

Lymphocyte Proliferation

Several studies (Kegley et al., 2001; Liu et al., 2008) reported a stimulating effect on the proliferation and function of peripheral lymphocytes in different species due to either dietary supplementation with P or increased P availability upon phytase addition. Though studies in pigs are rare, the present work determined a supporting effect of the high CaP diets on the adaptive arm of the immune system due to higher cell numbers of the cellular and humoral adaptive immune response and a higher *in vivo* response to KLH. The reactivity of blood and mesenteric lymph node lymphocytes to concanavalin A in these pigs was impaired, indicating modulating effects of other origin such as migration patterns of antigen-presenting cells or cytokine secretion profiles. Future studies should investigate migration patterns and activity of innate immune cells such as phagocytic activity and cytokine secretion profiles.

The present work demonstrated that sufficiently high amounts of CaP are required to support the innate and adaptive immune response and finally the animal health. Since results of the present work determined contradictory effects of a low CaP level on immune cell numbers and lymphocyte reactivity *in vitro* and *in vivo*, further studies should focus on cell signalling such as cytokine production profiles. One study with weaned piglets (Metzler et al., 2012) already demonstrated that as a consequence of P deficiency conditions the expression of intestinal interleukin-1β was higher, indicating potential modulating effects on intestinal permeability and nutrient transport (McKay & Baird, 1999). In addition, the results of the present work showed that the dietary treatment has local and systemic effects on the porcine immune response. Further studies are therefore needed to elucidate, whether other effector sites of the intestinal immune system, such as immune cell distribution in other compartments of the GIT or IgA production, are also affected by immune modulations caused by dietary treatment. Nevertheless, further research on potential mechanisms of P modulating immune parameters are still required, with special focus to be directed to the purpose of individual InsPs for the immune functions of the host. InsPs are essential for several cell biological processes in mammalian species. There is rising scientific evidence that InsPs have to be considered as part of an integrated approach to support immune functions. However, studies on the interaction between InsPs and the immune system have been performed only in humans and rodents, it can be suggested that similar effects in pigs can occur due to the anatomical and physiological similarities to humans. The impact of the interaction of dietary InsPs and cellular InsPs needs to be further elaborated. Moreover, it can be suggested that components of P metabolism, such as calcitriol and receptor activator of nuclear factor-κB ligand (RANKL), have a direct impact on immune cell function. RANKL is a member of the tumour-necrosis factor family essential for osteoclastogenesis.

Takayanagi et al. (2000) determined an interaction between RANKL and IFN-γ resulting in a negative feedback between T-cell activation and bone resorption. Furthermore, the impact of calcitriol had been studied by Engstrom et al. (1985), who investigated whether a low P diet fed to pigs enhances the concentration of plasma calcitriol. According to Lemire et al. (1984) and Tsoukas et al. (1984), an increased plasma level of calcitriol might stimulate monocytes, thereby enhancing antigen presentation, but inhibiting several lymphocyte functions including the proliferation response to mitogen stimulation. This suggests that calcitriol, with its modulatory properties on P absorption, has a direct impact on immune cell function and migration. However, further research is required to verify this hypothesis.

6.3.2 INFLUENCE OF PHOSPHORUS ON THE GUT MICROBIOME

Numbers of caecal and colonic saccharolytic bacteria, such as the important butyrate producers *Eubacterium rectale* and *Roseburia* spp., were higher in pigs fed the high CaP and soybean meal diets, whereas caecal and colonic *Bifidobacterium* spp. and jejunal *Lactobacillus* spp. were higher for the low CaP and soybean meal diets. The main carbohydrate fermentation end-products are short-chain fatty acids (SCFA) decreasing pH in the colon and thereby preventing the growth and activity of pathogenic bacteria (Conlon & Bird, 2015). The abundance of proteolytic and optionally pathogenic bacteria, such as *Enterobacteriaceae* and *Bacteroides-Prevotella-Porphyromonas*, was therefore higher in different segments of the GIT in pigs fed the pea diets in combination with low dietary CaP content that might increase the risk of intestinal disturbances. In caecal digesta, the dissimilarity between the low and the high CaP content was 55% effected to more than 5% to these dissimilarities: terminal restriction fragment (TRF) 92 (member of the *Clostridiales*), TRF 219 (not assigned to a known sequence) and TRF 521 (uncultured bacterium related to unclassified *Porphyromonadacea*e). In the caecum, the abundance of TRF 92, assigned to a member of the *Clostridiales* affiliated with *Clostridium sensu stricto* and *Clostridium* cluster XI, with the two main representatives *Clostridium perfringens* and *Clostridium difficile*, was higher for pigs fed the low CaP diets, indicating a less healthy microbiota (Songer & Uzal, 2005) for these pigs.

The effects of P on the intestinal microbiota might be associated with a reduced P digestibility affecting bacterial structure and bacterial metabolic processes (Durand & Komisarczuk, 1988; Lengeler et al., 1999). The P digestibility might be reduced due to the insolubility of mineral Ca-InsP_6 complexes at pH levels usually prevalent in the small intestine (Selle et al., 2009), the limited efficiency of mucosal phytase and a reduced abundance of phytase-producing bacteria

such as *Lactobacillus* spp. (Steiner et al., 2007). In addition, the colonization of mucosal areas might be modulated due to an increased Ca concentration and competition for the same adhesion sites with other bacterial species such as *Escherichia coli* (*E. coli*), reducing the adhesion potential of strains such as *Lactobacillus* (Larsen et al., 2007). Moreover, differences in slowly digestible and resistant starch originating from grain legumes (Jezierny et al., 2011; Aumiller et al., 2015) and corn, may affect selectively specific bacterial groups such as certain *Bifidobacterium* species (Ryan et al., 2006). For example, Rist et al. (2014) showed that an increased supply of cornstarch may have been in favour of starch escaping digestion in the small intestine to be subsequently utilized by the microbiota in the large intestine. As a consequence, a shift from protein-fermenting to carbohydrate-fermenting bacterial groups occurs. These results are in agreement with our present work with higher caecal gene copy numbers of *Enterobacteriaceae* for pigs fed the pea-based diets compared to the soybean meal diets. However, caecal numbers of *Eubacterium rectale* and *Roseburia* spp. were higher for the soybean meal diets, indicating health promoting effects due to butyrate (Aminov et al., 2006). Several interactions between CaP content and protein source were determined for jejunal total SCFA and acetate, indicating that the low CaP content increased their concentration when combined with the feeding of soybean meal diets. In the caecum, pigs fed the low CaP diets showed lower concentrations of total SCFA, acetate and propionate. Jejunal propionate, caecal ammonia, total SCFA, acetate and butyrate concentrations were higher for pigs fed the soybean meal diets. According to an *in vitro* study of Komisarczuk et al. (1987), the available P in the surrounding medium affects the activity of bacterial fibrolytic enzymes. This might be an indication for a reduced SCFA synthesis due to a decreased fermentation of cellulose by microorganisms. Besides the impact of P, variations in fermentable substrates between diets due to different protein sources and corn contents affect the concentration of bacterial metabolites. Compared to soybeans, peas and pea fibre increased *in vitro* bacterial protein synthesis in comparison to other fibre sources (Jha et al., 2011), suggesting that nitrogen incorporated into bacterial mass would not be immediately available for metabolite production such as ammonia. This suggests that increased bacterial nitrogen assimilation might be beneficial for host health (Mosenthin et al., 1992; Nahm, 2003). The bacterial shift from protein fermenting to carbohydrate fermenting bacteria might have modulated the bacterial metabolite production, resulting in a lower total SCFA, acetate, and butyrate concentration in caecal digesta for pigs fed the pea diets compared to the soybean meal diets.

The overall picture emerging from the current study on P availability, fermentable substrates and the intestinal microbiota indicates a minimum requirement of P maintain a stable microbial

ecosystem in the GIT. Results demonstrated that especially the high CaP content and the soybean meal diets increased the number of butyrate-producing bacteria, such as *Eubacterium rectale* and *Roseburia* spp. and increased the concentration of various SCFA in the small and large intestine. In addition, potentially harmful bacteria, such as *Enterobacteriaceae* and *Bacteroides-Prevotella-Porphyromonas*, were decreased by the high CaP level and soybean meal diets. These results demonstrate that both, CaP supply and the amount of fermentable substrates, may beneficially affect gut health through modulation of the intestinal microbiota composition and activity. However, further studies should evaluate the role played by dietary CaP and fermentable substrates on specific bacteria known to produce toxic products creating a direct link to the immune system and animal health.

6.3.3 INFLUENCE OF DIETARY PHOSPHORUS ON HEALTH

Several studies already investigated the interactions between dietary CaP supply, fermentation activity and microbial composition of the GIT in pigs (Metzler et al., 2009; Metzler-Zebeli et al., 2011; Metzler-Zebeli et al., 2013), whereas studies on the impact of CaP supply on immune parameter are rare. Given the close relationship between specific microbial metabolites, intestinal barrier function, and immune regulation (Macfarlane & Macfarlane, 2012), interactions between the intestinal microbiota, the immune system, and the P content of the diet can be suggested. The intestinal immune system controls the bacterial exposure to the host tissue, in particular of bacterial pathogens, by two main immunological mechanisms called stratification and compartmentalisation (Hooper et al., 2012). Thus, it can be speculated that due to modulations of CaP supply these mechanisms might be reduced, indicating a negative effect on the host's ability to prevent colonisation with intestinal pathogens (Heyer et al., 2015). Future research should focus on the role of active microbial fractions and their functions, possibly causing activation of the immune system due to, for example, lipopolysaccharides present on the outer membrane of gram-negative bacteria. In the current study, the impact of the dietary treatment on the immune system and the intestinal microbiota was investigated to describe the effects on animal health. In addition, haematological parameters and BW were determined possibly affecting animals' performance. In vertebrates, erythrocytes are principal means of delivering oxygen to the body tissue. In the present work, the number of red blood cells, the concentration of haemoglobin and hematocrit were higher in pigs fed the low CaP diets, whereas the spleen weight was lower for the low CaP diets. Nakao et al. (1962) examined in a human study possible interactions between ATP levels and *in vivo* viability of erythrocytes. A modulation in the ATP concentration in P-depleted human erythrocytes resulted in a reduced

osmotic resistance of erythrocytes, spherocytosis and intravascular haemolysis. However, the low CaP diets promoted the delivery of oxygen possibly due to effects of InsPs on erythrocytes (Irvine and Schell, 2001). In vertebrate species, a decreased affinity of haemoglobin for O_2 due to Ins(1,3,4,5,6)P_5 has been suggested (Coates, 1975), indicating a negative effect on delivery of oxygen. Concerning the BW, the number of various immune cells in the blood and tissue, such as blood B cells, neutrophils and monocytes, were higher with increasing BW, whereas the number of antigen-experienced Th and regulatory T cells in the blood were lower. Concerning intestinal bacterial composition, the gene copy numbers of jejunal *Roseburia* spp., jejunal and colonic *Bifidobacterium* spp., and caecal *Lactobacillus* spp. were higher with an increased BW. In caecal and colonic digesta, gene copy numbers of *Eubacterium rectale*, as well as jejunal isovalerate and caecal acetate concentration were lower with an increased BW. The abundance of TRF 521 was higher in pigs fed the high CaP diets. In human studies by Manichanh et al. (2006) and Gupta et al. (2011), an increased abundance of members of *Porphyromonadaceae* were found in human intestine of Crohn's disease patients as well as in malnourished children. Ryan et al. (2014) determined in mice a negative correlation between BW gain and *Porphyromonadaceae* abundance. Evidence exists that animal growth may decrease upon feeding of low P diets (Murphy et al., 1995). In addition, immune cell function and migration seems to be associated with growth-related hormones, such as somatotropin or cortisol (Welniak et al., 2002; Webster & Glaser, 2008). Although no consistent effect of BW gain on immunological and microbiological values could be observed, further research is needed to elucidate the impact of animal growth and growth-related hormones.

In the current study, dietary supplementation with CaP and the use of the corn-soybean meal diets have been shown to potentially support pig health due to higher numbers of saccharolytic and especially butyrate-producing bacteria, higher concentrations of SCFA, lower number of proteolytic and potentially harmful bacteria, and the promotion of compartments of the innate and adaptive immune response both locally and systemically. A limited first line of defence and activation of the cellular and humoral adaptive immune response due to P deficiency might therefore negatively affect the host's ability to prevent colonisation with intestinal pathogens. However, hematological parameters were higher for the low CaP diets without apparent effect on animal performance. Results of the present work on P availability, immune functioning and the microbial ecosystem indicate that the high CaP and corn-soybean meal diets have a supporting effect on pig health, whereas several parameters, such as the lymphocyte proliferation to ConA, showed an opposite effect. Further studies are needed to evaluate P availability, InsP isomers, immune system and the intestinal microbiota.

6.4 CONCLUSION

In conclusion, high dietary CaP concentrations and the corn-pea diets increased the P net absorption. Almost no $InsP_6$ degradation was determined, and mainly $InsP_5$ isomers were measured in jejunal, caecal digesta and faecal samples. In particular, the high CaP diets showed higher $InsP_6$ and $InsP_5$ concentrations, thus indicating a reduction of the initial step of P release from $InsP_6$ and a further breakdown of $InsP_5$ isomers. Concerning the immune system, results demonstrated that sufficiently high amounts of CaP are required to support the innate and adaptive immune response. Since results showed contradictory effects of the low CaP level on immune cell numbers, antibody response and lymphocyte reactivity *in vitro*, further studies should focus on cell signalling such as cytokine production profiles. In addition, the intestinal microbiota was affected by dietary treatment. The high CaP content and the soybean meal diets increased the numbers of butyrate-producing bacteria such as *Eubacterium rectale* and *Roseburia* spp., and increased the concentration of various SCFA in the small and large intestine. Potentially harmful bacteria, such as *Enterobacteriaceae* and *Bacteroides-Prevotella-Porphyromonas*, were moreover decreased by the high CaP level and soybean meal diets. The overall picture emerging from the current work on P availability and animal health indicates a minimum requirement of P to ensure a stable intestinal ecosystem and immune functioning. Although most parameters of the present work indicate a positive effect of the high CaP diet, not all values show a consistent effect on animal health, such as immune cell numbers and lymphocyte proliferation *in vitro*. Nevertheless, further studies are needed to adjust dietary formulations including potential effects of differences in InsP hydrolysis, including evaluation of potential available P supply and lower InsP isomers affecting the immune system.

6.5 SUGGESTIONS FOR FURTHER RESEARCH

Studies on the effects of different amounts of mineral P and $InsP_6$ in pigs are rare, measurements of phosphate, $InsP_6$ and $InsP_6$ hydrolysis products intake, digestion, retention and excretion, such as P and InsP concentration in different tissues (bone, kidney), fluids (blood, urine) and P transporter expression in the gut are required to describe the complex mechanisms of modulating effects of P on immune parameters and the microbial ecosystem. Additional determination of Ca and calcitriol measurements would contribute to the description of the close relationship to the P metabolism. *In vitro* studies with porcine immune cells incubated in different media with various concentrations of InsPs would be a first step to evaluate the modulating impact on immune cell functioning, such as cytokine production, lymphocyte proliferation or activity of phagocytes. Furthermore, several studies demonstrated that individual InsPs or phosphate affect bacterial

properties, such as metabolism or virulence (Kröger & Fuchs, 2009).

For piglets, interest has been focused on the time of weaning, which is often very stressful for the young animals and is accompanied by morphological, histological, microbial, and immunological changes along the GIT (Pluske et al., 1997). Due to these changes, the time after weaning is characterized by a reduced growth rate and diarrhoea, both of which cause severe economic losses for the farmers. The role of P on possible anti-diarrhoeal effects due to the prevention of pathogen adhesion, and the production of toxins needs to be further evaluated. Furthermore, no study has so far investigated the interaction between dietary P, stress and bacterial infections in pigs or other species, although weaning especially imposes tremendous stress on piglets. Stress is described by Dhabhar & McEwen (1997) as a constellation of events resulting in a release of fast-acting catecholamines (epinephrine and norepinephrine) and slow-acting glucocorticoids (cortisol and corticosterone) (Dhabhar, 2009). Chronic stress affects a cytokine shift from a Th 1 response to a Th 2 response that stimulates the humoral immune response, whereas the cellular immune response is limited. Due to these changes, the course of an infection and/or the susceptibility to pathogens is modulated (Elenkov & Chrousos, 1999; Verbrugghe et al., 2012). In addition, stress alters the intestinal barrier function and results in a higher exposure to antigen and pathogen passage (Verbrugghe et al., 2012). Lyte et al. (2011) reviewed the profound effects of stress, in particular of norepinephrine, on host cells and host-microbe interactions that might engage the intestinal microbiota. Possible mechanisms of norepinephrine influencing the gut microbiota are intestinal motility, colonic transit and transepithelial ion transport (Enck et al., 1989; Mizuta et al., 2006; Freestone et al., 2008). In addition, direct effects of catecholamines on bacteria, such as growth promotion and virulence, increase the potential to cause diseases (Freestone et al., 2008; Verbrugghe et al., 2012). In conclusion, it can be suggested that dietary P might limit stress-related effects due to a support of the innate immune response and a decrease of the abundance of proteolytic and potentially harmful bacteria, probably promote the animal's protection against pathogens.

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CHAPTER 7

SUMMARY

7 SUMMARY

Phosphorus (P) represents a crucial input for agriculture and food industries as a mineral present in ingredients used for livestock feeding as well as in mineral fertilisers. In the current systems, P is primarily derived from the finite mined phosphate rock resource. Thus, a critical challenge of global P scarcity is directly linked to future food security and sustainable resource management, especially in the European Union which is dependent on raw P from outside Europe. Apart from other future activities in animal nutrition, new dietary formulations of livestock diets emerged as a potential approach to increase the digestibility of plant P, phytate (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate, $InsP_6$), and to reduce the supplementation with mineral phosphate. In non-ruminant animals, such as the pig, $InsP_6$ hydrolysis is incomplete, as the small intestine lacks sufficient enzymes such as endogenous mucosal phytase and phosphatase. As a consequence, there is rising scientific interest to improve the understanding of $InsP_6$ degradation in the digestive tract as well as the effects on nutritional factors and finally animal performance and health.

The aim of the present thesis was to investigate the impact of dietary P, $InsP_6$ and $InsP_6$ hydrolysis products in combination with different fermentable substances (protein, carbohydrate) on the porcine immune system, the intestinal microbiota and animal health. First, a comprehensive literature overview describes the impact of P on the immune system and the microbiota along the gastrointestinal tract (GIT), including potential effects on host health with special focus on the pig. Secondly, an *in vivo* study with growing pigs was conducted to examine the effects of diets with varying mineral calcium-phosphorus (CaP) levels as well as different fermentable substrates on intestinal CaP concentration, $InsP_6$ hydrolysis, the intestinal microbial ecosystem, and the peripheral and gut-associated immune system. In 2 consecutive experiments, 31 growing pigs (55 ± 4 kg) were allotted to a 2×2 factorial arrangement with 4 treatment groups, fed either a corn-soybean meal or a corn-pea based diet, each with 2 different CaP levels (low, 66% of the CaP requirement; high, 120% of the CaP requirement) supplemented with monocalcium phosphate and calcium carbonate. After 3 weeks of adaptation to the diets, all pigs were immunized twice with keyhole limpet hemocyanin (KLH). Blood and faeces samples were taken. After slaughtering, immunological tissue (jejunal, ileal mesenteric lymph nodes, spleen) as well as jejunal, ileal, caecal and colonic digesta were taken. Faecal and digesta samples were examined for P, Ca, inositol phosphate (InsP) isomers and for the marker titanium dioxide. The number of different leukocyte subpopulations analysed by flow cytometry, mitogen-induced lymphocytes proliferation *in vitro* were assessed. In addition, concentrations of plasma

anti-KLH IgM and plasma anti-KLH IgG analysed by ELISA and haematological parameters analysed by an automated hematology system have been measured in blood and tissue samples. In digesta samples, bacterial 16S rRNA gene copy numbers were determined by quantitative real-time PCR. The concentration of short chain fatty acids (SCFA) and ammonia was assessed. In addition, the use of terminal restriction fragment length polymorphism has been proven to characterize the structure of porcine gut microbiota.

Results of the current study demonstrated that CaP and fermentable substrates had a distinct effect on the peripheral and gut-associated immune system, as well as on microbial composition and activity in growing pigs. High dietary CaP concentrations and the corn-pea diets increased P net absorption. Almost no $InsP_6$ degradation could be observed in the GIT, and mainly *myo*-inositol pentakisphosphate ($InsP_5$) isomers were measured in jejunal, caecal digesta and faecal samples. In particular, the high CaP diets showed higher $InsP_6$ and $InsP_5$ concentrations, indicating a reduction of the initial steps of P release from $InsP_6$ and a further breakdown of $InsP_5$ isomers. The low CaP content might cause an impaired first line of defence and activation of the cellular and humoral adaptive immune response. As an example, the high CaP content affected the outcome of the adaptive immune response including a higher number of antigen-experienced T-helper cells in the blood as well as higher plasma anti-KLH IgG concentrations. The reactivity of blood and mesenteric lymph node lymphocytes to Concanavalin A in these pigs was impaired, indicating modulating effects of other origin such as migration patterns or activity of antigen-presenting cells. Since results of the present study suggest contradictory effects of CaP level on immune cell numbers and lymphocyte reactivity *in vitro* and *in vivo*, further studies are needed to determine effects on cell signalling such as cytokine production profiles. Moreover, the high CaP content and the soybean meal diets increased the number of butyrate-producing bacteria, such as *Eubacterium rectale* and *Roseburia* spp. and increased the concentration of various SCFA in the small and large intestine, thereby contributing to improve gut health. Potentially harmful bacteria, such as *Enterobacteriaceae* and *Bacteroides-Prevotella-Porphyromonas*, were increased by the low CaP level and pea diets, indicating a less healthy microbiota. Results demonstrated that both, CaP supply and the amount of fermentable substrates, may beneficially affect gut health due to modulations of the composition and activity of the intestinal microbiota. Further studies should evaluate the impact of CaP on specific pathogenic bacteria known to produce toxic products creating a direct link to the immune system and animal health.

Although most parameters of the present study indicate a positive effect of the high CaP diet, not

all values showed a consistent effect on animal health, such as immune cell numbers and lymphocyte proliferation *in vitro*. In conclusion, variations in P availability and the formation of individual InsPs have to be considered when formulating diets in support of a stable intestinal microbial ecosystem and immune functions of the host.

CHAPTER 8

ZUSAMMENFASSUNG

8 ZUSAMMENFASSUNG

Phosphor (P) leistet als Bestandteil von Futterrationen sowie von mineralischen Düngern einen bedeutenden Beitrag für die Landwirtschaft und Nahrungsmittelindustrie. Allerdings sind die globalen Rohphosphatvorkommen limitiert. Die globale P-Knappheit zieht entscheidende Herausforderungen im Bereich der nachhaltigen Nutzung dieser Ressource, einschließlich der damit verbundenen Ernährungssicherheit, nach sich. Von den Veränderungen betroffen ist vor allem die Europäische Union, die vollständig von Importen abhängig ist. Im Bereich der Tierernährung existieren zahlreiche Ansätze, die nachhaltige Nutzung von P zu fördern. Ein Ansatz liegt in der Formulierung neuer Rationen zur Steigerung der Verdaulichkeit von pflanzlichem P, Phytat-P (*myo*-Inositol 1,2,3,4,5,6-Hexakisphosphate, $InsP_6$), und der Reduktion der Supplementierung mit mineralischem P. Nichtwiederkäuer wie das Schwein können nur einen geringen Teil des $InsP_6$ hydrolysieren, da sie nur teilweise über die entsprechende enzymatische Ausstattung endogener mukosaler Phytasen und Phosphatasen im Verdauungstrakt verfügen. Aus diesem Grund besteht ein großes wissenschaftliches Interesse daran, das Verständnis über die Vorgänge beim Abbau von $InsP_6$ im Verdauungstrakt und die damit verbundenen Effekte auf die Ernährung sowie letztendlich die Leistung und Gesundheit des Tieres zu verbessern.

Das Ziel der vorliegenden Arbeit war es, den Einfluss von P, $InsP_6$ und $InsP_6$-Hydrolyseprodukten und unterschiedlich fermentierbaren Substanzen (Proteine, Kohlenhydrate) in der Ration auf das Immunsystem, die intestinale Mikrobiota und die Tiergesundheit beim Schwein zu untersuchen. Auf Basis einer umfangreichen Literaturauswertung wurden mögliche Einflussfaktoren von P auf das Immunsytem und die Mikrobiota entlang des gastrointestinalen Verdauungstraktes mit Fokus auf das Schwein identifiziert. Zusätzlich wurden mögliche Effekte von P auf die Tiergesundheit abgeleitet. Auf dieser Grundlage wurde eine 2×2 faktoriell angelegte *in vivo* Studie mit Mastschweinen durchgeführt. Ziel war es, Effekte unterschiedlicher mineralischer Calcium-Phosphor (CaP) Konzentrationen und fermentierbarer Substanzen auf die intestinale CaP Konzentration, die $InsP_6$-Hydrolyse, das intestinale mikrobielle Ökosystem und das darmassoziierte sowie periphere Immunsystem zu untersuchen. In zwei aufeinanderfolgenden Versuchen wurden 31 Mastschweine (55 ± 4 kg) entweder mit einer auf Mais-Sojaschrot oder Mais-Erbse basierten Ration gefüttert, die jeweils mit zwei unterschiedlichen CaP Konzentrationen (niedrig, 66% der CaP Empfehlung; hoch, 120% der CaP Empfehlung) in Form von Monocalciumphosphat und Calciumcarbonat ergänzt wurden. Nach einer dreiwöchigen Adaptation an die Futterration

wurden alle Schweine zweimalig mit *keyhole limpet hemocyanin* (KLH) immunisiert. Blut- und Faecesproben wurden entnommen. Nach der Schlachtung wurde immunologisches Gewebe (jejunale, ileale mesenterische Lymphknoten, Milz) sowie Digesta aus Jejunum, Ileum, Caecum und Colon entnommen. P, Ca, Inositolphosphat (InsP) Isomere und der Marker Titandioxid wurden im Faeces und der Digesta bestimmt. Die Anzahl der Leukozytensubpopulationen wurde mittels Durchflusszytometrie gemessen, außerdem wurde die mitogen-induzierte Lymphozytenproliferation *in vitro* bestimmt. Zusätzlich wurde die Konzentration von anti-KLH IgM und anti-KLH IgG im Plasma mittels ELISA ermittelt und die hämatologischen Parameter mit einem Hämatologie-Analysator im Blut und Gewebe gemessen. In Digestaproben wurde die bakterielle 16S rRNA Genkopienanzahl mittels quantitativer real-time PCR und die Konzentration an flüchtigen kurzkettigen Fettsäuren und Ammoniak bestimmt. Zusätzlich wurde mittels *terminal restriction fragment length polymorphism* die Struktur der porcinen Darmmikrobiota charakterisiert.

Die Ergebnisse zeigen, dass CaP und fermentierbare Substanzen einen Effekt auf das periphere und darmassoziierte Immunsystem, aber auch auf die mikrobielle Zusammensetzung und Aktivität bei Mastschweinen haben. Durch die hohe CaP Ration und die Mais-Erbsen Ration konnte eine Steigerung der P Nettoabsorption festgestellt werden. Der Abbau von $InsP_6$ im gastrointestinalen Trakt war sehr gering, so konnten hauptsächlich *myo*-Inositol Pentakisphosphat ($InsP_5$) Isomere im Faeces, in jejunaler und caecaler Digesta ermittelt werden. Vor allem die hohe CaP Ration führte zu höheren $InsP_6$ und $InsP_5$ Konzentrationen, die auf eine Hemmung des initialen $InsP_6$-Hydrolyseschrittes und die Entstehung von $InsP_5$ Isomeren hindeuten. Tiere, die mit der geringen CaP Ration gefüttert wurden, zeigten eine schwächere angeborene Immunantwort, zusätzlich konnte eine veränderte zelluläre und humorale Immunantwort festgestellt werden. Die Fütterung der hohen CaP Ration führte zu einer Beeinflussung der adaptiven Immunantwort, welche sich in einer höheren Anzahl an Antigen-erfahrenen T-Helferzellen im Blut und einer höheren anti-KLH IgG Konzentration im Plasma darstellte. Darüber hinaus konnte eine niedrigere Proliferation nach Stimulation mit Concanavalin A für Lymphozyten aus dem Blut und den mesenterischen Lymphknoten beobachtet werden, die auf modulierende Effekte anderen Ursprungs, beispielsweise Zellmigration oder die Aktivität von Antigen-präsentierenden Zellen, hindeuten kann. Da die Ergebnisse der vorliegenden Arbeit teilweise widersprüchliche Effekte der CaP Konzentration auf die Immunzellanzahl und Lymphozytenaktivität *in vitro* und *in vivo* vermuten lassen, sind weitere Studien nötig, um Effekte durch an der Zellkommunikation beteiligte Zytokine darzustellen. Im Hinblick auf die Mikrobiota konnte gezeigt werden, dass die Anzahl der

Butyrat-produzierenden Bakterien, wie *Eubacterium rectale* und *Roseburia* spp., und die Konzentration an unterschiedlichen kurzkettigen Fettsäuren im Dünn- und Dickdarm durch die hohe CaP Ration sowie die Sojaschrot Ration erhöht waren. Diese Effekte können auf eine Verbesserung der Darmgesundheit hindeuten. Die Anzahl potenziell schädlicher Bakterien, wie *Enterobacteriaceae* und *Bacteroides-Prevotella-Porphyromonas*, wurde durch die niedrige CaP Ration sowie die Erbsen Ration gesteigert, was auf eine weniger gesunde Mikrobiota hinweisen könnte. Die CaP Versorgung und der Gehalt an fermentierbaren Substanzen können möglicherweise einen positiven Effekt auf die Darmgesundheit haben, verursacht durch die Modulation der Zusammensetzung und Aktivität der intestinalen Mikrobiota. Weitere Studien sind daher notwendig, um Effekte von CaP auf spezielle pathogene Bakterien und deren toxische Substanzen zu untersuchen, um eine direkte Verbindung zwischen der intestinalen Mikrobiota, dem Immunsystem und der Wirkung auf die Tiergesundheit herzustellen.

Obwohl die meisten Parameter der vorliegenden Studie auf einen positiven Effekt der hohen CaP Ration hindeuten, zeigten nicht alle Ergebnisse einen einheitlichen Effekt auf die Tiergesundheit, wie die Anzahl an Immunzellen und die Lymphozytenproliferation *in vitro*. Zusammenfassend ist festzuhalten, dass Unterschiede in der P Verfügbarkeit und der Bildung einzelner InsPs bei Rationsformulierungen berücksichtigt werden sollten, um das mikrobielle Ökosystem und die Immunfunktionen des Tieres zu fördern.

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Place, Date Signature

Eidesstattliche Versicherung
gemäß § 8 Absatz 2 der Promotionsordnung der Universität Hohenheim zum Dr.sc.agr.

1. Bei der eingereichten Dissertation zum Thema

Impact of dietary phosphorus and fermentable substrates on the immune system and the intestinal microbiota of the pig

handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung oder -beratung in Anspruch genommen.

4. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt.

Die Richtigkeit der vorstehenden Erklärung bestätige ich. Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Stuttgart, den 28.05.2016

Ort, Datum Unterschrift

PEER REVIEWED ARTICLES

Heinritz SN, Weiss E, Eklund M, Aumiller T, Heyer CME, Messner S, Rings A, Louis S, Bischoff SC, Mosenthin R (2016) Impact of high-fat or high-fiber diet on intestinal microbiota and metabolic markers in a pig model. *Nutrients* **8**, 1-16.

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