EVALUATING THE FACTORS THAT CONTROL THE GROWTH PERFORMANCE OF PIGS FED WITH LOW PROTEIN DIETS SUPPLEMENTED WITH A CORN-EXPRESSED PHYTASE OR PHYTOGENIC FEED ADDITIVE

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Abstract: The objective of Study 1 was to assess the effect of a novel corn-expressed phytase (CEP) on growth, nutrients digestibility, bone characteristics, and fecal microbiota of pigs fed with very low-protein, -calcium (Ca) and -phosphorous (P) diets. Forty-eight barrows were subjected to 6 groups for 4 weeks: positive control-adequate protein, negative control-reduced protein (NC), NC + low-dose CEP, i.e., 2000 FTU/kg (LD), NC + high-dose CEP, i.e., 4000 FTU/kg (HD), LD with 0.12% unit reduced Ca and 0.15% unit reduced available P, and HD with 0.12% unit reduced Ca and 0.15% unit reduced available P. Supplementation of protein-restricted diets with a CEP decreased their negative effects on average daily gain and gain:protein ratio, increased the digestibility of Ca and P regardless of the levels of these minerals in the diet, improved bone characteristics and produced differential effects on fecal bacterial population. The objective of Study 2 was to investigate whether supplementation of very low protein (VLP), low-Ca, and low-P diets with a CEP can influence the blood amino acids (AA) and proteomics profiles in pigs. The experiment design and diets were the same as Study 1. CEP improved the blood profile of some essential AA and affected the expression of proteins involved in regulation of growth in pigs fed with VLP diets. Further, alterations in expression of serum proteins that are important for bone mineralization and muscle structure development likely contributed to beneficial effects of CEP on growth, body composition and bone measurements in pigs fed with VLP diets. The objective of Study 3 was to assess the effect of a phytogenic water additive (PWA) on growth performance and underlying factors involved in pigs fed with low-protein (LP)/high-carbohydrate diets. Forty-eight weaned barrows were allotted to 6 treatments for 4 weeks: control (CON) diet-no PWA, CON diet-low dose PWA (4 mL/L), CON diet-high dose PWA (8 mL/L), LP diet-no PWA, LP diet-low dose PWA and LP diet-high dose PWA. In this study, PWA improved the performance of weaned pigs fed with protein-adequate diets likely through increased blood essential AA and affected the muscle composition when dietary protein was deficient.

TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION……..……....1

1. Environmental Concerns Associated with Swine Production 1

2. Effect of Low Protein Diets on Nitrogen Excretion from Swine Production 1

3. Effect of Low Protein Diets on Growth Performance of Pigs and Their Mechanisms of Action..... 3

3.1. Growth Performance & Feed intake *3*

3.2. Blood Metabolites..... *4*

3.3. Bone Parameters and Metabolism *4*

3.4. Gut Microbiota *5*

3.5. Body Composition Component *6*

3.6. Blood Amino Acids *6*

3.7. Proteomics..... *7*

3.8. Nutrients Digestibility *8*

3.9. Amino Acids Transporters..... *8*

References 10

II. REVIEW OF THE LITERATURE....……..19

1. Phytase and its Benefits for Swine Production 20

2. Effect of Phytase on Growth Performance of Pigs Fed Dies with Variable Dietary Protein Content and its Mechanisms of Action 21

2.1 Growth Performance & Feed intake *21*

2.2. Blood Metabolites..... *22*

2.3. Bone Parameters and Metabolism *22*

2.4. Gut Microbiota *23*

2.5. Blood Amino Acids *24*

2.6. Nutrients Digestibility *25*

Chapter page

2.7. Amino Acid Transporters *28*

3. Effect of Phytogenic Feed Additives on Growth Performance of Pigs Fed with Dies with Variable Dietary Protein Content and their Mechanisms of Action 30

3.1. Growth Performance & Feed Intake *30*

3.2. Blood Metabolites *32*

3.3. Gut Microbiota *33*

3.4. Blood Amino Acids *35*

3.5. Nutrients Digestibility *35*

3.6. Amino Acid Transporters *36*

3.7. Total Antioxidant Capacity *37*

References *39*

III. A NOVEL CORN-EXPRESSED PHYTASE IMPROVES DAILY WEIGHT GAIN, PROTEIN EFFICIENCY RATIO AND NUTRIENTS DIGESTIBILITY AND ALTERS FECAL MICROBIOTA IN PIGS FED WITH VERY LOW PROTEIN DIETS 56

Abstract 56

1. Introduction 57

2. Materials and Methods 59

2.1. Animals, housing, and diets *59*

2.2. Growth performance parameters *64*

2.3. Feed, fecal, and blood samples collection *64*

2.4. Bone mineral density and bone mineral content analysis *65*

2.5. Proximate analysis of feed samples *65*

2.6. Nutrients apparent fecal digestibility *66*

2.7. Fecal DNA isolation, amplicon sequencing, sequence data analysis and taxonomic classification *66*

2.8. Serum osteocalcin, calcium, phosphorus, and alkaline phosphatase *68*

2.9. Statistical analysis *68*

3. Results 69

3.1. Body weight, feed intake and feed efficiency *69*

Chapter page

3.2. Bone mineral content and density *76*

3.3. Apparent fecal digestibility of calcium, phosphorus, and nitrogen *76*

3.4. Serum osteocalcin, calcium, phosphorus, and alkaline phosphatase *78*

3.5. Fecal microbiota *80*

4. Discussion 87

5. Conclusions 92

References 93

Appendices 105

IV. CORN-EXPRESSED PHYTASE MODULATES BLOOD AMINO ACIDS AND PROTEOMICS PROFILES IN NURSERY PIGS FED WITH LOW-PROTEIN, -CALCIUM, AND -PHOSPHOROUS DIETS 107

Abstract 107

1. Introduction 108

2. Material and Methods 110

2.1. Animals and Experimental Design *110*

2.2. Serum Triglycerides, Glucose, and Cholesterol *110*

2.3. Plasma Nitrogen-Containing Compounds Analysis *111*

2.4. Serum Proteomics Analysis *111*

2.5. Bioinformatics Analysis of the Differentially Abundant Proteins *113*

2.6. Statistical Analysis *114*

3. Results 114

3.1. Plasma Metabolites and Body composition *114*

3.2. Plasma Nitrogen-Containing Compounds *116*

3.3. Differentially Abundant Proteins in Serum of Pigs *119*

4. Discussion 142

5. Conclusion 145

References 146

Chapter page

V. EFFECT OF A PHYTOGENIC WATER ADDITIVE ON GROWTH PERFORMANCE, BLOOD METABOLITES AND GENE EXPRESSION OF AMINO ACID TRANSPORTERS IN NURSERY PIGS FED WITH LOW-PROTEIN/HIGH-CARBOHYDRATE DIETS 153

Abstract 153

1. Introduction 154

2. Materials and Methods 156

2.1. Animals, Housing, and Diets *156*

2.2. Growth Performance *160*

2.3. Feed and Fecal Samples Collection *161*

2.4. Blood and Tissue Samples Collection *161*

2.5. Feed and Fecal Samples Composition Analysis *161*

2.6. Plasma Nitrogen-Containing Compounds Analysis *162*

2.7. Muscle Composition Analysis *162*

2.8. Serum Metabolites Analysis *163*

2.9. RNA Isolation and RT-qPCR Analysis *163*

2.10. Total Antioxidant Capacity *167*

2.11. Apparent Fecal Digestibility of Nutrients *167*

2.12. Statistical Analysis *167*

3. Results 168

3.1. Growth Performance 168

3.2. Muscle Fat and Lean Content *175*

3.3. Plasma Nitrogen-Containing Compounds *175*

3.4. Apparent Fecal Digestibility of Calcium, Phosphorus, and Nitrogen *179*

3.5. Serum Calcium, Phosphorus, and Alkaline Phosphatase and Blood Urea Nitrogen *181*

3.6. The mRNA Abundance of Amino Acid Transporters in the Jejunum and Skeletal Musc. 183

3.7. Serum Total Antioxidant Capacity *188*

4. Discussion 188

5. Conclusions 192

References 196

LIST OF TABLES

Chapter III

Table page

1. Ingredients and composition of experimental diets1 (% as-fed basis).……...…63

2. Overall growth performance and bone minerals of pigs fed with low-protein diets supplemented with a corn-expressed phytase…….....72

3. Weekly growth performance of pigs fed with low-protein diets supplemented with a corn-expressed phytase……...…….75

4. Table S1. Digestibility of calcium, phosphorous and nitrogen of pigs fed with low-protein diets supplemented with a corn-expressed phytase……..……..108

Chapter IV

Table page

1. Plasma metabolites and body composition of nursery pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase……..…117

2. The composition (nmol/mL) of plasma nitrogen-containing compounds of nursery pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase……..119

3. Proteins identified with significantly differential abundance in serum of nursery pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase ……...……..122

4. Paired-wise comparison of proteins identified with significantly differential abundance in serum

of nursery pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase……131

Chapter V

Table Page

1. Ingredients and chemical composition of experimental diets (as-fed basis)……...…….161
2. Primer sequences, forward (F) and reverse (R), location on the template, amplicon size (bp), and GenBank accession numbers for both target and reference genes for reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)…….....167
3. Growth performance and muscle fat and lean content of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive……..…….171
4. Weekly growth performance of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive……...…….175
5. The concentration (nmol/mL) of plasma nitrogen-containing compounds of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive……179
6. Ingredients and chemical composition of the phytogenic water additive (Herbanimals®) used in this study (as-fed basis)…….203

LIST OF FIGURES

Chapter III

Figure page

1.Nutrients' digestibility in pigs fed with low-protein diets supplemented with a corn-expressed phytase. ……...79

2. Serum metabolites and hormones in pigs fed with low-protein diets supplemented with a corn-expressed phytase……81

3. Beta diversity of fecal bacterial community in pigs fed with low-protein diets supplemented with a corn-expressed phytase. ……...83

4. The fecal bacterial composition in pigs fed with low-protein diets supplemented with a corn-expressed phytase…….……...85

5. Fecal microbiota composition histograms in pigs fed with low-protein diets supplemented with a corn-expressed phytase.…….…….....87

7. Figure S1. Fecal rarefaction curve analysis for pigs fed with low-protein diets supplemented with a corn-expressed phytase…….…..107

Chapter IV

Figure page

1. Venn diagrams of total identified proteins in serum of pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase……..…124

2. Principal Component Analysis (PCA) score plots of differentially expressed protein in serum of

pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase. ……..…..……..….126

3. Hierarchical clustering of differentially expressed protein in serum of pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase.…….127

4. Network analysis of differentially expressed proteins in serum of nursery pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase. ……..…..…….....140

5. Pearson correlation analysis of overall differentially expressed proteins (protein ID) in serum of pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase identified by proteomics approach and growth performance related measurements……..……..139

Chapter V

Figure page

1. Apparent fecal digestibility (AFD) of (A) calcium (Ca), (B) phosphorus (P) and (C) nitrogen (N) of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive (PWA)…….……...182

2. Serum (A) calcium (Ca), (B) phosphorus (P), (C) alkaline phosphatase (ALP) and (D) blood urea nitrogen (BUN) of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive

(PWA)…….…….....184

3. Relative mRNA abundance of (A) solute carrier family 15 member 1 (*SLC15A1*), (B) solute carrier family 7 member 11 (*SLC7A11*), (C) solute carrier family 6 member 19 (*SLC6A19*), (D)

solute carrier family 3 member 1 (*SLC3A1*), (E) solute carrier family 7, member 1 (*SLC7A1*), (F) solute carrier family 7 member 9 (*SLC7A9*), and (G) solute carrier family 7 member 8 (*SLC7A8*) in the jejunum of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic 8ater additive (PWA)…….....……...187

4. Relative mRNA abundance of (A) solute carrier family 15member 1 (*SLC15A1*), (B) solute carrier family 7 member 8 (*SLC7A8*), (C) solute carrier family 6 member 19 (*SLC6A19*), (D) solute carrier family 3 member 1 (*SLC3A1*), (E) solute carrier family 7, member 1 (*SLC7A1*), and (F) solute carrier family 7 member 11 (*SLC7A11*) in the skeletal muscle of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive (PWA)…….....…189

5. S1. Body weight (A) and feed intake (B) of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive (PWA)……...……204

CHAPTER I

INTRODUCTION

1. Environmental Concerns Associated with Swine Production

Intensive animal production is associated with environmental and sustainable agriculture concerns attributed to the production of large amounts of waste including nitrogen (N) or nitrogen-containing compounds (Jongbloed and Lenis, 1998; Aarnink and Verstegen, 2007). Nitrogenous compounds are in the form of nitrous oxide, ammonia, and nitrates. They contribute to greenhouse gas emissions and their source in pig production is a dietary protein (Wood et al., 2013). It is estimated that around 137 million tons of total global N from livestock was discharged into the environment in 2006 (Mauricio et al. 2006) and N pollution in the pork industry has led to a severe environmental problem requiring a reduction in N excretion (Pierce et al., 2007). Therefore, in order, to reduce the costs associated with protein waste and N excretion to the environment, new strategies for the reduction of N excretion to the environment from the swine industry are required to be developed.

2. Effect of Low Protein Diets on Nitrogen Excretion from Swine Production

Slightly low protein (SLP) diets are used to reduce N excretion and other toxic nitrogenous compounds in pigs (Le Bellego and Noblet, 2002; Heo et al., 2008).

Moreover, these diets have been reported to reduce feed cost and alleviate the incidence of diarrhea in the early weaning period in pigs (Deng et al., 2007; Le Bellego & Noblet, 2002; Nyachoti et al., 2006; Pluske, 2013). SLP diets supplemented with limiting amino acids (AA) (*i.e.* lysine, methionine, tryptophan, and threonine) do not appear to deteriorate performance in swine production (Le Bellego and Noblet, 2002; Yin et al., 2010; Toledo et al., 2014). However, further research is needed to improve the growth performance of pigs fed with SLP diets.

As previously mentioned, low protein (LP) diets decrease the incidence of diarrhea in piglets, which can help in reducing stress in weaned pigs, because at this stage, piglets undergo stressful events, such as environmental, physiological, and immunological challenges. Therefore, the incidence of diseases and diarrhea can negatively affect performance. Moreover, the change in their diet from liquid milk to solid feed, and change in environment, from the farrowing barn to the nursery building, and transport are some of the challenges weaned pigs have to overcome during the weaning period (Campbell et al., 2013). Piglets are weaned when their maternal immunity is still high. At birth, piglets are healthy, and maternal milk intake provides immunity to the piglets. The type of post-weaning diet influences intestinal morphology (Leiting et al., 1998). Makkink et al., (1994) reported that the weaning period is followed by physiological and architectural changes such as the development of the intestines. Because of the change in form of feed and physiological changes in structure and function of intestinal enzymes at weaning, thus affecting feed intake negatively.

To minimize the stress occurring during the weaning period, researchers are aiming to decrease dietary protein levels. Severe reduction of dietary crude protein (CP) or very low protein (VLP) might be more beneficial for health and decreasing the nutrients excretion. However, these diets, although supplemented with limiting AA, decrease the feed efficiency and growth performance of nursery and growing pigs (Manjarin et al., 2012; Peng et al., 2016; Li et al., 2017a).

3. Effect of Low Protein Diets on Growth Performance of Pigs and Their Mechanisms of Action

3.1. Growth Performance & Feed intake

Previous research reported that severe reduction in dietary CP along with supplementing crystalline AA helped to reach an ideal AA (AA) profile, and reduce nitrogen excretion and diarrhea in the pigs (Manjarin et al., 2012; Li et al., 2017b). However, VLP diets negatively impact feed intake and growth performance of pigs and compromise digestive function and tissue protein synthesis (Deng et al., 2007; Peng et al., 2016). Peng et al. 2016 demonstrated that reducing the dietary CP level from 20% to 15.3% along with essential AA supplementation had no significant effect on growth performance in 10 to 35 kg pigs. However, negative growth performance and altered immunological parameters were reported when CP level was reduced to 13.9%, although essential AA was supplemented (Peng et al., 2016). Overall, unlike a slight reduction in dietary protein, VLP diets supplemented with essential AA reduce feed intake (FI), feed to gain, and body weight (BW) and increase fat deposition (Le Bellego and Noblet 2002; Hansen and Burgoon 1993; Li et al. 2017b; Liao and Regmi 2015)

3.2. Blood Metabolites

Very low protein diets not only affect the feed intake and growth performance but also the blood metabolites. Restricted intake of protein (Ruusunen et al., 2007) or AA (Fabian et al., 2004) decreased the levels of total protein and urea N in blood. Other studies reported a linear increase in blood glucose in pigs fed lysine-restricted grower diets, suggesting that it may be due to the decreased insulin concentration associated with protein-restricted diets (Yang et al., 2008). Fabian et al. (2004) reported an increase in the blood glucose in pigs fed low lysine diets (Fabian et al., 2004).

3.3. Bone Parameters and Metabolism

Protein intake is an important factor for the better development of bone and bone health in humans and rodents (Rouy et al., 2014). Deficient protein intake leads to growth retardation during the early stages of life and poor bone quality as an adult (Rizzoli et al., 2010). The influence of protein on bone metabolism has for the large part been related to the capacity to provide essential AA for the synthesis of the bone collagen matrix (Rouy et al., 2014). Protein restriction is also associated with an inhibition of the insulin-like growth factor 1 (IGF-1), an anabolic factor that is important for bone and muscle development (Bourrin et al., 2000).

Severe reduction of dietary CP decreased bone mass density and bone mass content in rats (Mehta et al., 2002). The detrimental effects of low protein diets on bone mass in humans have been reported previously (Kerstetter and Insogna 2003).

3.4. Gut Microbiota

The importance of the gut microbiota in the physiology of the host is well known (Xiao et al., 2016). The microbial composition, along with a wide range of microbial metabolites, has a complex function in several host processes, such as energy harvest, recovery from inflammation and infection, resistance to autoimmunity, and endocrine signaling that affects brain function through the gut-brain axis (Hollister and Versalovic 2014; Hooper, and Macpherson 2012; Zhao et al. 2015). Gut microbiota has also been suggested to act as source of AA in animals (Miller and Ullrey, 1987). Several factors can influence the composition and activity of gut microbiota including environment, age, and diet; among the list, diet is the most critical factor in swine (Chen et al. 2018; Fan et al. 2015). Diet plays an important influential role in altering the gut microbiota composition and activity. Moderate dietary protein restriction could affect the composition of gut microbiota and improve ileal barrier activity in growing pigs (Fan et al. 2015).

Reduced-protein diets were reported to alter the intestinal bacterial community of growing pigs (Chen et al., 2018). According to a previous study, *Streptococcus* and *Escherichia-Shigella* are involved in AA utilization (Dejong, and Rensen 2015). Chen et al., 2018 observed a lower Shannon index in the ileum of growing pigs fed low protein diets (12% CP) suggesting that severe reduction in protein may affect the diversity of intestinal bacteria (Chen et al., 2018).

Recent data suggest that low-protein diets have the potential to affect the abundance and diversities of the jejunum microbiome and also improve the biological functions of the jejunum microbiome of the offspring of sows fed low protein diets. The above reports provide a foundation for understanding of the gut microbiota homeostasis. Additionally, a 2% reduction in dietary protein does not appear to negatively affect the reproductive performance of sows. However, the reduction in dietary protein did enhance the gut environment of piglets; therefore, reduce the incidence of diarrhea (Jin et al., 2019).

3.5. Body Composition Component

Low protein diets have been reported to promote fat deposition in pigs and especially to increase intramuscular fat or termed marbling fat (Teye et al., 2006). The body composition of pigs exhibiting compensatory growth may also be affected by feed quality, protein levels and feeding strategy (Pond and Mersmann, 1990).

3.6. Blood Amino Acids

There is enough evidence suggesting that blood AA levels are highly associated with animal health and growth performance (Wu et al., 2014; Li et al., 2016). Restricted protein diets have various effects on the concentration of blood AA. Previous studies showed an increase in serum AA concentrations when a reduced protein-diet was fed to pigs (Guay and Trottier, 2006). Reduction of dietary CP concentration beyond 12% increased ileal digestibility of both protein-bound essential and non-essential AA (Otto et al., 2003). AA metabolism, especially metabolism of Gly, Ser, Phe, Cys, Met, Arg, Pro, Try, Asp, Glu, His, and Ala, is highly associated with dietary protein restriction (Li et al. 2018; Yin et al. 2017).

Dietary AA has a positive effect on the deposition of protein in skeletal muscle. Specific transporters control the process of dietary AA absorption. AA transporters, found in membranes of many cell types act as gatekeepers for muscle cells, sense AA availability, send nutrients signals to the cell, regulate uptake and efflux of AA, and launch a series of cascade responses. Therefore, they function as transporter and receptor (Bröer et al., 2004). Li et al., 2016 reported that offering VLP diet (14% CP) supplemented with limiting AA results in poor growth and feed conversion efficiency, and reduced muscle weight in growing pigs (Li et al., 2017a). The protein synthesis process has been reported to be regulated by the intracellular presence of available AA (Miyazaki

and Esser, 2009). When the body protein synthesis process is reduced this has been suggested to be an indication of a reduction in performance of pigs fed with VLP diets (Deng et al., 2009; Morales et al., 2013).

3.7. Proteomics

Proteomics is a very powerful technique that has helped scientists to fill the gap between genome sequence and cellular behavior (Kalia and Gupta, 2005). Currently, a major shortcoming of developing the pig as a model for human-related health problems is a lack of detailed information on similarities and differences between the human and pigs from the molecular standpoint including gene expression and the constituents of the proteome. However, genome-wide technologies for transcript and profile of protein composition enables quick accumulation of molecular data to close the gap between the pig and the more traditional biomedical mouse and rat models. High-throughput and shotgun-based proteomic techniques are increasingly utilized to improve proteome coverage and produce protein catalogs (Omenn et al., 2005; Hamacher et al., 2006). For example, a more recent analysis using HPLC-ESI-MS/MS increased by a factor of almost tenfold the number of proteins identified in human muscle and quantification of proteins (Højlund et al., 2008). An understanding of how dietary protein restriction affects host metabolism and protein expression will provide an important basis for nutritional strategies for disease prevention and treatment and improving feed formulation strategies (Yin et al., 2017). Yin et al., (2017) used an iTRAQ-based quantitative proteomic approach to identify changes in proteins profile in pigs fed low protein diets. Following KEGG pathway analysis, the authors observed marked changes in the expression of proteins involved in PI3K-Akt signaling pathway, lysosome, spliceosome, oxidative phosphorylation, phagosome, and DNA replication, oxytocin signaling pathway, ribosome, tight junction, cardiac muscle contraction, and protein digestion and absorption(Yin et al., 2017).

3.8. Nutrients Digestibility

Amino Acids and phosphorous (P) are two key regulators and components of skeletal muscle growth. Gene expression of the active transporter of P in the small intestine is downregulated by CP restriction (Xue and Adeola 2016). Moreover, Xue et al. showed that ileal digested P decreased in growing pigs fed with LP diet compared with those fed with a high protein diet, which indicates a limiting effect of dietary CP level on ileal P digestion. A possible reason may be that limited protein intake impairs the active transportation system of P (Xue et al., 2017). An earlier study reported higher ileal digestibility of fat and saturated fatty acids 14:0 and 18:0 with increasing dietary CP (Jorgensen and Eggum 1992). A relationship between high dietary CP content and decreased fat digestibility suggests that free fatty acids can be attached to the undigested protein to form an aggregate of molecules in a colloidal solution (micelle) that are unavailable for absorption (Adams and Jensen, 1985).

3.9. Amino Acids Transporters

Recent evidence suggests that modification in the composition of the nutrient can alter the intestinal and muscular AA transporters' activity (Li et al. 2012a; Zhang et al. 2013). Dietary protein intake will appear in extra-intestinal tissues as single AA following their absorption in the small intestine (Rezaei, et al.,2012). Thus free AA is transported into the cell by specific transporters. These transporters carry specific types of AA, such as basic AA, neutral AA, and acidic AA. Dipeptides or tripeptides are also transported by peptide transporters (Leibach and Ganapathy, 1996; Kanai and Hediger, 2003). Reducing dietary CP and supplementing essential AA may provide a good balance of AA for uptake across the epithelial cells by reducing competition for AA transport by transporters. AA carrier systems can interact with many AA in poultry (Torras-Llort et al., 1996; Soriano-García et al., 1998) and swine (Matthews et al., 1996). For example, it has been reported

that the AA transporter solute carrier family 7 members 1 (SLC7A1) contributes to lysine, histidine, and arginine uptake in the intestine (Li, et al. 2018; Ren et al. 2017), and upregulations of SLC7A1 is expected to increase abundances of lysine, histidine, and arginine in the serum. Additionally, Li et al., (2018) reported that the AA transporters such as solute carrier family 1 member 5 (SLC1A5), solute carrier family 7 member 7 (SLC7A7), and solute carrier family 7 member 9 (SLC7A9) correspond to the absorption of neutral AA and the mechanisms by which protein restriction influences these transporters and relative AA metabolism (Li, et al. 2018). The intracellular availability of dietary AA was reported to be regulated by coordinated activity of certain transporters of AA found in the cellular membrane that allow the movement of AA in and out of the cells (Reidy et al., 2014).

The overall aim of this dissertation is to identify the dietary strategies that could improve the growth performance of pigs fed with very low protein diets.

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CHAPTER II

REVIEW OF THE LITERATURE

In order to improve growth performance of pigs fed with LP diets, multiple strategies have been proposed. Previous reports suggested that adding a mixture of branched-chain amino acids (BCAA) to LP diets improved the growth performance of nursery pigs fed with these diets (Zheng et al., 2016; Tian et al., 2019). Phytase and phytogenic feed additives (PFA) have been extensively used for swine production due to their positive effects on animals' production and health (Papatsiros et al., 2011; Bartoš et al., 2016, Adeola et al., 2009; Zeng et al., 2014; Moran et al., 2019). The precise mechanisms of action of phytase and PFA on growth performance are still unclear and therefore further research is required to elucidate how these additives modulate the performance of pigs.

The objective of this literature review is to evaluate the status of previous data on the effect of supplemental phytase and PFA on growth performance, blood metabolites, bone parameters, gut microbiota, body composition component, plasma AA, proteomics, nutrients digestibility, and AA transporters of pigs fed with LP diet.

1. Phytase and its Benefits for Swine Production

Phosphorus is an essential nutrient for growth in animal nutrition because it is involved in several metabolic processes (National Research Council, 2012). It is essential for the growth of bones, cellular integrity, and many enzymatic functions. Meeting the P requirements of pigs for growth could be difficult largely because approximately 60 to 82% of P from plant sources used in swine diets is trapped in an unavailable molecule called phatic acid. Phytase can break the insoluble complex of phytate to make the P available for absorption. Non-ruminant animals lack the sufficient amount of phytase activity to release P from phytate molecule (Ravindran and Sivalogan 1994). Since pigs cannot produce adequate phytase in their gastrointestinal tract for digestion of P, excess amounts of dietary P are excreted in feces (Cromwell and Cowieson, 2011). Therefore, exogenous phytase has been widely used in the swine diet to decrease the excretion of nutrients such as Ca and P in the manure through improving the utilization of these minerals (Thomas et al., 1991; Adeola et al., 1995; Jongbloed and Lenis, 1998). As insoluble complexes of phytic acid and protein may also occur in the gastrointestinal tract of pigs (Adeola and Cowieson, 2011), using high doses of exogenous phytase may improve the digestibility of protein, minerals, and energy (She and Stein 2018; Singh et al. 2013).

Microbial phytase is produced by fermentation from yeasts, fungi, and several strains of bacteria (Pandey et al., 2001). In particular, two strains *of Aspergillus sp.*, *A. vacuum*, and *A. niger* have been commonly used for the commercial production of microbial phytase (Pandey et al., 2001). Microbial phytase has shown beneficial effects on growth performance of pigs (Jendza et al., 2005; Gourley et al., 2018; Lu et al., 2019a) and broilers (Lee et al., 2017; Walk et al., 2018). A cheaper option for phytase production is through transgenic plants that produce extensive amounts of recombinant phytase (Greiner and Konietzny 2003). Similar to microbial phytase,

recombinant phytase has been shown to be effective in improving the growth performance of pigs fed with normal protein diets (Cromwell et al., 1995).

2. Effect of Phytase on Growth Performance of Pigs Fed Dies with Variable Dietary Protein Content and its Mechanisms of Action

2.1 Growth Performance & Feed intake

There is enough evidence that phytase supplementation improves P digestibility and reduces its excretion in nursey piglets fed with P deficient corn-soybean meal diets, without negatively impacting growth (Hong et al., 2001; Jendza et al., 2005; Zeng et al., 2014). Adeola et al (1998) reported a 10% improvement in growth performance of pigs fed low-Ca and low-P corn-soybean meal diets supplemented with microbial phytase (Adeola et al., 1998). It has also been reported that phytase improves the digestibility of phytin-bound P, which can positively affect AA digestibility and protein utilization (Adeola and Sands 2003; Kemme et al. 1999; Jongbloed and Kemme 1994; Sands et al. 2009).

Jendza et al. (2005) reported that the addition of *Escherichia coli*-derived phytase to diets deficient in P improved the growth performance of pigs. The growth performance was improved by addition of *Escherichia coli*-derived phytase which was in line with previous research using phytases from different sources (Jendza et al., 2005). A dose-dependent improvement in growth performance was observed when microbial phytase Natuphus E 5,000 was fed to piglets at 1,000 FTU/kg (Gourley et al., 2018).

In poultry, supplementation of phytase improved the growth performance, bone quality, and P utilization in broiler chickens fed diets with reduced Ca and P (Shang et al., 2015). Walk et al. (2018) observed a significant increase in weight gain in broilers supplemented with phytase at 4,500 FTU/kg. Additionally, Lee et al. (2017) observed an improvement in feed conversion ratio when broilers were superposed with phytase at 1,500 FTU/kg of diet. Phytase supplementation have been

reported to improve growth performance in pigs fed low protein diets (Biel et al., 1996; Sands et al., 2009).

2.2. Blood Metabolites

Moran et al., (2019) reported that pigs fed diets with a high dose of phytase (2,500 FTU/kg) had a greater concentration of serum Cu than pigs fed diets without phytase (2.06 vs. 1.82 mg/L). Phytate is a chelating agent of divalent cations, forming the strongest to the weakest mineral-phytin complex (Cheryan, 1980). These complexes reduce the availability of the minerals in the gut of pigs. Thus, phytase supplementation is not limited to P release but several minerals can be resealed from the phytate molecule, allowing them to be more digestible. Mineral concentrations in the serum have been utilized as an indicator of the mineral status of animals (Walk et al., 2013). Several studies observed that the addition of phytase increased serum concentrations of P, Ca, Mg, Fe, Zn, and Cu in pigs (Adeola et al., 1995; Gebert et al., 1999; Walk et al., 2013) and broiler chickens (Sebastian et al., 1996). Also, Kies et al., (2005) reported an increase in mineral absorption by addition of phytase to diet of pigs. Also, portal glucose concentration was 21% higher in phytase-treated piglets than control piglets (Kies et al., 2005).

2.3. Bone Parameters and Metabolism

There is enough evidence on the positive effect of phytase on bone parameters. Zeng et al., (2014) reported that with increasing the dose of phytase, bone parameters including metacarpal bone breaking strength, fresh and fat-free dry bone weight, and bone ash weight were also increased (Zeng et al., 2014). The addition of microbial phytase Natuphus E 5,000 at 500, 1000, 2000, 3000, or 4000 FTU/kg increased the percentage of bone ash in a dose-dependent manner in the negative

control group with 0.40, 0.30, or 0.25% aP for phases 1, 2, and 3, respectively (Gourley et al., 2018). Also, microbial phytase Natuphus E 5,000 at 2000 FTU/kg added to the positive control with 0.55, 0.45, or 0.40% aP in phases 1, 2, and 3, respectively, improved the growth performance (Gourley et al., 2018). Additionally, the observed improvements in bone measurements following addition of phytase to the diets was suggested to be linked to the improvement in the total tract digestibility of ash, P, and Ca (Gourley et al., 2018). Phytase addition has been shown to increase bone density and mineral content (Zeng et al., 2014; Duffy et al., 2018). Broomhead et al. (2019) observed a dose-dependent (500, 1,000, 2,000, or 4,000 FTU/ kg) increase in bone ash, P digestibility, and average daily gain (ADG) responses, when a corn-expressed phytase (Graizyme) was fed to nursery pigs, suggesting that with 4,000 FTU/kg of phytase added to diets bone measurements were improved. In another study (Veum et al., 2006) when phytase was supplemented great than 500 FTU/ kg of diet, bone ash weight and strength were similar to the positive control. Similarly, Broomhad et al. (2019) observed that feeding 1,000 or 2,000 FTU/kg Graizyme phytase resulted in equivalent bone ash weight and bone strength as the pigs fed with positive control.

A previous study reported a reduction in bone ash in nursery pigs fed with diets with reduced P at 0.1% that was improved with phytase supplementation (Braña et al. 2006). In addition, 750 FTU/kg of *E. coli* phytase is needed to get higher bone ash percentage as compared to the non-supplemented pigs (Braña et al. 2006). In another study by Varley et al. (2011) where two levels of phytase Natuphos 5000 (0 and 500 FTU/kg) were used, phytase addition reduced the amount of dicalcium phosphate added in swine diets leading to reduced diarrhea, less P excreted, and improved feed efficiency in weaned pigs and increased bone ash in growing pigs (Varley and O'Doherty 2011).

2.4. Gut Microbiota

Bacteroidetes and Firmicutes phyla contribute to more than 90% of total bacterial populations in the gut of weaning and growing pigs (Schokker et al., 2014; Ramayo-Caldas et al., 2016). Microbial phytase supplementation improves Ca and P digestibility, and changes in intestinal availability of these minerals affect the activity of the intestinal microbiota in growing pigs (Hooda et al., 2010; Klinsoda et al., 2019). The microbiota composition in the feces is similar to the bacterial composition of gut; however, feces cannot fully represent the microbial profile of gastro intestinal (GI) tract (Zhao et al., 2015). Therefore, characterization of the fecal microbiota community is a informative way to better understand the interaction between the dietary nutrient and the microbiota contributing to the exploration of cause-and-effect relationships between dietary changes and expression and shedding of microbiota composition in pigs. Moreover, the different microbial communities in the gut are involved in many digestive activities including proteolytic, starch-degrading, and fibrolytic reactions (Klinsoda et al., 2019). In addition, the composition and metabolic activity of the intestinal microbiota are prone to fermentable carbohydrates, and to the changes in the availability of Ca and P in the intestines. It was suggested that an increase in intestinal phytate-P availability in the small intestine after addition of phytase stimulated the growth of strictly anaerobic bacteria while a greater amount of intestinal Ca was suggested to reduce the population of some specific bacterial species belonging to lactobacilli, enterococci, and the *C. leptum* cluster (Metzler-Zebeli et al., 2010).

2.5. Blood Amino Acids

Phytase supplementation has been shown to have a positive effect on nutrients digestibility, thus, the AA digestibility may be improved by phytase supplementation (Kies et al., 2005).

In a study conducted by Gagne et al. (2002), feeding finishing pigs with diets containing 647 and 522 FTU/kg phytase increased the plasma concentrations of nitrogen-containing compounds few

hours following a meal. Authors suggested that this increase in concentration of nitrogen-containing compounds is an indication of the positive effect of phytase in enhancing the absorption of AA (Gagné et al., 2002). There are some reports indicating that phytate may interfere with intestinal uptakes of AA and glucose via sodium (Na)-dependent transport systems (Selle and Ravindran, 2008; Woyengo et al., 2011). Kies et al. (2005) reported that phytase supplementation increased the plasma concentrations of glucose post-meal and the digestibility of Na and potassium (K) was also improved (Kies et al., 2005). Moreover, Woyengo et al. (2010) reported that phytate increased the Na secretion in the jejunum and reduced the apparent ileal digestibility of Na in piglets (Woyengo et al., 2011). In poultry, Cowieson et al. reported an increase in endogenous loss of Na in broilers fed diets supplemented with phytase (Cowieson et al., 2004). About 3.1 percentage units increase in apparent ileal digestibility of AA was observed when phytase was added up to 20,000 FTU/kg (Zeng et al., 2014). Authors suggested that the improved digestibility of AA following phytase supplementation can improve the growth performance of pigs (Zeng et al., 2014).

2.6. Nutrients Digestibility

Phytase addition in the diet increases the solubility of nutrients in the small intestine through reducing the chelating capacity of isopropyl (IP) esters in the gut of non-ruminants. Some speculations suggest that the effect of phytase beyond the P release is linked to the release of myo-inositol and to destroy the anti-nutritive inositol phosphate esters (Walk et al., 2013). Myo-inositol-1,2,3,4,5,6-hexa*kis*phosphate (Ins P(6)) is a common constituent of eukaryotic cells, containing several functions beyond its role in storing P and other minerals in seeds and other plant tissues (Raboy, 2003). Additionally, myo-inositol plays an important role in cellular processes and cell function as phospholipids or inositol phosphates (Lu et al., 2019b). Several studies have suggested that myo-inositol supplementation could improve the growth performance of chickens

(Sommerfeld et al., 2018). Phytase supplementation increased plasma myo-inositol concentration in pigs, suggesting that the improvement in growth performance in pigs supplemented with phytase could be linked to the increase in myo-inositol levels (Aaron J. Cowieson et al., 2017). It has been shown that increasing the concentrations of an *E. coli* phytase in corn–soybean meal diet improves the apparent total tract digestibility (ATTD) of Ca and P and several other minerals that may have been restrained in the phytate molecule in the gut of pigs (Lu et al., 2019b).

Zeng et al., (2014) reported that *E. coli* phytase used was efficacious at 20,000 FTU/kg for swine, based on the growth performance, bone parameters, the apparent ileal digestibility of gross energy (GE) and CP, and the total tract digestibility of Ca, P, Na, and magnesium (Mg) measurements. Another study reported that super dosing phytase at 20,000 FTU/kg further enhanced the degradation of phytic acid in the gut and other than improvement of mineral digestibility, the CP and AA utilization were also improved (Zeng et al., 2014).

Previous studies have reported inconsistencies in the apparent fecal digestibility coefficients data related with phytase supplementation (Harper and Schell 1997; Yin et al. 2001; Zeng et al. 2014). These discrepancies can be attributed to the variations in sources and concentration of phytase and available P in the diets, but also to the different Ca:P ratio. It is well known that a wide range of Ca:P ratios can result in adverse effects of Ca on the digestibility of P (Qian and Conner 1996). According to Fernández (1995), the differences in absorption between different P sources is the reason for the variation in digestibility coefficients and also the physiological stage of the animals.

There is a need to develop accurate and uniformly expressed requirements for available P and Ca in phytase-supplemented pig diets. This, considering the known dietary phytate levels and good recognition of the amount of P released by phytase, would allow producers in improving dietary formulations to reduce excretion (Selle and Ravindran, 2008). Létourneau-Montminy et al.,

(2008) investigated the effect of reduced dietary Ca concentration and phytase supplementation on Ca and P utilization in weanling pigs with modified minerals level, with animals receiving depletion diets (DD) that consisted of a corn-soybean meal with either 1.42% Ca and 0.80% P (DD+) or 0.67% Ca and 0.43% P (DD−), designed to achieve the same Ca: digestible P ratio, concluded that phytase was more efficient in P retention and bone mineralization in the diet with a ratio of Ca:P of 1.9 than that with a ratio of 1.3. This study suggested that dietary Ca be adjusted to an amount that maximizes P digestibility without impairing P retention in swine diets (Letourneau-Montminy et al., 2008). A previous study reported an improvement in protein and P digestibility in pigs supplemented with phytase regardless of the deficiency in some of the amino acids in the diets (Biel et al., 1996). Lala et al., (2020) reported an improvement in ATTD of Ca, P, ether extract and GE obtained with growing pigs fed with low protein diet (14% CP) supplemented with phytase. Additionally, the ileal digestibilities of Lys, Thr and Trp was enhance in growing pigs fed with low protein diet with phytase addition, suggesting the involvement of endogenous protein in phytate/phytase nutrition. Low protein diets are mostly associated with more available dietary energy and free fatty acids (Kerr et al., 2003).

In poultry, Cowieson et al., (2017) found an interactive effects of dietary protein source and exogenous protease on growth performance, immune competence, and jejunal health of broilers, providing a strong evidence that phytase increases apparent ileal AA digestibility in chickens. When the dose of phytase was increased above 1,000 FTU/kg there will likely be a disconnection between liberated P (which may continue moderately strongly to 2000–3000 FTU/kg or beyond) and all other nutrients (Cowieson et al., 2017). It was concluded that phytase was effective in improving AA digestibility in broilers and that these effects originate from the removal of the anti-nutritional effects of phytate (Cowieson et al., 2017; walk et al., 2019). The authors indicated that their results will enable the producers in including the right amount in phytasein the diet while considering the amount of P that will be released by phytase, to reduce the confusion currently

present on the market about phytase inclusion levels (Cowieson et al., 2017). Another study by Lee et al. (2017) reported that 1,500 FTU/kg phytase improved the feed conversion ratio in broilers compared with the negative control in the absence of myo-inositol, while only a minor improvement was occurred in its presence suggesting that part of improvement in growth performance with feeding high levels of phytase is related with myo-inositol production. Theses authors concluded that the phytase supplementation and myo-inositol have a less beneficial effect on bone measurements and were only effective on feed conversion ratio (Lee et al., 2017). The phytase effect on Na digestibility and excretion and ileal digestibility of Na has been shown in poultry (Cowieson et al., 2004). In broilers fed with corn-soybean meal diets, ileal digestibility of Na was depressed when dietary phytate was increased (Selle and Ravindran, 2008).

A large number of studies have demonstrated the magnitude of phytase supplementation in diets of non-ruminant animals and the concentration of phytase needed to maximize the ATTD of Ca and P, which is about 700 to 1,000 FTU/kg. However, there are variations in the amount of phytase required in the diet due to the differences in source and types of phytase available in the market (Almeida et al., 2013; AOAC 2007; Lee et al., 2017).

2.7. Amino Acid Transporters

Amino acids are sensed and transported by specific AA transporters in the gut. It is well established that nutrients composition alters the intestinal AA transporters expression in pigs fed protein-deficient diets (Zhang et al., 2013). Phytase addition to swine diet increases the expression of Na-dependent glucose cotransporters 1 (SGLT1) in the upper gut. The increased relative abundance of SGLT1 in the jejunum by the addition of phytase suggests that phytase reduce the negative effects of phytic acid through increased expression of SGLT1 (Woyengo et al., 2011).

A study by Vigors et al. (2014) concluded that the addition of phytase stimulated the upregulation of oligopeptide transporter PEPT1 (solute carrier family 15 members 1, peptide transporter), suggesting that phytase might have a positive effect on AA and peptides absorption in the gut of pigs. Additionally, supplementation of phytase in the diet increased the gene expression Ca transporter TRPV6, the P transporter SLC34A2 and the fatty acid transporter FABP2 compared with the control diet. The upregulation of these nutrient transporters shows that intestinal nutrient transporters expression is a mechanism involved in the uptake of nutrients following the degradation of the phytic acids molecule in the gut of pigs (Vigors et al., 2014).

The claudin family members that regulate the barrier function contribute to the regulation of epithelial homeostasis and are expressed in a spatiotemporal manner in the intestinal and crypt-luminal axis. The claudins are part of tight junction proteins that regulate the paracellular permeability of nutrients (Garcia-Hernandez and Nusrat 2017). When the expressions of claudins are reduced it can compromise the tight junctions and impair the performance of animals (Xia et al., 2019). Phytase addition has been shown to change the composition of the tight junction claudins by upregulating the expression of claudin 3 in the upper small intestines and increase it in the ileum with the higher level of phytase. Phytase can protect the intestinal barrier through reducing the anti-nutritional effect of phytic acids-induced mucin loss (Onyango and Adeola, 2009), which is a suggestive mechanism by which phytase enhances the intestinal integrity. Lu et al., (2020) investigated the effect of phytase on nutrient digestibility and expression of intestinal tight junction and nutrient transporters in nursery pigs and concluded the use of elevated dietary phytase levels (3,000 FTU/kg) in low-P diets resulted in improved growth performance and P digestibility in nursery pigs, which was linked to a breakdown of InsP6 and increased levels of myo-inositol concentrations in blood circulation. Phytase may also be involved in glucose transport through the upregulation of GLUT2 expression (Lu et al., 2020). Finally, phytase may change the intestinal

brush border mucin and tight junction composition by changing the Mucin 2, claudin 1, and claudin 3 gene expression (Lu et al., 2020).

3. Effect of Phytogenic Feed Additives on Growth Performance of Pigs Fed with Dies with Variable Dietary Protein Content and their Mechanisms of Action

3.1. Growth Performance & Feed Intake

Phytogenic feed additives (PFA) plant-based compounds are added in diets or water to enhance animal productivity by improving feed palatability, improving gut health, and immune system (Windisch et al., 2008). PFA have received increasing attention to be used for swine production lately due to their positive effects on animals' production and health (Bartoš et al. 2016; Karásková et al., 2015; Papatsiros et al. 2011; Zeng et al. 2015). Improvement in growth performance was observed when adequate-protein diets were supplemented with PFA such as Chinese herbal powder in early-weaned piglets (Kong et al., 2007) and herbal extract mixture (buckwheat, thyme, curcuma, black pepper, and ginger) in growing pigs (Yan et al., 2011a). Manzanilla et al. (2009). found that supplementing a plant extract mixture to a low protein diet (18% CP) in weaned pigs had differential positive or negative effects on various variables measured (Manzanilla et al., 2009).

The PFA are plant-derived compounds that unlike synthetic antibiotics are residue-free and are mostly considered safe to be incorporated in the animal diet and used as a growth promoter (Lan et al., 2016). The PFA used to enhance the growth performance of animals contain several bioactive compounds including, alkaloids, bitters, flavonoids, thymol, cineole, linalool glycosides, mucilage, saponins, tannins phenolics, polyphenols, terpenoids, and polypeptide, anethole, allicin, capsaicin, vitamins, and minerals (Windisch et al., 2008; Grashorn, 2010; Kiczorowska et al., 2016). Therefore, the expected effects of these PFA are various, depending on which plant extract is used. Furthermore, PFA have a large number of bio-active compounds depending on their

chemical composition, influences of location, weather conditions, harvest conditions, and their mode of action (Huyghebaert and Immerseel 2011). Some other factors contributing to the efficacy of the PFA includes the part of the plant and their physical properties, the various dosage used, and method of extraction (Yang et al., 2009). Additionally, the beneficial effect of PFA can also be influenced by the type of diets fed to animals, health status, and the environmental condition. The mode of action of some PFA is still undergoing research. However, growth-promoting, the antimicrobial, immunomodulatory, and anti-oxidative effects of PFA in livestock have been previously reported (Windisch et al., 2008; Alagawany and Abd El-Hack, 2015).

Bartos et al. (2016) investigated the effects of PFA on growth performance and ammonia and greenhouse gases emissions in growing-finishing pigs, using two commercial PFA containing a blend of essential oils (≥1.5% of the pure product), with caraway and lemon oil as the main components, dried herbs and spices, and quillaja saponins. The authors reported an improvement in growth performance of growing pigs, confirming the positive effect of PFA as growth promoters (Bartoš et al., 2016). Additionally, a significant reduction in NH3 emissions was observed, an indication that PFA can be used for reducing NH3 emissions from pig production (Bartoš et al., 2016). PFA have been successfully used to improve the growth performance of pigs (Cho and Kim 2014; Krauze and Ognik 2020; Windisch et al. 2008).

Cho et al., (2014) studied the effects of PFA containing essential oils of thyme and star anise as main active components on growth performance, energy and nutrient apparent total tract digestibility, and relative organ weight after oral challenge with *C. perfringens* in broilers. The authors concluded that supplementation of PFA, containing essential oils of thyme and star anise as main active components, improved growth performance and inhibited *C. perfringens* and *E. coli* proliferation in the small and large intestine as well as the intestinal lesion score of chick challenged with oral *C. perfringens* (Cho et al., 2014). Park et. al., (2020) assessed the effects of dietary *Achyranthes japonica* extract supplementation on the growth performance, total tract

digestibility, cecal microflora, excreta noxious gas emission, breast meat quality, and organ weight in broiler chickens, and concluded that that feeding *Achyranthes japonica* extract to broilers improved their growth performance, dry matter and nitrogen digestibility, cecal *Lactobacillus* population, and breast meat production, whereas it decreased the *E. coli* and *Salmonella* population in the large intestine, excreta ammonia emission, and abdominal fat. They suggested that *Achyranthes japonica* extract can be used in broilers as growth promoters (Park and Kim, 2020).

3.2. Blood Metabolites

Previous studies have indicated that the inclusion of herbal extract (curcumin, black pepper, and thymol) could enhance blood circulation, metabolic process, and the immune system of animals (Chakravarty and Yasmin, 2005; Trevisi et al., 2007). The report by Yan et al. (2011) investigating the effect of an herb extract mixture (including thyme, curcuma, buckwheat, black pepper, and ginger) on growth performance, nutrient digestibility, blood characteristics, and fecal noxious gas content in growing pigs, concluded that supplementation of this mixture to the diet of pigs increased growth performance, lymphocyte count, red blood cell and white blood cell concentration, and decreases the fecal noxious gas content (NH_3 and H_2S). The authors suggested that the addition of a mixture of buckwheat, thyme, curcuma, black pepper, and ginger at a ratio of 10:15:30:10:35) to diets of growing pigs could be considered as a potential antibiotic alternative in growing pigs due to its positive effect on feed intake (Yan et al., 2011b).

Some blood metabolites are changed when the animals are under stress (Korošec et al., 2009). An increase in red blood cells was reported in the growing-finishing phase of pigs fed diets supplemented with Coptis Chinensis extract a (Zhou et al., 2013). Furthermore, Li et al. (2012) reported an increase in lymphocytes in nursery pigs fed diets supplemented with essential oils (Li

et al., 2012a). In contrast, no changes in blood metabolites, leukocytes and lymphocytes were reported in growing-finishing pigs supplemented with PFA (artichoke, celery, beet, onion, garlic, spinach, avocado, oats, and parsley) during the summer heat. An improvement in growth performance was reported in these pigs under heat stress, suggesting that this PFA could be used as a strategy to minimize the negative effects of a high temperature (Dávila-Ramírez et al., 2020). In contrast, no change in blood metabolites was reported when weaned pigs diet supplemented with gel-based PFA (Upadhaya and Kim 2016).

In broiler, it was reported that supplementation of PFA (containing essential oils of thyme and star anise) reduced blood total cholesterol and increased high-density lipoprotein-cholesterol concentration (Cho et al., 2014). Similarly, Hong et al. found that dietary addition of essential oil decreased serum levels of total cholesterol and increased high-density lipoprotein-cholesterol with no effects on serum low-density lipoprotein-cholesterol (Hong et al., 2012). Calislar et al.,(2009) reported that PFA (*Origanum vulgare* ssp. hirtum) supplementation had no positive effects on triglycerides and blood cholesterol. It was suggested that the use of most of the essential oils might be often associated with hypocholesterolemic properties (Calislar, A. 2009).

3.3. Gut Microbiota

Numerous studies have demonstrated that PFA has antibacterial effects (Dorman and Deans, 2000; Mitsch et al., 2004). It has been shown that good microbial communities such as lactobacilli and bifidobacteria promote host protective responses against pathogenic microorganisms. Li et al. (2018) investigated the effects of dietary essential oils on intestinal microbial composition and metabolic profiles in weaned piglets and showed intestinal microbiome–metabolome responses to essential oils supplementation in weaned piglets. The results from that study indicated that essential oils influenced the microbiota composition in the intestines of weaned piglets by promoting the

growth of some good bacterial species such as *Lactobacillales, Streptococcaceae*, *Veillonellaceae,* and *Megasphaera* in the colon. Additionally, several metabolic pathways, including AA, lipid, and carbohydrate metabolism of intestinal microbiota were also shaped by the addition of essential oils in nursery pigs (Li et al., 2018). A recent report by Sanchez et al., (2020) studying the effect of Alliaceae extract (*Allium* spp. extract rich in organosulfur compounds, such as propyl thiosulfonate) supplementation on performance and intestinal microbiota of growing-finishing pigs indicated that the addition of *Allium* spp. extract at 30 ppm of propyl thiosulfonate to the diets of pigs had positive effects on gut microbiota, resulting in a selective increase in certain good bacteria such as *Enterobacteriaceae and Lactobacillus* spp. and decrease in the numbers of pathological bacteria such as *Salmonella* spp and *Clostridium* spp in the feces of pigs. Also, the addition of garlic and onion extract in the diet altered the short-chain fatty acid composition, improving the levels of isobutyric propionic, and isovaleric acids and the percentage of total branched fatty acids in the feces of pigs (Sánchez et al., 2020).

A group of researchers studied the effects of dietary supplementation of *Achyranthes japonica* extract on the growth performance, total tract digestibility, cecal microflora, excreta noxious gas emission, breast meat quality, and organ weight in broiler chickens (Park and Kim, 2020). They concluded that that feeding *Achyranthes japonica* extract to broilers improved their cecal *Lactobacillus* population, and breast meat production, however, a decrease in *E. coli* and *Salmonella* population in the large intestines considered as unhealthy bacteria was observed (Park and Kim, 2020). The cecum plays a major role in gut health, breakdown of some carbohydrates, synthesis of microorganisms of vitamins, and absorption of additional nutrients. Addition of plant preparations was found to reduce the intestinal pH and increase the number of lactic acid bacteria in the ileum and cecal contents that are known to significantly decrease the *E. coli* and *C. perfringens* counts in chickens (Vidanarachchi et al., 2006). Previous reports have suggested that beneficial microbial communities such as lactobacilli and bifidobacteria stimulate

host protective responses against pathogenic microorganisms. The addition of *Achyranthes bidentata* extract decreased the incidence of diarrhea in nursery piglets, suggestive of the inhibition of gut pathogens. Moreover, broilers fed with diets supplemented with *Achyranthes japonica* extract had higher levels of good bacterial components such as *Lactobacillus* and decreased *E. coli* and *Samonella* concentration in ceca. In another study, the effect of supplementation of essential oil (*Zataria multiflora*) on the microbes composition and peroxidation of breast fillets in broiler chicken was studied (Jebelli et al. 2013). It was indicated that the essential oil prolonged the peroxidation and microbial spoilage of chicken breast fillets (Jebelli et al., 2013).

3.4. Blood Amino Acids

Some important metabolic pathways involved in the growth performance and health of livestock are controlled by AA (Jobgen et al., 2006; van Faassen et al., 2009). An elevated levels of AA in the portal system can stimulate the synthesis of proteins in the tissues of weaned piglets (Wu et al., 2007). Supplementing ultra-fine Chinese herbal powder in the diet enhanced serum concentrations and apparent ileal digestibility of most AA in weaned piglets as well as the growth performance in nursery pigs (Kong et al., 2009). Liu et al., (2017) reported that the addition of dietary *saccharomyces cerevisiae* cell wall extract to diets of weaned pigs mitigated the oxidative stress and altered the blood AA profiles. Furthermore, the addition of this PFA was effective in improving serum concentration of some AA, and gut morphology (Liu et al., 2017).

3.5. Nutrients Digestibility

There is evidence supporting that PFA improve nutrients digestibility and absorption of AA in weaned piglets (Kong et al., 2009). The improvement in nutrient digestibility when PFA are fed has been attributed to the improvement in intestinal morphology and gut health in swine (Yan et

al., 2012; Upadhaya et al., 2016). Upadhaya et al. (2016) showed that the gel-based PFA feed supplement did not affect the feed intake. However, it increased the gain to feed (G: F) and ADG by improving the dry matter and energy digestibility and significantly improved jejunum and ileum villi length (Upadhaya et al., 2016). The improvement in growth performance, nutrient digestibility as well as improvement in intestinal morphology was associated with the phytogenic component of the gel-based feed supplement. Similar results were found by Amad et al. (2011), where they reported that addition of essential oils to diet of broilers enhanced the ileal digestibility of CP, dry matter, and energy (Amad et al., 2011). Other studies have reported that some PFA are capable of stimulating the secretion of endogenous digestive enzymes in the gut, and increase bile flow; therefore, improving nutrients digestibility (Lee et al., 2003; Jamroz et al., 2005; Yan et al., 2010; Cho et al., 2014).

3.6. Amino Acid Transporters

The membrane of many cell types contains amino acids transporters (Duan et al., 2015). There are specific transporters for each AA, for example, the L-type AA transporter (LAT) family is in charge of transmitting and caring the majority of cellular branched-chain amino acids (BCAA) (Li et al., 2016). There is enough evidence suggesting that blood AA levels are highly associated with animal health and growth performance (Wu et al., 2014; Li et al., 2016).

In boilers, supplementation of PFA (Quillaja saponin blend) increased the mRNA level of AA transporters in chickens fed with PFA and correlated with differences in digestibility, indicating an enhanced absorption of nutrient components in the small intestine. The intestinal presence of saponins has been previously shown to affect membrane permeability and the cellular transport of molecules such as macromolecules (So et al., 2002). Particularly, the altered changes in the relative abundance of AA transporters including, peptides transporter 1 (PEPT1) and excitatory amino acid

transporter 3 (EAAT3) in the intestines are known to be linked to the growth and feed conversion efficiency in broilers (Mott et al., 2008; Zhang et al., 2013). Supplementation of essential oils and saponins significantly increased the relative abundance of PEPT1 in the membrane of broilers. Additionally, it was concluded that feeding chickens with PFA including an essential oil blend from star anise, thyme, rosemary, and oregano, and a Quillaja saponin resulted in an improvement in the digestibility of crude protein and AA as well as the performance of growing broilers. The most prominent molecular alterations induced by both PFA and their combination seem to affect the balance of carbohydrate and lipid metabolism (Reyer et al., 2017).

3.7. Total Antioxidant Capacity

Several studies reported that PFA enhance the total antioxidant capacity and gut health in pigs (Manzanilla et al., 2006; Windisch et al., 2008; Li et al., 2012b) and broilers (Paraskeuas et al., 2017; Krauze et al., 2020). An investigation by Li et al, indicated that administration of encapsulated essential oils containing thymol and cinnamaldehyde (50, 100 or 150g/ton of feed) enhanced the immune system by increasing in lymphocyte proliferation rate, phagocytic rate as well as blood immunoglobulins concentrations in piglets (Li et al., 2012a). Other studies observed no effects of PFA supplementation on the markers of antioxidant capacity in serum or saliva (Dávila-Ramírez et al., 2020).

In broilers, supplementation of 20g PFA per liter water from plants including Withania somnifera containing withanolides as a bioactive compound enhanced the hemoglobin, white blood cells count, packed cell volume, and antibody titer against viral disease, indicating the enhancement in the immune system status of birds (Mushtaq et al. 2012). Others showed that addition of PFA (6 g of anise seed) per kg diet increased antibody titer against infectious bronchitis and Newcastle disease virus in poultry (Alhajj and Al-Mufarrej 2015).

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CHAPTER III

A NOVEL CORN-EXPRESSED PHYTASE IMPROVES DAILY WEIGHT GAIN, PROTEIN EFFICIENCY RATIO AND NUTRIENTS DIGESTIBILITY AND ALTERS FECAL MICROBIOTA IN PIGS FED WITH VERY LOW PROTEIN DIETS

This chapter is based on: *Shili CN, Broomhead JN, Spring SC, Lanahan MB, Pezeshki A. A Novel Corn-Expressed Phytase Improves Daily Weight Gain, Protein Efficiency Ratio and Nutrients Digestibility and Alters Fecal Microbiota in Pigs Fed with Very Low Protein Diets. Animals (Basel). 2020 Oct 20;10(10):1926. doi: 10.3390/ani10101926. PMID: 33092137; PMCID: PMC7590218.*

Abstract

The objective was to assess the effect of a novel corn-expressed phytase (CEP) on growth, nutrients digestibility, bone characteristics and fecal microbiota of pigs fed with very low-protein, -calcium (Ca) and -phosphorous (P) diets. Forty-eight barrows were subjected to 6 groups for 4 weeks: positive control-adequate protein (PC), negative control-reduced protein (NC), NC+low-dose CEP, *i.e*. 2,000 FTU/kg (LD), NC+high-dose CEP, *i.e.* 4,000 FTU/kg (HD), LD with 0.12% unit reduced Ca/0.15% unit reduced available P (LDR), and HD with 0.12% unit reduced Ca/0.15% unit reduced available P (HDR). Compared to NC, LD and HDR had higher average daily gain (ADG) and gain:protein ratio (G:P), HD and HDR had greater apparent fecal digestibility of Ca and P and bone mineral density and LDR and HDR had lower serum osteocalcin. The feces of LD was enriched in Lachnospiraceae, while the HD had a higher abundance of *Succinvibrio* and LDR had higher abundance of *Bifidobacterium* and Actinobacteria.

In conclusion, supplementation of protein-restricted diets with a CEP decreased their negative effects on ADG and G:P ratio, increased the digestibility of Ca and P regardless of the levels of these minerals in the diet, improved bone characteristics and produced differential effects on fecal bacterial population.

Keywords: Low-protein diets; low calcium/phosphorous diets; corn-expressed phytase; growth; nutrients digestibility; bone characteristics; fecal microbiota; pigs.

1. Introduction

Sustainability of the swine industry is challenged by increased feed cost and environmental concerns associated with excessive excretion of pollutants such as nitrogen (N) and phosphorus (P) from swine production [1,2]. Feeding pigs with low protein diets with reduced P may help decrease the nutrients excretion [3,4]. Slightly low protein diets, supplemented with limiting amino acids (*i.e.* lysine, methionine, threonine, and tryptophan), can be used to decrease the excretion of N and feed cost and mitigate the incidence of diarrhea after weaning without negative impact on performance or feed efficiency in pigs [5–8]. Severe reduction of dietary CP, *i.e.* > 25%, may decrease the nutrients excretion more than slight protein restriction; however, very low protein diets while supplemented with limiting amino acids, decrease the feed efficiency and growth performance of pigs [9–11]. Similarly, diets with low P: calcium (Ca) ratio compromise the growth measures such as daily gain and bone development and increase the feed conversion rate in pigs [12]. Thus, there is a need to develop strategies for improving the N and P utilization in pigs fed with very low-protein and -P diets to minimize their negative effects on animals' growth.

Exogenous phytase has been widely used in swine diet to improve the utilization of nutrients through the breakdown of insoluble complexes formed between nutrients and phytate (*myo*-inositol hexaphosphate) [2,13]. The phytate is a polyanionic molecule and unavailable form of phosphorus that can chelate divalent cations such as Ca with a high capacity and create mineral-phytate complexes [2]. Similar insoluble complexes are formed between phytate and proteins

[14], inhibiting the activity of proteolytic enzymes [15] and reducing the utilization of protein. The most common type of exogenous phytase used in the swine diet is microbial phytase. There is a plethora of evidence that digestibility and utilization of P and Ca are increased in pigs when microbial phytase was supplemented in their diets formulated with adequate Ca and P [16–19] or reduced Ca- and P [20–25]. However, there are conflicting reports on the effects of microbial phytase on digestibility of N and amino acids in pigs [26,27]. Some studies have shown promising outcomes on growth performance when microbial phytase was used for pigs receiving amino acids-deficient diets [28,29], but some others have reported no positive effects on growth of animals [30,31].

Microbial phytase is produced by fermentation from yeasts, fungi and multiple strains of bacteria [32]. In particular, two strains of *Aspergillus* sp., *A. ficuum* and *A. niger* have been commonly used for commercial production of microbial phytase [32]. An alternative cost-effective strategy for commercial production of phytase is through its expression in transgenic plants that due to their large biomass they can express the transferred genes and produce recombinant phytase extensively [33]. There are limited animal studies to test the efficacy of recombinant phytase as an alternative for microbial phytase. Recently, a novel corn-expressed phytase (CEP) was shown to improve the growth performance, bone characteristics and digestibility of Ca and P in weaned pigs fed with Ca- and P-deficient diets [34,35]. However, it remains to be determined whether the CEP improves the growth performance of young pigs when they are fed with very low protein diets. Further, little is known on the mechanisms by which the CEP improves the growth performance of pigs. The beneficial effect of the CEP on the growth performance of pigs has been mainly attributed to improved digestibility of Ca and P [34,35]. There is some evidence that changes in intestinal availability of Ca and P as a result of using microbial phytase can affect the activity of the intestinal microbiota in pigs [36,37]; however, little is known whether dietary supplementation of CEP influences the performance of pigs through changes in fecal microbiota. Further, the effect of CEP on bone mineral density and content and blood metabolites associated with Ca and P metabolism is not known.

Therefore, the objective of the current study was to investigate the effect of two levels of a CEP on growth performance, nutrient digestibility, fecal microbiota composition, bone mineral density and content and blood metabolites associated with Ca and P metabolism in young pigs fed with very low protein diets with reduced Ca and P.

2. Materials and Methods

2.1. Animals, housing, and diets

The experimental procedures used during this entire study were performed in accordance with FASS Guide for the Care and Use of Agricultural Animals in Research and Teaching, and all the experimental procedures were approved by the Institutional Animal Care and Use Committee at Oklahoma State University (Animal Care and Use Protocol # AG-17-22). A total of forty-eight weanlings (three weeks old; 6.3 ± 1.2 kg body weight (BW) crossbred barrows (Duroc sire line and Large White X Landrace dam) were used (Seaboard, Hennessey, OK). The general animal husbandry procedures were undertaken according to our previous publications [38, 39]. Upon arrival, the pigs were group-housed and acclimated to the environment in a controlled temperature and ventilation facility. The temperature was set at 31 °C during the first week and it was reduced by 1 °C every week. Feed was provided in one-hole stainless steel feeders, and water was provided by cup waterers (Aqua Chief™) with single 1/2" nipples (Lixit® Nipple Waterer - L-70). Both feed and water were provided *ad libitum*. All pigs used in this study were barrows (male). Following two weeks of adaptation, all pigs were weighed, individually housed in a 60 x 167 cm pen and assigned to one of six groups (n=8/group) while keeping the mean body weight consistent for all groups (10.2 ± 1.5 kg). Each group then was randomly allotted to one of dietary treatments including, 1) positive control with normal protein content (PC); 2) negative control with low protein content (NC); 3) NC+ low dose of CEP (LD); 4) NC+ high dose of CEP (HD); 5) LD with reduced Ca/P (LDR); 6) HD with reduced Ca/P

(HDR). The ingredients and composition of diets used are given in Table 1. The dose of CEP used for LD and HD diets was 2,000 one phytase unit (FTU)/kg and 4,000 FTU/kg. The used doses for CEP was chosen based on recent research published on corn-expressed phytase (GraINzyme®), the same product used in this study, where 4,000 FTU/Kg phytase was shown to increase the growth performance of pigs fed with diets with normal protein content [35]. Both LDR and HDR diets had reduced Ca and available P by 0.12 and 0.15 units [35], respectively compared to the rest of the diets (Table 1). All diets were corn-soybean based and prepared according to Nutrient Requirements of Swine-National Research Council [40]. Phase feeding was applied according to requirements of pigs during the nursery period [40] with providing the commercial nursery phase 1 pelleted diet (United Animal Health, Sheridan, IN) for one week (days 1-7), nursery phase 2 diet for two weeks (days 8-21) and nursery phase 3 diet for three weeks (days 22-42), the dietary treatments were fed from day 14 of the study (Table 1).

All diets were isocaloric with low protein diets being isonitrogenous (Table 1). The desired energy and protein content of the diets were achieved with manipulating the amount of soybean, cornstarch, corn, and limiting amino acids (*i.e.* lysine, methionine, threonine, and tryptophan). The Ca and P levels in LDR and HDR diets were obtained by reducing the amount of dicalcium phosphate and limestone. All diets contained 0.5% chromium oxide (AquaPhoenix Scientific Inc, Hanover, PA) as an indigestible marker. The CEP used in this study (GraINzyme®) was provided by Agrivida Inc. (St Louis, MO). The specific activity of GraINzyme® was 3,200 FTU/g.

Table 1. Ingredients and composition of experimental diets1 (% as-fed basis).

	Phase 2[2]						Phase 3[3]					
Ingredients[4] %	**PC**	**NC**	**LD**	**HD**	**LDR**	**HDR**	**PC**	**NC**	**LD**	**HD**	**LDR**	**HDR**
Corn, yellow dent	48.49	73.77	73.71	73.67	74.23	74.20	56.80	87.10	86.95	86.89	87.57	87.52
Fish meal, menhaden	5.44	5.30	5.27	5.27	5.31	5.30	2.06	2.49	2.49	2.48	2.50	2.50
Soybean meal, 47.5% CP	35.13	3.17	3.16	3.16	3.18	3.18	38.22	4.97	4.97	4.97	5.00	5.00
Whey, dried	5.44	6.32	6.32	6.31	6.36	6.36	-	-	-	-	-	-
Corn starch	2.97	6.32	6.32	6.31	6.36	6.36	-	-	-	-	-	-
Dicalcium phosphate, 18.5%	0.70	0.95	0.95	0.95	0.13	0.13	0.98	1.18	1.18	1.17	0.38	0.38
Limestone	0.50	0.64	0.63	0.63	0.77	0.77	0.62	0.72	0.72	0.72	0.85	0.85
Vitamin premix[5]	0.19	0.18	0.18	0.18	0.18	0.18	0.18	0.15	0.15	0.15	0.15	0.15
Trace mineral premix[6]	0.05	0.07	0.08	0.07	0.07	0.07	0.07	0.10	0.10	0.10	0.10	0.10
Lysine, sulfate	-	1.60	1.60	1.60	1.60	1.60	-	1.58	1.57	1.57	1.57	1.57
DL-methionine	-	0.16	0.16	0.16	0.16	0.16	-	0.16	0.16	0.16	0.15	0.15
L-threonine	-	0.41	0.41	0.41	0.41	0.41	-	0.40	0.40	0.40	0.40	0.40
L-tryptophan	-	0.13	0.13	0.13	0.13	0.13	-	0.12	0.12	0.12	0.13	0.13
Salt	0.60	0.53	0.53	0.53	0.53	0.53	0.57	0.62	0.62	0.62	0.63	0.63
Chromium oxide	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Grainzyme (phytase)	-	-	0.06	0.12	0.06	0.12	-	-	0.06	0.12	0.06	0.12

Calculated Chemical Composition[7]

Dry matter, %	90.40	90.70	90.70	90.70	90.65	90.65	89.66	89.54	89.54	89.54	89.48	89.48
Crude protein, %	24.87	13.41	13.41	13.41	13.50	13.49	24.26	12.85	12.84	12.83	12.91	12.91
Crude fat, %	3.52	3.54	3.54	3.53	3.56	3.56	3.57	3.79	3.79	3.79	3.82	3.82
Calcium, %	0.80	0.80	0.80	0.80	0.68	0.68	0.70	0.70	0.70	0.70	0.58	0.58
Phosphorus, %	0.71	0.61	0.61	0.61	0.46	0.46	0.67	0.57	0.57	0.57	0.43	0.43
aP, %	0.40	0.40	0.40	0.40	0.25	0.25	0.33	0.33	0.33	0.33	0.18	0.18
Nitrogen, %	3.98	2.15	2.15	2.15	2.16	2.16	3.88	2.05	2.05	2.05	2.06	2.06
SID Lysine	1.35	1.34	1.35	1.35	1.35	1.35	1.24	1.23	1.23	1.23	1.23	1.23
SID Methionine	0.39	0.39	0.39	0.39	0.39	0.39	0.36	0.37	0.37	0.37	0.36	0.36
SID Threonine	0.84	0.79	0.79	0.79	0.80	0.80	0.80	0.74	0.74	0.74	0.74	0.74
SID Tryptophan	0.27	0.22	0.22	0.22	0.22	0.22	0.27	0.21	0.21	0.21	0.21	0.21
Grainzyme, FTU/kg	-	-	2000	4000	2000	4000	-	-	2000	4000	2000	4000
ME, Mcal/kg	3.45	3.40	3.39	3.39	3.39	3.42	3.34	3.34	3.34	3.34	3.36	3.36

Analyzed Chemical Composition[8]

Dry matter, %	87.80	87.80	88.20	87.60	88.20	86.90	87.30	87.00	87.40	87.20	87.70	86.50
Crude protein, %	24.20	13.60	13.50	13.70	13.40	14.00	23.10	12.00	11.90	12.20	13.20	11.60
Crude fat, %	2.20	2.90	2.60	3.00	3.00	3.00	2.40	2.70	2.80	2.50	2.80	3.10
Chromium, %	0.21	0.23	0.22	0.20	0.27	0.26	0.23	0.20	0.20	0.20	0.24	0.21
Calcium, %	0.86	0.63	0.79	0.75	0.56	0.63	0.69	0.77	0.60	0.69	0.63	0.59

Phosphorus, %	0.74	0.60	0.61	0.58	0.42	0.43	0.63	0.58	0.52	0.58	0.46	0.43
Nitrogen, %	3.90	2.20	2.20	2.20	2.20	2.20	3.70	1.90	1.90	2.00	2.10	1.90

[1]PC (positive control): normal protein, adequate Ca and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 FTU/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced calcium (Ca) and phosphorus (P); HDR: HD with reduced Ca and P. *n=8* for each dietary group.

[2]Fed for two weeks of nursery phase, from day 7 to 21 (from 28 day of age and 7-11 kg body weight)

[3]Fed for three weeks of nursery phase, from day 21 to 42 (from 42 day of age and 11-25 kg body weight).

[4]Corn, fish meal, soybean meal, whey, corn starch, dicalcium phosphate, limestone, and salt were obtained from Nutra Blend, LLC (Neosho, MO). DL-methionine (MetAMINO®) and lysine, sulfate (Biolys®) were obtained from Evonik (Kennesaw, GA). L-threonine and L-tryptophan were obtained from Ajinomoto (Overland Park, KS). Grainzyme was obtained from Agrivida (Woburn, MA).

[5]Vitamin premix was obtained from Ralco Animal nutrition (Marshal, MN). Each kilogram of mix contained: vitamin A, 22,044 IU; vitamin D, 3,330 IU; vitamin E, 143 IU; vitamin K, 8.83 mg; vitamin B6, 2.75 mg; vitamin B12, 18.50 mcg; niacin, 99,33 mg; pantothenic acid, 90.50 mg; riboflavin 19.86 m, Thiamine 4.41 mg; Folic Acid 2.42mg.

[6]Trace mineral premix was purchased from Nutra Blend, LLC (Neosho, MO). Each bag (22.68 kg) of mix contained: iron, 7.3%; zinc, 7.3%; manganese, 2.2%; copper, 1.1%; iodine, 198 ppm; selenium, 198 ppm.

[7]Values were calculated using National Swine Nutrition Guide (NSNG; V 2.0).

[8]Diets chemical composition was analyzed by Servitech (Dodge city Ks).

2.2. Growth performance parameters

The individual feed intake and BW were recorded daily and weekly, respectively. The average daily gain (ADG) was calculated by dividing the weight gain of each pig during the experimental period to 28 days (weeks 2-6). The average daily feed intake (ADFI) was calculated by dividing the cumulative feed intake (CFI) of each pig during the treatment feeding period to 28 days (weeks 2-6). Body weight gain (BWG) to feed intake ratio considered as gain to feed ratio (G:F) and BWG to protein intake ratio defined as gain to protein ratio (G:P) were computed by dividing the overall weight gain to cumulative feed intake or cumulative protein intake, respectively. The BWG, CFI, cumulative protein intake (CPI), and G:F and G:P ratios were calculated weekly.

2.3. Feed, fecal, and blood samples collection

Approximately 500 g of feed samples were collected after mixing each diet and stored at -20 ˚C for further analysis. Fecal samples were collected at weeks 5 and 6 of the study by transferring the pigs to metabolic crates. Each pig was housed in a metabolic crate for two consecutive days with free access to feed and water. Collected fecal samples in plastic bags were pooled and stored at -20 ˚C for nutrients digestibility tests.

At the end of the study (week 6), blood samples were drawn from the anterior vena cava (jugular) of each pig in the supine position using a 20-gauge vacutainer needle in 10 mL sterile serum tubes (BD, Franklin Lakes, NJ). Blood samples were placed on ice after collection, transferred to the laboratory and centrifuged for 10 minutes at 4 ˚C and at 2,000 x *g* to collect the serum. The collected serum was stored at -80 ˚C until further analysis.

At week 6 of the study, fresh fecal samples were collected from the rectum of all pigs in the fecal collection tubes (Global Scientific, Wilmington, NC) as previously described [38,39], placed on ice, transferred to the laboratory, and stored at -80 ˚C for bacterial DNA extraction and sequencing.

2.4. Bone mineral density and bone mineral content analysis

At the end of the study, all pigs were euthanized via CO2 asphyxiation method and the whole carcass of each pig was scanned in ventral position with extended limbs by dual-energy X-ray absorptiometry (DXA) (Hologic, Discovery QDR Series, Bedford, MA) in the same day by the same operator as previously explained [23]. The DXA scanner provided the measurements of bone mineral density (BMD) and bone mineral content (BMC). The standards procedure of manufacturer was used for calibration of the scanner and to obtain the DXA scans and for analysis. were Previous studies have shown a high correlation between bone measurements using DXA and bone ash content in pigs suggesting that DXA can be used accurately for evaluation of bone and skeletal status through measuring the mineral content and density in live animals [41].

2.5. Proximate analysis of feed samples

All feed samples were analyzed by Servi-Tech laboratories (Dodge City, KS). As we previously described [38,39], experimental diets were analyzed for moisture, crude protein, crude fiber, crude fat, nitrogen, Ca, and P, and chromium using official methods of analysis of AOAC [42].

2.6. Nutrients apparent fecal digestibility

Fecal samples were analyzed for Ca, P, N, and chromium as indicated above for feed samples by Servi-Tech (Dodge City, KS). The apparent fecal digestibility (AFD) for Ca, P, and N were calculated for individual animals using the marker method, which is based on the differential concentrations of analyzed chromium (used as an external marker) and the nutrient in feed and feces, according to the following formula: AFD = 100 – (100 x (marker concentration in feeds/marker concentration in feces) x (nutrient concentration in feces / nutrient concentration in feed) [43].

2.7. *Fecal DNA isolation, amplicon sequencing, sequence data analysis and taxonomic classification*

The DNA from fecal samples was isolated using the QIAamp DNA stool mini kit (Qiagen, Inc., Germantown, MD) as previously described [38,39], and following the instructions of the manufacturer. Isolated DNA samples concentration and quality were determined (Epoch, Biotek; Winooski, VT) and stored at -80 ˚C. The samples with DNA concentration greater than 6 ng/μl with the OD 260/280 of 1.8-2 were used for PCR amplification and microbial amplicon sequencing (Novogene Corp., Sacramento, CA).

As we previously described [38,39], for amplicon sequencing, the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') and Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA) were used for amplifying the 16S rRNA V4 region by PCR. The identical volume of PCR products was mixed with 1X loading buffer containing SYBR green and loaded on 2% electrophoresis agarose gel for quality control and quantification. The PCR products were extracted from the agarose gel using GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, MA), and the sequencing library was prepared using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) per manufacturer's instructions. Index codes were

added and the library quality was determined by using the Qubit@2.0 Fluorometer (Thermo Scientific, Waltham, MA) and Agilent Bioanalyzer 2100 system. The library was then sequenced using the Illumina HiSeq 2500 platform (Illumina, Inc., San Diego, CA), and 250bp paired-end raw reads were generated.

For sequence data analysis, paired-end reads were assigned to samples based on their unique barcode and then the barcode and primer sequence was truncated. Paired-end reads were then merged using FLASH (V1.2.7,http://ccb.jhu.edu/software/FLASH/) [44] to obtain splicing sequences called raw tags. Using specific filtering conditions of QIIME (V1.7.0 **http://qiime.org/index.html**) [45], quality filtering was performed on the raw tags, which produced high-quality clean tags. To detect chimeric sequences and remove them [46], the tags were compared with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using UCHIME algorithm (UCHIME Algorithm , **http://www.drive5.com/usearch/manual/uchime_algo.html**) [47], and Effective Tags were obtained. The obtained Effective Tags were clustered by Uparse software (Uparse v7.0.100http://drive5.com/uparse/) [48] and assigned to Operational Taxonomic Units (OTU) based on at least 97% similarity.

For taxonomic classification, OTU representative sequences were classified using the QIIME-based wrapper of the Ribosomal Database Project (Version 2.2 , **http://sourceforge.net/projects/rdp-classifier/**) [49] and naive Bayesian classifier retrained on the Greengenes 16S rRNA gene database (**http://greengenes.lbl.gov/cgi-bin/nph-index.cgi**) [50], using a 0.80 confidence threshold. Rarefaction curves were generated through a random selection of certain sequencing data. Further, the beta diversity of fecal bacterial communities was assessed by Principal Coordinate Analysis (PCoA) and weighted and unweighted UniFrac methods [51] using QIIME (Version 1.7.0).

2.8. Serum osteocalcin, calcium, phosphorus, and alkaline phosphatase

Serum osteocalcin concentration was determined using a porcine enzyme-linked immunosorbent assay kit (Cloud-clone corp., Katy, Texas) according to the manufacturer's instructions. The absorbance values were measured using a microplate reader (Epoch from Biotek, Winooski, VT) at 450 nm. Serum Ca, P, and alkaline phosphatase (ALP) were analyzed using a chemical chemistry analyzer (Carolina Liquid Chemistry, Brea, California). Prior to analyzing the samples, the analyzer was tested using the calibrator as instructed by the manufacturer.

2.9. Statistical analysis

The sample size was calculated with Al-Therapy Statistics (https://www.ai-therapy.com/psychology-statistics/sample-size-calculator) using data from our previous study [38]. For 8 nursery pigs/group, a difference in BW of 7.90 kg (SD: control diet=4.27 kg, low protein diet=4.26 kg) can be detected with 93% power (α=0.05; effect size=1.852). Overall growth performance, bone measurements, minerals digestibility, and concentration of serum metabolites and hormones data were analyzed using the univariate ANOVA procedure of SPSS® (IMB SPSS Statistics version 23, Armonk, NY, USA). Repeated measures on weekly growth performance were analyzed with a linear mixed model. Diet, time, and the interaction of diet and time were included in the model as fixed, and the animal was a random variable. Based on the smallest values of fit statistics for corrected Akaike's Information Criterion and Bayesian Information Criterion, the covariance structure of the repeated measurements for each variable was modeled as either first-order antedependence, autoregressive, heterogenous autoregressive, compound symmetry, heterogenous compound symmetry, or toeplitz. Means of dietary groups were separated by Tukey's post hoc analysis. $P \leq 0.05$ and $0.05 < P \leq 0.1$

were considered as statistical significance and trends, respectively. For quantitative analysis of gut microbiota composition within dietary groups, linear discriminant analysis (LDA) with effect size measurements (LEfSe) was used using a tool hosted in the Galaxy (server) instance of Huttenhower lab (https://huttenhower.sph.harvard.edu/galaxy/) and the scores were normalized by log10. The populations with LDA score (log10) > 2 were considered as bacterial with markedly increased numbers. To determine the significantly different beta diversity among dietary groups pairwise Wilcoxon test was performed with differences being considered significant at P value ≤ 0.05.

3. Results

3.1. Body weight, feed intake and feed efficiency

The initial BW was not different ($P = 0.56$) among groups, with an average BW of 10.18 kg for experimental groups (Table 2). The overall effect of diet on final BW, ADG, ADFI, G:F and G:P was significant ($P \leq 0.05$). Pigs fed positive PC had higher final BW, ADG, ADFI, and G:F than other groups (Table 2). Relative to NC, pigs fed LD and HDR tended to have 26% higher BW. Pigs fed LD and HDR had 84% and 80% higher ADG than the NC, respectively. In addition, pigs fed with LDR tended to have a 66 % higher ADG than those fed with NC (Table 2). No differences were detected on final BW and ADG when CEP supplemented groups with reduced Ca and P (*i.e.* LDR and HDR) were compared to the CEP supplemented diets with adequate Ca and P (*i.e*. LD and HD). Similarly, there were no differences in final BW and ADG when the groups with two doses of CEP (*i.e.* LD vs. HD or LDR vs. HDR) were compared (Table 2). Except week 1, there was no difference in BWG of PC and LD, positive control had higher BWG compared to all other treatments during the study (Table 3). In the second week, pigs in HDR group gained 129% more weight than those in NC group (Table 3).

Table 2. Overall growth performance and bone minerals of pigs fed with low-protein diets supplemented with a corn-expressed phytase**.**

	Diets[1]							
Item	**PC**	**NC**	**LD**	**HD**	**LDR**	**HDR**	**SEM[2]**	***P*-value**
Initial BW, kg	9.85 ± 2.02	10.21 ± 1.39	10.15 ± 1.66	10.32 ± 0.99	10.10 ± 0.58	10.44 ± 2.23	0.21	0.56
Final BW, kg	28.60 ± 2.40^{a}	14.87 ± 2.68$^{b*\#}$	18.73 ± 3.82 ^{b*}	17.53 ± 1.13^{b}	17.82 ± 2.02 ^{b}	18.80 ± 3.76$^{b\#}$	0.73	< 0.01
ADG[3], g/d	669 ± 81^{a}	166 ± 80^{c*}	306 ± 101^{b}	257 ± 53^{bc}	276 ± 78^{bc*}	299 ± 80^{b}	26	< 0.01
ADFI[3], g/d	953 ± 64^{a}	602 ± 174^{b}	714 ± 20^{b}	654 ± 141^{b}	617 ± 118^{b}	633 ± 48^{b}	23	< 0.01
G:F[3], g/g	0.70 ± 0.13^{a}	0.28 ± 0.11$^{b*\#\$}$	0.43 ± 0.14^{b*}	0.39 ± 0.09^{b}	0.45 ± 0.12$^{b\#}$	0.47 ± 0.08$^{b\$}$	0.02	< 0.01
G:P [3], g/g	3.05 ± 0.42^{ab}	2.25 ± 0.67^{a*}	3.50 ± 1.12^{ab*}	3.31 ± 1.09^{ab}	3.31 ± 1.21^{ab}	3.91 ± 0.79^{b}	0.15	0.03
BMD[4], g/cm^{2}	0.64 ± 0.06$^{ac\#}$	0.53 ± 0.03^{b*}	0.61 ± 0.04^{bc*}	0.62 ± 0.04^{cd}	0.56 ± 0.04$^{bcd\#}$	0.63 ± 0.09^{cd}	0.01	0.01
BMC[5], g	378.92 ± 64^{ac}	287.33 ± 36^{b}	371.67 ± 63^{cd}	344.14 ± 57^{bc}	292.02 ± 19^{b}	326.84 ± 22^{bc}	9.21	< 0.01

[1]PC (positive control): normal protein, adequate Ca and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at a low dose, i.e. 2,000 FTU/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced calcium (Ca) and phosphorus (P); HDR: HD with reduced Ca and P. Values are means ± standard deviations. n=8 for each dietary group.

[2]SEM: standard errors of means

[3]ADG: average daily gain; ADFI: average daily feed intake; G:F: gain:feed; G:P: gain:protein

[4]BMD: Bone mineral density

[5]BMC: Bone mineral content

[a-d]Within a row, values without a common superscript letter differ ($P \leq 0.05$)

[*#$]Within a row, values with a common superscript symbol are tended to be different ($0.05 < P \leq 0.1$).

The ADFI of pigs fed with NC was not different compared to groups fed LD, HD, LDR and HDR (Table 2). The ADFI of treatments LD and HD did not differ compared to LDR and HDR, respectively. Further, the ADFI was not different for LD vs. HD and LDR vs. HDR (Table 2). CFI and CPI were significantly higher for PC pigs than those in other treatments during this whole study (Table 3). Pigs fed LD tended to have 25% and 23% higher CFI than NC and LDR, respectively, during the first week of the study (Table 2).

The LD, LDR, and HDR treatments tended to have a 53%, 61%, and 68% higher G:F than NC (Table 2). The LD also tended to have a 55% higher G:P and HDR had a 74% higher G:P compared to NC. LDR and HDR were not different from LD and HD on their G:F and G:P, respectively (Table 2). Also, when two different doses of CEP used were compared (*i.e.* LD vs. HD and LDR vs. HDR), no differences in G:F and G:P were detected. In the first week of the study, although pigs in groups NC tended to have lower G:F than those in group PC, no differences in G:F was detected when groups LD, HD, LDR, and HDR were compared with PC (Table 3). In week 2, except HDR that was not different from PC, all other groups had lower G:F than PC. In week 3, pigs fed with LD, HD and HDR were not different in G:F from those fed PC. At week 4, although NC had lower G:F than PC, none of the other groups showed a significant difference in G:F compared to PC. Further, LDR and HDR pigs had 156% and 170% higher G:F than those fed with NC. In weeks 1 and 3, no differences in G:P were detected among dietary groups when G:P was assessed on a weekly basis. In week 2, the HDR and in week 4, the LDR and HDR had a 132% and 172% higher G:P compared to NC, respectively (Table 3).

Table 3. Weekly growth performance of pigs fed with low-protein diets supplemented with a corn-expressed phytase.

	Diets[1]							
Item	**PC**	**NC**	**LD**	**HD**	**LDR**	**HDR**	**SEM[2]**	***P-value***
BWG [3], g								
Wk 1	3242 ± 1515[a]	851 ± 511[b]	2157 ± 1157[ab]	1192 ± 872[b]	1475 ± 1022[b]	1232 ± 934[b]	185	< 0.001
Wk 2	4994 ± 741[a]	1021 ± 630[b]	1589 ± 594[bc]	1702 ± 321[bc]	1702 ± 1131[bc]	2334 ± 1241[c]	220	< 0.001
Wk 3	4994 ± 945[a]	1646 ± 415[a]	2724 ± 1372[b]	1816 ± 908[b]	1645 ± 1110[b]	1816 ± 1112[b]	224	< 0.001
Wk 4	5513 ± 714[a]	1135 ± 1864[b]	2100 ± 1349[b]	2497 ± 1329[b]	2894 ± 1371[b]	2983 ± 1591[b]	278	< 0.001
CFI [3], g								
Wk1	5714 ± 80[a]	3746 ± 1063[b#]	4665 ± 158[b#$]	3950 ± 799[b]	3768 ± 688[b$]	4190 ± 375[b]	135	< 0.001
Wk 2	6557 ± 458[a]	3995 ± 1183[b]	4937 ± 169[b]	4461 ± 928[b]	4256 ± 785[b]	4404 ± 376[b]	161	< 0.001
Wk 3	6726 ± 550[a]	4222 ± 1317[b]	4880 ± 105[b]	4574 ± 1014[b]	4381 ± 956[b]	4248 ± 324[b]	171	< 0.001
Wk 4	7686 ± 897[a]	4903 ± 1363[b]	5522 ± 417[b]	5335 ± 1481[b]	4881 ± 942[b]	4884 ± 631[b]	203	< 0.001
CPI [3], g								
Wk 1	1378 ± 18[a]	509 ± 144[b]	630 ± 21[b]	541 ± 109[b]	516 ± 94[b]	587 ± 52[b]	44	< 0.001
Wk 2	1491 ± 93[a]	479 ± 142[b]	588 ± 20[b]	544 ± 113[b]	562 ± 103[b]	511 ± 43[b]	51	< 0.001
Wk 3	1526 ± 113[a]	507 ± 158[b]	581 ± 12[b]	558 ± 123[b]	578 ± 126[b]	493 ± 37[b]	53	< 0.001
Wk 4	1716 ± 149[a]	588 ± 163[b]	657 ± 49[b]	651 ± 180[b]	644 ± 124[b]	567 ± 73[b]	59	< 0.001
G:F [3], g/g								
Wk 1	0.56 ± 0.26[*]	0.23 ± 0.16[*]	0.46 ± 0.25	0.30 ± 0.24	0.39 ± 0.23	0.29 ± 0.21	0.36	0.10

Wk 2	0.76 ± 0.15[a]	0.26 ± 0.15[b]	0.32 ± 0.12[b]	0.38 ± 0.14[b]	0.40 ± 0.23[b]	0.53 ± 0.25[ab]	0.35	< 0.001
Wk 3	0.74 ± 0.14[a]	0.40 ± 0.09[b]	0.56 ± 0.27[ab]	0.40 ± 0.27[ab]	0.38 ± 0.21[b]	0.43 ± 0.26[ab]	0.36	0.02
Wk 4	0.72 ± 0.13[a]	0.23 ± 0.25[bc]	0.38 ± 0.23[acd]	0.47 ± 0.23[ac]	0.59 ± 0.35[ad]	0.62 ± 0.24[ad]	0.42	< 0.001
G:P [3], g/g								
Wk 1	2.36 ± 1.08	1.67 ± 1.23	3.42 ± 1.90	2.20 ± 1.76	2.86 ± 1.97	2.10 ± 1.52	0.24	0.36
Wk 2	3.35 ± 0.67[ab]	2.13 ± 1.25[a]	2.70 ± 1.03[ab]	3.13 ± 1.19[ab]	3.03 ± 1.96[ab]	4.58 ± 2.21[b]	0.23	0.11
Wk 3	3.27 ± 0.64	3.25 ± 0.82	4.69 ± 0.2.27	3.25 ± 2.27	2.85 ± 1.65	3.68 ± 2.26	0.26	0.43
Wk 4	3.21 ± 0.58[ab]	1.93 ± 3.13[a]	3.20 ± 1.95[ab]	3.84 ± 1.89[ab]	4.49 ± 2.69[b]	5.26 ± 2.14[b]	0.36	0.03

[1]PC (positive control): normal protein, adequate Ca and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 FTU/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced calcium (Ca) and phosphorus (P); HDR: HD with reduced Ca and P.

The *P* values for the overall model effects for diet, week and diet × week for BWG were < 0.01, < 0.01 and 0.03, respectively, for CFI were < 0.01, < 0.01 and < 0.01, respectively, for CPI were < 0.01, < 0.01 and < 0.01, respectively, for G:F were < 0.01, 0.01 and 0.11, respectively, and for G:P were < 0.01, 0.05 and 0.22, respectively. Values are means ± standard deviations. *n=8* for each dietary group.

[2]SEM: Standard error of the mean

[3]BWG: body weight gain; CFI: cumulative feed intake; CPI: cumulative protein intake; G:F: gain:feed; G:P: gain:protein.

[a-c] Within a row, values without a common superscript letter differ ($P \leq 0.05$)

[*#$] Within a row, values with a common superscript symbol tend to be different ($0.05 < P \leq 0.1$).

3.2. Bone mineral content and density

Pigs fed with NC had significantly lower BMD compared to PC; however, none of the CEP supplemented groups were different from PC in BMD (Table 2). Pigs in LD group tended to have higher BMD and the ones in HD and HDR had significantly (17% and 18%) higher BMD than those in NC group, respectively. No differences in BMD were detected for: LD vs. HD, LDR vs. HDR, LD vs. LDR and HD vs. HDR. NC and LDR had lower BMC than PC; however, no differences in BMC were detected when LD, HD and HDR were compared to PC. Further, LD had 29% and 27% higher BMC than NC and LDR, respectively. No differences in BMC were detected when LD vs. HD, LDR vs. HDR and HD vs. HDR were compared (Table 2).

3.3. Apparent fecal digestibility of calcium, phosphorus, and nitrogen

The AFD of Ca was not different between PC and NC groups. HD, LDR and HDR had higher AFD of Ca than PC (Figure 1A and Table S1). The AFD of Ca for HD and HDR was higher than NC (Figure 1A). The AFD of Ca for Ca- and P-deficient diets supplemented with CEP (*i.e.* LDR and HDR) was not different compared to that for Ca and P-adequate diets supplemented with CEP (*i.e.* LD and HD). Further, the AFD of Ca was not different for LD vs. HD and LDR vs. HDR comparisons.

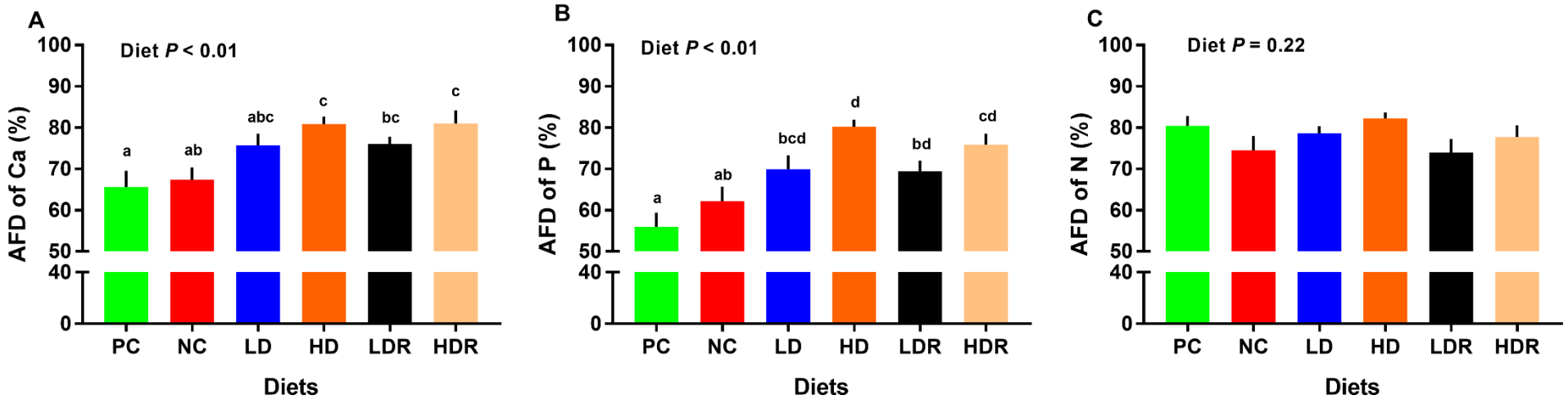

Figure 1. Nutrients' digestibility in pigs fed with low-protein diets supplemented with a corn-expressed phytase. (**A**) Apparent fecal digestibility (AFD) of calcium (Ca), (**B**) AFD of phosphorus (P), (**C**) AFD of nitrogen (N). Pigs are grouped based on their dietary treatments: PC (positive control): normal protein, adequate Ca and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 FTU/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced calcium (Ca) and phosphorus (P);

HDR: HD with reduced Ca and P. The values are means ± standard errors of means. Different letters in the bar plots indicate significant differences ($P \leq 0.05$, Tukey's test). *n=8* for each dietary group.

All experimental groups (*i.e.* LD. HD, LDR and HDR) had higher AFD of P compared to PC (Figure 1B and Table S1). Also, a significant increase in AFD of P was observed for HD and HDR compared to NC, while the LDR and HDR were not different compared to LD and HD, respectively. Additionally, the AFD of P for LD v. HD and LDR vs. HDR was not different (Figure 1B). No differences across treatments were detected for AFD of N (Figure 1C and Table S1).

3.4. Serum osteocalcin, calcium, phosphorus, and alkaline phosphatase

Compared to PC, pigs fed diets with NC and LD had significantly higher ($P < 0.01$) concentration of serum osteocalcin (Figure 2A). Relative to NC, no differences in serum osteocalcin were observed in LD, HD and LDR, but pigs fed with HDR had significantly lower serum osteocalcin ($P < 0.01$). There was no difference in serum osteocalcin when LD vs. LDR, HD vs. HDR and LDR vs. HDR were compared (Figure 2A). No differences among dietary groups were detected for serum Ca, P and ALP concentrations (Figure 2B-D). Serum ALP tended to be significantly higher for HDR relative to PC (Figure 2D).

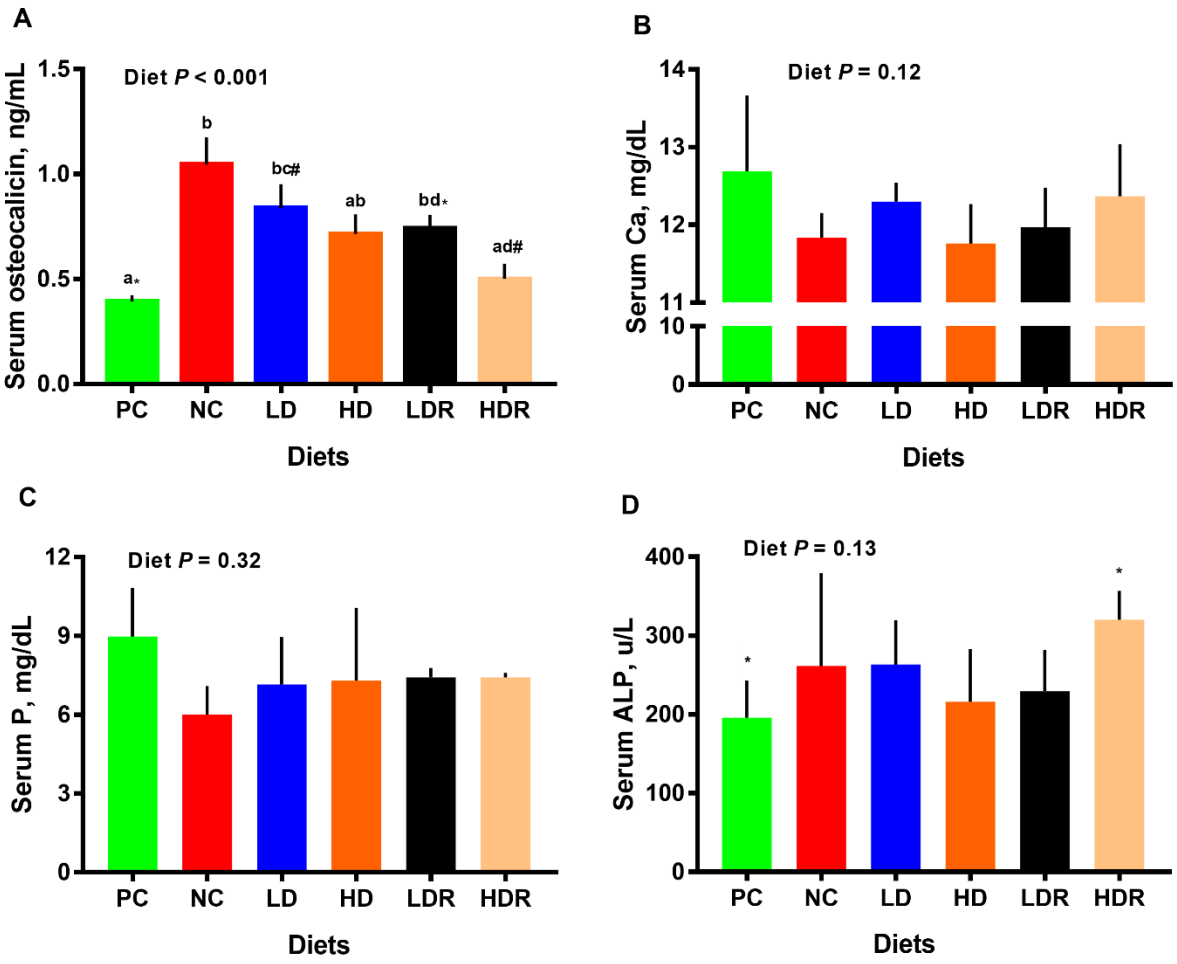

Figure 2. Serum metabolites and hormones in pigs fed with low-protein diets supplemented with a corn-expressed phytase. (**A**) serum osteocalcin, (**B**) serum calcium (Ca), (**C**) serum phosphorus (P), (**D**) serum alkaline phosphatase (ALP). Pigs are grouped based on their dietary treatments: PC (positive control): normal protein, adequate Ca and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 FTU/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced calcium (Ca) and phosphorus (P); HDR: HD with reduced Ca and P. The values are means ± standard errors of means. Different letters in the bar plots indicate significant differences ($P \leq 0.05$, Tukey's test) and

common superscript symbols indicate a trend ($0.05 < P \leq 0.1$). *n=7-8* for each dietary group.

3.5. Fecal microbiota

As shown in the rarefaction curve analysis, at 30,000 reads and 600-1,200 OTUs, all fecal samples analyzed reached a stable plateau (Figure S1A and S1B) suggesting a sufficient sequencing depth for capturing the species richness of the samples assessed.

PCoA and unweighted and weighted UniFrac distances are shown in Figure 3A-D. There was a significant difference in the beta diversity of bacterial communities between CEP supplemented groups (*i.e.* groups LD, HD, LDR and HDR) with NC and PC when unweighted UniFrac distances analysis was applied (Figure 3B). No differences in beta diversity were seen for PC vs. NC, LD vs. LDR, HD vs. HDR and LDR vs. HDR, but there was a difference between LD and HD (Figure 3B). When weighted UniFrac distances analysis was applied, beta diversity was significantly different between HD and PC (Figure 3D). LD and LDR had significantly different beta diversity than NC, but no differences were detected for NC vs. HD and NC vs. HDR. Further, LD and LDR was different when compared to HD and HDR, respectively (Figure 3D).

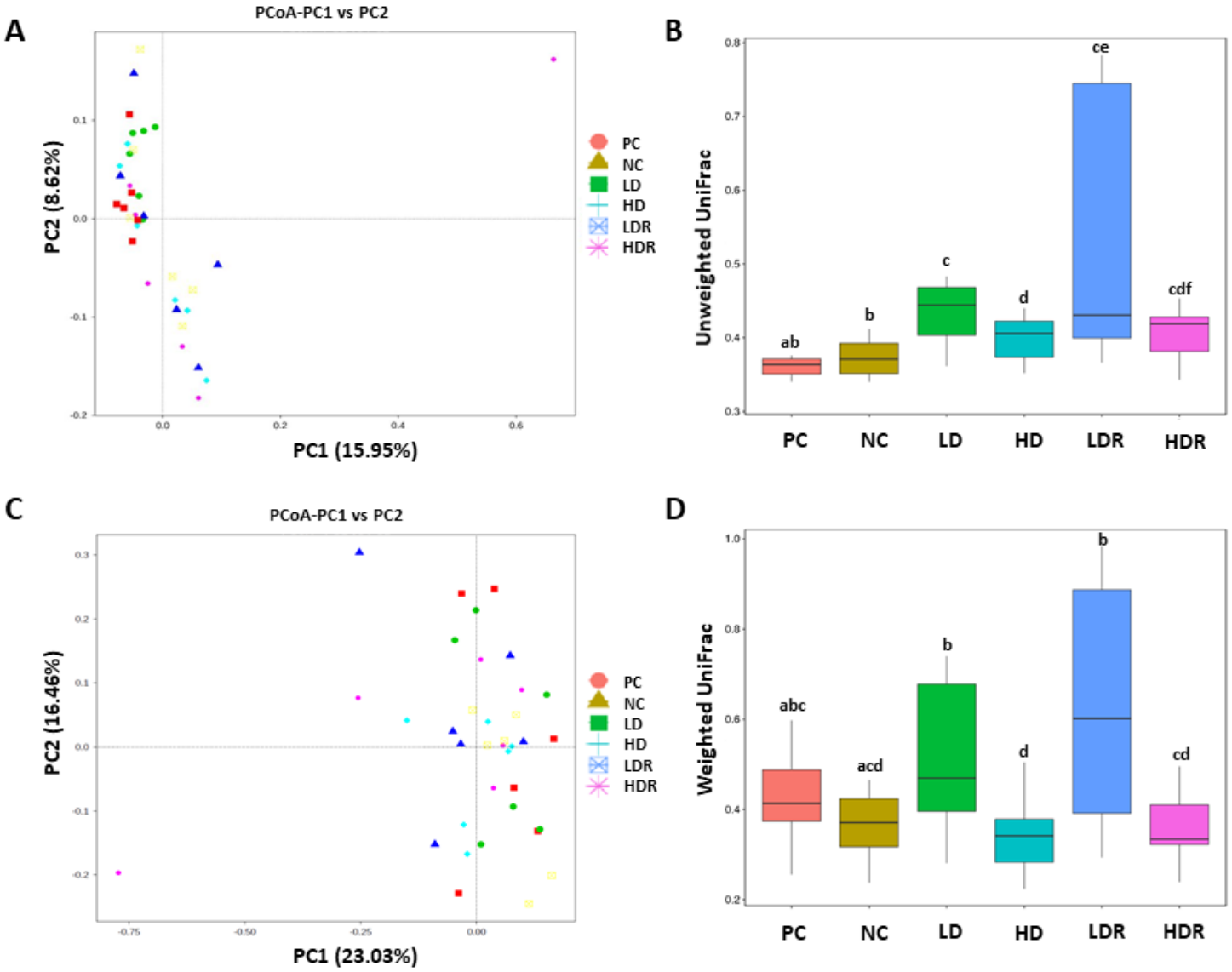
A
PCoA-PC1 vs PC2
PC2 (8.62%)
PC1 (15.95%)
PC
NC
LD
HD
LDR
HDR
B
Unweighted UniFrac
ab
b
c
d
ce
cdf
PC
NC
LD
HD
LDR
HDR
C
PCoA-PC1 vs PC2
PC2 (16.46%)
PC1 (23.03%)
PC
NC
LD
HD
LDR
HDR
D
Weighted UniFrac
abc
acd
b
d
b
cd
PC
NC
LD
HD
LDR
HDR

Figure 3. Beta diversity of fecal bacterial community in pigs fed with low-protein diets supplemented with a corn-expressed phytase. (**A**) Principal Coordinates Analysis (PCoA) of unweighted UniFrac distances showing the diversity of fecal microbiota across individual animals assigned to 6 dietary groups. Each node represents an individual pig, (**B**) Unweighted UniFrac distances shown for each dietary group, (**C**) PCoA of weighted UniFrac distances showing the diversity of fecal microbiota across animals assigned to 6 dietary groups. Each node represents an individual pig, (**D**) Weighted UniFrac distances shown for each dietary group. Pigs are grouped based on their dietary treatments: PC (positive control): normal protein, adequate Ca and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 FTU/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced calcium (Ca) and phosphorus (P); HDR: HD with reduced Ca and P. The values are means ± standard errors. Different letters in the box plots indicate significant differences ($P \leq 0.05$, Wilcoxon test). *n=6* for each dietary group.

Overall, the three main phyla in all dietary groups were Firmicutes, Bacteriodetes, and Proteobacteria (Figure 4A). The most abundant bacterial community at phylum level for all six groups was Firmicutes (Figure 4A). At genus level, *Succinivibrio, Prevotella*, *Lactobacillus*, *Megasphaera,* and *Ruminococcaceae-UCG-002* were the most abundant bacteria across all diets (Figure 4B). There were differential differences among groups on the abundance of *Streptococcus, Treponema, Bifidobacterium, Clostridium*, and *Succiniclasticum* (Figure 4B).

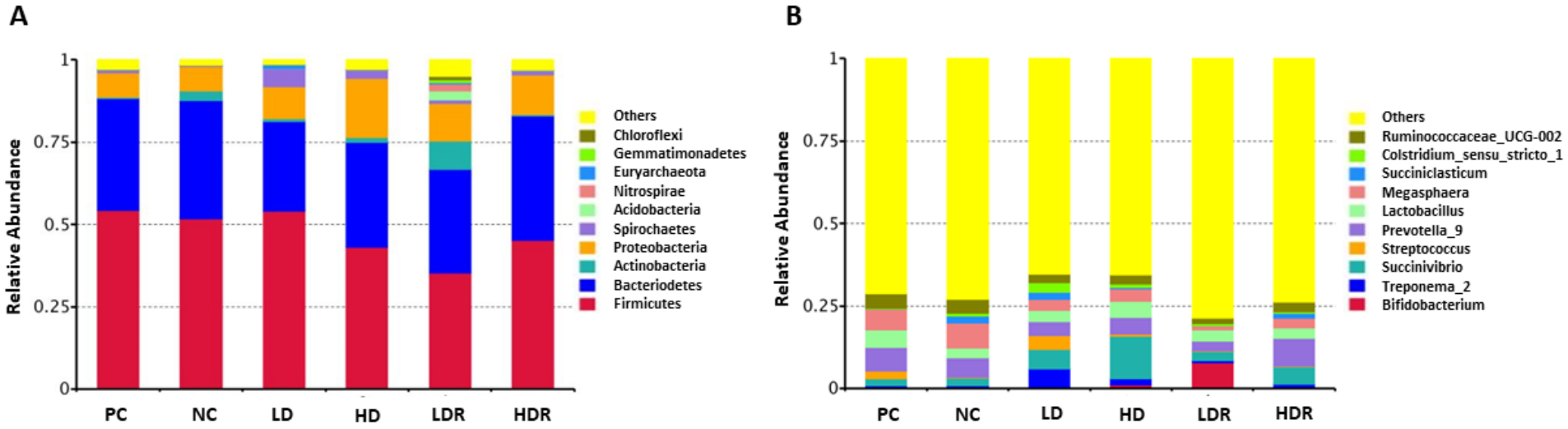

Figure 4. The fecal bacterial composition in pigs fed with low-protein diets supplemented with a corn-expressed phytase. (**A**) the relative abundance of bacterial community composition at phylum level, (**B**) the relative abundance of bacterial community composition at genus level. Pigs are grouped based on their dietary treatments: PC (positive control): normal protein, adequate Ca and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 FTU/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced calcium (Ca) and phosphorus (P); HDR: HD with reduced Ca and P. Only the top 10 phyla and genera are shown for clarity. n=6 for each dietary group.

To identify the differences in gut bacterial abundances across dietary groups, LDA with LEfSe was performed (Figure 5). Pigs fed NC had a higher proportion of family Erysipelotrichaceae and family Coriobacteriaceae compared to PC that had a higher abundance of family Lachnospiraceae (LDA [log_{10}] score > 2.0; Figure 5A). Pigs fed with LD had a higher abundance of family Erysipelotrichaceae compared to PC (Figure 5B). Compared to PC, the feces of HD pigs was more enriched in genus *Succinvibrio*, whereas PC pigs had a higher abundance of family Lachnospiraceae (Figure 5C). The feces of pigs fed with LDR was enriched in phylum Actinobacteria, genus *Bifidobacterium*, phylum Acidobacteria, class Deltaproteobacteria, and genus *Nitrospira,* whereas pigs fed with PC had a higher abundance of genus *Streptococcus* and genus *Megasphaera* (Figure 5D). Compared to PC, class Deltaproteobacteria and genus *Desulfovibrio* were predominant in feces of group HDR (Figure 5E). Compared to NC, group LD had a higher abundance of family Lachnospiraceae (Figure 5F). Feces of pigs fed with treatment HD were enriched in family Spirochaetaceae, whereas NC had a higher proportion of family Acidaminococcaceae (Figure 5G). Compared to LDR, the pigs in group LD had higher numbers of genus *Streptococcus* in their feces (Figure 5H). Pigs fed with LDR had a higher abundance of genus *Bifidobacterium* and phylum Actinobacteria compared to those fed with HDR, which had more family *Acidaminococcaceae* (Figure 5I).

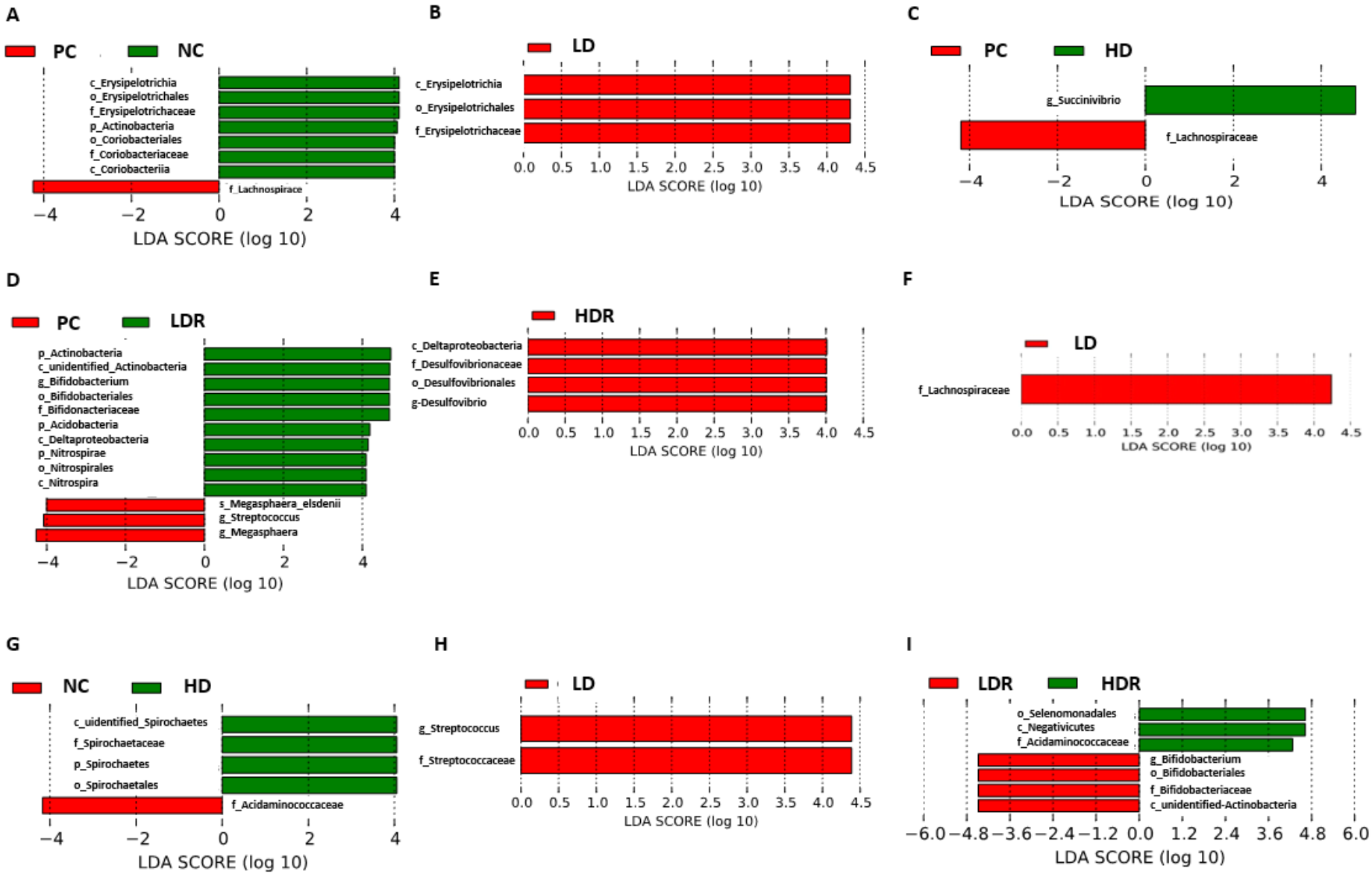
A
PC
NC
c_Erysipelotrichia
o_Erysipelotrichales
f_Erysipelotrichaceae
p_Actinobacteria
o_Coriobacteriales
f_Coriobacteriaceae
c_Coriobacteriia
f_Lachnospirace
−4 −2 0 2 4
LDA SCORE (log 10)
B
LD
c_Erysipelotrichia
o_Erysipelotrichales
f_Erysipelotrichaceae
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
LDA SCORE (log 10)
C
PC
HD
g_Succinivibrio
f_Lachnospiraceae
−4 −2 0 2 4
LDA SCORE (log 10)
D
PC
LDR
p_Actinobacteria
c_unidentified_Actinobacteria
g_Bifidobacterium
o_Bifidobacteriales
f_Bifidonacteriaceae
p_Acidobacteria
c_Deltaproteobacteria
p_Nitrospirae
o_Nitrospirales
c_Nitrospira
s_Megasphaera_elsdenii
g_Streptococcus
g_Megasphaera
−4 −2 0 2 4
LDA SCORE (log 10)
E
HDR
c_Deltaproteobacteria
f_Desulfovibrionaceae
o_Desulfovibrionales
g-Desulfovibrio
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
LDA SCORE (log 10)
F
LD
f_Lachnospiraceae
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
LDA SCORE (log 10)
G
NC
HD
c_uidentified_Spirochaetes
f_Spirochaetaceae
p_Spirochaetes
o_Spirochaetales
f_Acidaminococcaceae
−4 −2 0 2 4
LDA SCORE (log 10)
H
LD
g_Streptococcus
f_Streptococcaceae
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
LDA SCORE (log 10)
I
LDR
HDR
o_Selenomonadales
c_Negativicutes
f_Acidaminococcaceae
g_Bifidobacterium
o_Bifidobacteriales
f_Bifidobacteriaceae
c_unidentified-Actinobacteria
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

Figure 5. Fecal microbiota composition histograms in pigs fed with low-protein diets supplemented with a corn-expressed phytase. Histograms of linear discriminant analysis (LDA) with effect size (LEfSe) on fecal microbiota composition (**A**) PC vs. NC, (**B**) PC vs. LD, (**C**) PC vs. HD, (**D**) PC vs. LDR, (**E**) PC vs. HDR, (**F**) NC vs. LD, (**G**) NC vs. HD, (**H**) LD vs. LDR, (**I**) LDR vs. HDR. Pigs are grouped based on their dietary treatments: PC (positive control): normal protein, adequate Ca and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 FTU/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced calcium (Ca) and phosphorus (P); HDR: HD with reduced Ca and P. There was no significant difference on species identified for C vs. D and D vs. F. n=*6* for each dietary group.

4. Discussion

Very low-protein and P deficient diets could be potentially used to reduce the environmental concerns associated with excretion of nutrients and feed cost in swine production. However, these diets have negative influence on the growth performance and health of pigs [9–12]. The objective of the current study was to investigate whether supplementation of very low-protein, -Ca and –P diets with a CEP would improve growth, digestibility of Ca, P and N, bone mineral density and content, serum metabolites associated with Ca and P metabolism, and fecal microbiota composition in nursery pigs. This study revealed several important findings: 1) severe reduction of dietary CP depressed the BW, ADG, ADFI, G:F and G:P; however, supplementation of these diets with CEP regardless of the doses used (*i.e.* 2,000 and 4,000 FTU/kg) with or without reduction in dietary Ca and P improved ADG and G:P ratio and tended to increase the final BW; 2) very low protein diets supplemented with CEP increased the AFD of Ca and P with or without reduction in dietary Ca and P, with a dose response of CEP on AFD of Ca and P; 3) reduction of dietary CP, decreased the BMD and BMC, but this decrease was completely recovered by supplementing the CEP to Ca and P-adequate diets; 4) in Ca- and P-deficient diets, supplementation of CEP at lower dose (*i.e.* 2,000 FTU/kg of diet) did not improve the BMC, but both high (*i.e.* 4,000 FTU/kg of diet) and low doses of added CEP completely reversed the negative effects of very low protein diets on BMD, 5) reduction in dietary CP increased the serum concentration of osteocalcin, but supplementing these diets with CEP at 4,000 FTU/kg of diet completely reversed the osteocalcin concentration in Ca- and P-deficient diet; 6) reducing the dietary CP content increased the abundance of families Erysipelotrichaceae, Acidaminococcaceae and Coriobacteriaceae, but supplementing a CEP at 2,000 FTU/kg increased the family Lachnospiraceae and at 4,000 FTU/kg increased the genus *Succinvibrio* in Ca and P-adequate diets. Supplementing phytase at 2,000 FTU/kg in Ca and P-deficient diets increased the genus *Bifidobacterium* and phylum Actinobacteria. Overall,

supplementation of very low protein diets with a CEP decreased the negative effects of these diets on average daily gain and protein efficiency ratio and increased the total tract digestibility of Ca and P regardless of the levels of Ca and P in the diet, improved bone characteristics and produced differential effects on fecal bacterial population.

Very low protein diet decreased the feed efficiency and growth performance of pigs, which was similar to previous studies [9–11]. The beneficial effects of microbial phytase as a feed additive on the growth performance of pigs fed with amino acids-deficient diets have been documented previously [28,29], but it is unknown whether the negative effects of low protein diets can be mitigated by using phytase produced in transgenic plants. Although both doses of CEP (i.e. 2,000 and 4,000 FTU/kg) showed some promising effects on certain parameters, 4,000 FTU/kg CEP seems to have added benefits. Using CEP as a supplement in very low protein diets, regardless of the doses applied, improved the ADG and G:P ratio and tended to increase the final BW; completely recovered the reduced BMD and BMC when dietary Ca and P was adequate and fully reversed the negative effects of very low protein diets on BMD when Ca and P was deficient. Compared to 2,000 FTU/kg CEP, using 4,000 FTU/kg CEP produced greater improvements on AFD of Ca and P, improved BMC in Ca- and P-deficient diets and completely reversed the increased serum osteocalcin concentration in Ca- and P-deficient diet when very low protein diets are fed.

For the first time, here we show that the negative outcome of protein-deficient diets on daily weight gain, final body weight and protein efficiency ratio of pigs is alleviated when these diets are supplemented with a CEP with no adverse effects of reduced dietary Ca and P. Similar positive effects for microbial phytase [20-25] and a CEP [34,35] were reported in the nursery, growing and finisher pigs fed with adequate protein but Ca and P deficient diets. The beneficial effect of CEP on the growth performance of pigs in both Ca and P adequate and deficient diets might be due to improvement in utilization of Ca, P, and other nutrients [34,35]. Our data showed that very low

protein diets supplemented with CEP at 4,000 FTU/kg had a higher AFD of Ca and P. Similarly, previous studies showed that digestibility and utilization of P, Ca and CP or amino acids were increased in pigs when microbial phytase was supplemented in the diets with adequate amounts of nutrients [16–18]. The data from this study provide evidence that a novel CEP can be used to improve the growth performance of pigs fed with reduced CP, Ca, and P possibly through improved utilization of these nutrients.

Severe reduction of dietary CP decreased bone mass density and bone mass content. The detrimental effects of low protein diets on bone mass in humans have been reviewed previously [52]. The reduced bone mass and density in pigs fed with low protein diets might be attributed to their effects on reducing the absorption of Ca and P and secondary hyperparathyroidism [52]. This decrease in bone mass density and content was accompanied by an increased concentration of osteocalcin. Osteocalcin is a bone gamma-carboxyglutamic acid-containing protein with 49 to 50 amino acid residues produced by the osteoblast and is considered as a measure of osteoblast activity [53-55]. Serum osteocalcin is negatively linked with bone mineralization in pigs receiving diets with varying levels of Ca and P [56]. The high concentration of osteocalcin in pigs fed with very low protein diets may be suggestive of less available Ca and P for bone mineralization in these animals.

When very low protein diets were supplemented with a CEP, their negative effects on bone parameters were completely reversed. In parallel with these results, others reported a positive effect of CEP on bone characteristics in protein adequate, but P deficient [35] or both Ca and P deficient diets [34]. Likewise, a linear improvement in bone parameters was observed by others when microbial phytase was added to the diets of pigs with reduced Ca and P [21,24,25,57,58]. Previously, using a dual-energy X-ray absorptiometry technique, it was shown that gilts received diets with no inorganic P but supplemented with 750 FTU/kg of microbial phytase had higher BMD

and BMC [23]. This positive effect on bone parameters can be attributed to the effect of phytase in facilitating the utilization of Ca and P, which are both required for optimal bone mineralization through building hydroxyapatite crystals [59]. In the current study, no effects of added phytase on serum Ca and P were observed; however, these diets depressed the serum concentration of osteocalcin which is suggestive of availability of Ca and P for bone mineralization [55,56,59]. Others reported a temporary drop in osteocalcin concentration in growing-finishing pigs when their diets were supplemented with a microbial phytase [60]. Our results are in line with previous reports in which no change in blood P concentration was observed when microbial phytase was used in the diet of nursery pigs [61]. However, other studies reported an increase in plasma Ca and P with supplementation of microbial phytase in diets of pigs [19,62-64].

Little is known whether the beneficial effects of CEP on growth performance are mediated by factors other than the stimulatory role of phytase in nutrients digestibility and utilization. There is some evidence on the modulatory influence of microbial phytase on intestinal microbiota in pigs [36,37] but there is a paucity of information on whether CEP can change the composition of gut organisms and hence influence the performance of pigs. This is while the importance of gut microbiota and feed efficiency in pigs has been reported [65]. The three main phyla in all dietary groups were Firmicutes, Bacteriodetes, and Proteobacteria, which is consistent with previous research where the above three populations were reported as the most dominant groups in porcine gut microbiota [66-69].

Here, for the first time, we showed that supplementing low protein diets with a CEP increased the family Lachnospiraceae and the genus *Succinvibrio* in Ca and P-adequate diets. Lachnospiraceae can produce a group of bioactive molecules that contribute to gut health [70]. Many members of this family can ferment various substrates and produce butyric acid [71]. Further, Lachnospiraceae has been reported as one of the abundant communities in pigs with the high feed conversion rate

[72], and their abundance was increased in healthy pigs compared to diarrheic pigs [73]. The increased abundance of Lachnospiraceae in pigs supplemented with phytase may be suggestive of better gut health in these animals as this family may protect the pigs from pathogen infections. *Succinvibrio* are mostly localized in the colon [74] and cecum [75] in swine and have fiber-degrading potential [76]. *Succinivibrio* species have not been well studied in monogastrics, but in ruminants they have been shown to be involved in hepatic gluconeogenesis via production of acetate and succinate required for propionate synthesis and improved feed efficiency [77,78]. Previous research has shown that *g. Succinivibrio* population is higher in the gut contents of pigs fed with wheat-based diet compared to those received the corn-based diet [79] and has been associated with backfat [80]. The *g. Succinivibrio* has been reported to be increased after weaning in pigs, which could be due to availability of cereal-based diet [81]. The Lachnospiraceae family along with *Succinvibrio*, may contribute to better growth performance in pigs receiving dietary phytase when the Ca and P level was adequate.

In the present study, supplementing a CEP in Ca and P-deficient diets increased the genus *Bifidobacterium* and phylum Actinobacteria. Bifidobacteria are saccharolytic organisms and use complex carbohydrates that escape the digestion in proximal intestine to be able to colonize in the gastrointestinal tract [81]. The higher abundance of Bifidobacteria in the gut microbiome of pigs received supplemented CEP is suggestive of higher carbohydrate utilization capacity, which may contribute to improved performance in these pigs. The increased abundance of *Bifidobacterium* has been linked with better gut health and improved feed conversion rate in broiler chickens [82] and rats [83], which might contribute to better animal performance in Ca and P-deficient groups supplemented with CEP in the current study. Actinobacteria members are capable of secreting microbial phytase which can hydrolyze the phytic acid, release P and increase P bioavailability for the pigs [84,85]. Therefore, the increased abundance of Actinobacteria in animals fed with low protein and deficient Ca/P diets supplemented with a CEP may help the function of exogenous

phytase used as a feed additive in making the P and Ca available and improving the growth performance as well as bone parameters.

5. Conclusions

In summary, supplementing very low protein diets with a corn-expressed phytase decreased the negative effects of very low protein diets on average daily gain, final body weight and protein efficiency ratio, and increased the total tract digestibility of Ca and P regardless of the levels of these minerals in the diet. Further, the negative effects of low protein diets on bone characteristics were completely recovered with supplementation of corn-expressed phytase when the dietary Ca and P levels were adequate. When dietary Ca and P were deficient the negative effects of low protein diets on bone characteristics were improved only at higher doses of added corn-expressed phytase, which was also accompanied by decreased osteocalcin concentration. Adding a corn-expresses phytase to low protein diets at a lower dose produced differential effects on the fecal bacterial population with increased family Lachnospiraceae, the genus *Bifidobacterium* and phylum Actinobacteria and at a higher dose enhanced the abundance of genus *Succinvibrio*. Thus, supplementation of very low protein diets with a corn-expressed phytase decreased the adverse effects of these diets on weight gain and protein efficiency ratio, increased the total tract digestibility of Ca and P, improved bone characteristics and produced diverse effects on fecal bacterial population. Overall, 4,000 FTU/kg phytase appeared to have added benefits compared to 2,000 FTU/kg phytase.

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Appendices

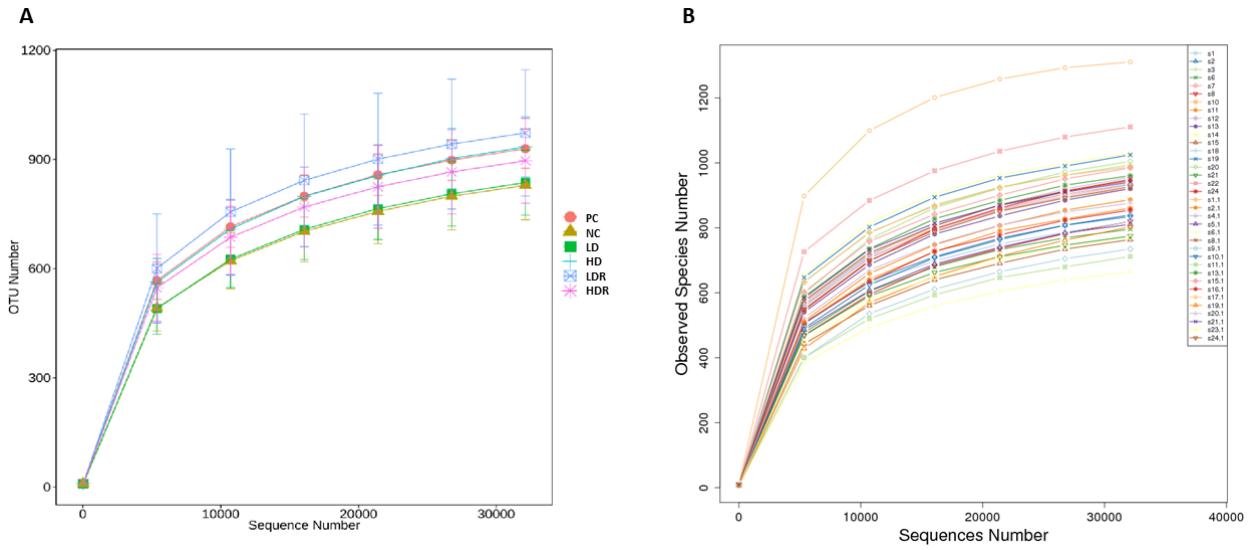

Figure S1. Fecal rarefaction curve analysis for pigs fed with low-protein diets supplemented with a corn-expressed phytase. The rarefaction curves from fecal samples collected show the number of operational taxonomic units (OTU's) found as a function of the number of reads sampled when data were analyzed based on (**A**) dietary groups and (**B**) individual pigs. Pigs are grouped based on their dietary treatments: PC (positive control): normal protein, adequate Ca and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 FTU/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced calcium (Ca) and phosphorus (P); HDR: HD with reduced Ca and P. Each line represents an individual pig. *n=6* for each dietary group

Table S1. Digestibility of calcium, phosphorous and nitrogen of pigs fed with low-protein diets supplemented with a corn-expressed phytase

	Diets[1]							
	PC	**NC**	**LD**	**HD**	**LDR**	**HDR**	SEM[2]	*P*-value
Calcium								
AFD[3], %	65.59 ± 10.34[a]	67.38±8.39[ab]	75.71±7.94[abc]	80.82±5.23[c]	76.03±4.68[bc]	80.97±8.53[c]	1.41	< 0.01
Phosphorus								
AFD[3], %	55.95±9.05[a]	62.21±9.82[ab]	69.90±9.55[bcd]	80.21±4.44[d]	69.42±6.63[bd]	75.87±7.04[cd]	1.67	< 0.01
Nitrogen								
AFD[3], %	80.42±6.32	74.50±9.99	78.61±4.83	82.23±3.74	73.98±8.53	77.70±7.57	1.11	0.22

[1]PC (positive control): normal protein, adequate Ca and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at a low dose, i.e. 2,000 FTU/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced calcium (Ca) and phosphorus (P); HDR: HD with reduced Ca and P. Values are means ± standard deviations. n=8 for each dietary group

[2]SEM: standard errors of means;

[3]AFD: Apparent fecal digestibility

[a-d]Within a row, values without a common superscript letter differ ($P < 0.05$).

CHAPTER IV

CORN-EXPRESSED PHYTASE MODULATES BLOOD AMINO ACIDS AND PROTEOMICS PROFILES IN NURSERY PIGS FED WITH LOW-PROTEIN, -CALCIUM, AND -PHOSPHOROUS DIETS

Abstract

Feeding pigs with very low-protein (VLP) and low-phosphorous (P) diets may be useful for decreasing the nutrient's excretion to the environment; however, this practice negatively impacts the animals' growth performance. A beneficial effect of corn-expressed phytase (CEP) on the growth performance of pigs fed with VLP diets was shown by our group recently. Little is known whether this improvement is related to alterations in the profile of blood proteins and amino acids (AA). The objective of this study was to investigate whether supplementation of VLP, low-calcium (Ca), and low-P diets with a CEP can influence the blood AA and proteomics profiles in pigs. Forty-eight weaned barrows were subjected into one of the following groups (n=8/group) for 4 weeks: positive control (PC), negative control-reduced protein (NC), NC+low-dose CEP, i.e. 2,000 FTU/kg (LD), NC+high-dose CEP, i.e. 4,000 FTU/kg (HD), LD with reduced Ca/P (LDR), and HD with reduced Ca/P (HDR). At week 4, blood samples were collected from all pigs. Compared to PC, NC reduced the plasma leucine and phenylalanine concentrations; however, LD recovered their levels. Using trypsinolysis and mass spectrometry, 703 serum proteins were identified and quantified, wherein 25 were found to be differentially expressed among groups.

Hierarchical clustering showed a clear separation in proteins identified among dietary groups. Compared to NC, 23 and 24 proteins were found to be differentially expressed in serum of LD and HD groups, respectively, with some important proteins in growth regulation such as SELENOP being upregulated and the IGFBP family being downregulated in these groups. A positive correlation was detected between growth and abundance of BGN, TLN1, PDLIM1, and COL1A2 that are involved in bone mineralization and muscle structure development. Thus, CEP improved the blood profile of some essential AA and affected the expression of proteins involved in the regulation of growth in pigs fed with VLP diets.

Keywords: very low-protein diet, corn-expressed phytase, amino acids, serum proteomics, pigs.

1. Introduction

Very low protein (VLP) diets with more than 25% reduced crude protein than suggested levels by nutrition requirement of swine (NRC, 2012) can be beneficial for animal health and decreasing the nutrients excretion and the incidence of diarrhea in pigs (Yue & Qiao, 2008; Lordelo et al., 2008; Manjarin et al., 2012). However, these diets, while supplemented with the first four limiting amino acids (AA; *i.e.* lysine, methionine, tryptophan, and threonine decrease the feed efficiency and growth performance of nursery (Nyachoti et al., 2006; Peng et al., 2016; Li et al., 2017) and growing pigs (Deng et al., 2007a; Manjarin et al., 2012). Further research is needed to develop effective and novel strategies to improve the growth performance of pigs fed with VLP diets so that they can be used in commercial swine production.

Microbial phytase supplemented to swine diets improve the digestibility of AA and protein utilization in pigs (Kies et al., 2005; Adeola & Sands 2003; She et al., 2018; Lu et al., 2019; Zeng et al., 2014, He et al., 2017). Therefore, supplemental phytase may have the potential to reduce the

need for high dietary protein content by improving the efficiency of protein utilization. Unlike microbial phytase, which is produced during the fermentation process, recombinant phytase is produced through its expression in transgenic plants. Recombinant phytase can be an economical alternative to microbial phytase due to the fact that large biomass of plants can express the transferred genes of phytase at a larger scale (Greiner et al., 2006). Supplementing normal protein, but low-phosphorus (P) diets with corn-expressed phytase (CEP) was shown to improve in average daily gain, P digestibility, and bone characteristics in the nursery (Broomhead et al., 2019) and growing pigs (Blavi et al., 2019). Importantly, we showed that supplementation of VLP diets with CEP, regardless of the doses used (i.e., 2000 and 4000 FTU/kg) also improved the average daily gain (ADG) and gain:protein (G:P) of weaned pigs (Shili et al., 2020).

In our previous study, we showed that the positive effect of CEP on the growth performance of pigs fed with VLP diets was associated with increased digestibility of calcium (Ca) and P, bone characteristics, and differential fecal bacterial population (Shili et al., 2020). There are some evidence that microbial phytase may improve the blood amino acid profile in broilers (Amerah et al., 2014; Moss et al., 2019). This improvement in the profile of amino acids has been shown to be linked with enhanced growth performance in pigs (Thongsong et al., 2019). Likewise, serum proteins such as heat-shock proteins can protect cells and play an important role in growth, development, differentiation, and other physiological activities (Arrigo and Simon, 2010). The serum proteome profile in pigs has been previously reported (Arrigo and Simon, 2010; Keshan Zhang, 2012), but no data is available on alterations in proteome profile when phytase is supplemented in the diet. Little is known whether profile of blood proteins and other nitrogen containing compounds such as amino acids are also related with enhanced growth performance of pigs fed with VLP diets supplemented with CEP. The objective of study was to investigate the effect of a CEP on blood metabolites, nitrogen containing compounds and serum proteomics profiles and body composition in nursery pigs with VLP diets.

2. Material and Methods

2.1. Animals and Experimental Design

The experimental procedures used in this study were according to FASS Guide for the Care and Use of Agricultural Animals in Research and Teaching (McGlone, 2010). All the experimental procedures were approved by the Oklahoma State University's Institutional Animal Care and Use Committee (Animal Care and Use Protocol # AG-17-22). The details on the animal experiments and diets were described previously (Shili 2020). Briefly, following 2 weeks of the acclimation period, forty-eight weaned barrows were weight-matched, housed individually, and randomly allotted to six dietary treatments (n=8/treatment) for 4 weeks: 1) positive control with normal protein content (PC); 2) negative control with low protein content (NC); 3) NC + low dose of CEP (2000 FTU/kg, LD); 4) NC + high dose of CEP (4000 FTU/kg, HD); 5) LD with reduced Ca/P (LDR); 6) HD with reduced Ca/P (HDR) (Shili 2020). At the end of the study (week 6), blood samples were collected, processed, and serum and plasma stored at -80 ºC, and pigs were euthanized and scanned by dual-energy X-ray absorptiometry (DEXA) as we previously described (Shili 2020).

2.2. Serum Triglycerides, Glucose, and Cholesterol

Serum triglycerides, glucose, and cholesterol were analyzed using an automated chemistry analyzer system (CLC 480/BioLis24i, Carolina Liquid Chemistries Corp., Brea, CA) as we described previously (Habibi 2021). The reagents for triglycerides (Catalogue #: BL213), glucose (Catalogue #: BL208), and cholesterol (Catalogue #: BL211) were obtained from Carolina Liquid Chemistries

Corp (Brea, CA). The triglyceride and cholesterol absorbance were detected at 505 nm, and glucose absorbance was detected at 340 nm.

2.3. Plasma Nitrogen-Containing Compounds Analysis

Plasma samples were analyzed for amino acids and other nitrogenous containing compounds at Molecular Structure Facility, Proteomics Core (UC Davis Genome Center, Davis, CA), as we previously described (Shili 2021). Briefly, samples were acidified with 2% sulfosalicylic acid and incubated at 25 ºC for 15 min. Following overnight storage of samples at -20 ºC, prior to injection (50ul), samples were diluted with Li sample diluent (Pickering Labs, Mountain view, CA). An ion-exchange chromatography method with using HITACHI L-8900 Amino Acid Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan) with a post-column ninhydrin reaction was applied to separate free amino acids. For the calibration of the AA analyzer, the AA standards (Sigma-Aldrich, St. Louis, MO) were utilized. To determine the response factor for each amino acid and amino acids concentrations relative to the known ones and related compounds standards, absorbance was measured at both 570 nm and 440 nm following the reaction with ninhydrin. To consider for variations in injection volume, the internal standard (AE-Cys, Sigma #A2636) was also included.

2.4. Serum Proteomics Analysis

To deplete abundant proteins with large molecular weight such as albumins and immunoglobulins from serum, samples were processed by the “depth filtration” approach as previously described (Alpert & Shukla 2003; Chertov et al., 2004, 2005). For this, 50 ul of serum was mixed with 75 ul of 100% acetonitrile, and the reaction was incubated at room temperature for 30 min. After incubation, tubes were centrifuged at 10,000 x g for 30 min at 22 ºC, the supernatant was collected

and loaded into the top segment of a commercial 200-ul pipet tip packed with monolithic C18 (ThermoFisher, Waltham, MA), which was then attached to a pipettor to force the samples through the packed bed. These filtrates were then dried in a vacuum centrifuge and re-dissolved for 30 min in buffered 8M urea containing 5 mM tris (2-carboxyethyl) phosphine. After dissolution and reduction, samples were alkylated with 10 mM iodoacetamide for 20 min, diluted with 3 volumes of 100 mM Tris-HCl (pH 8.2), and digested at 37 ºC with 1 ug of trypin/LysC (Promega Madison, WI). After overnight digestion, an additional 0.5 ug aliquot of protease was added and the reactions were incubated for an additional 6 h. Digestions were desalted by solid-phase extraction using monolithic C18 pipet tips (ThermoFisher, Waltham, MA), dried by vacuum centrifugation, and analyzed by LC-MS/MS.

For LC-M/MS analyses, samples were dissolved in 45 ul of mobile phase A (0.1% aqueous formic acid), diluted further with 2.4 volumes of mobile phase A, and 10 ul of peptides were injected onto a 75 um x 50 cm C18 analytical column using a trap column configuration (Thermo PN 164942 and PN 164535, resp.). Peptides were then eluted using a gradient of 2.5-30% acidified acetonitrile delivered at 250 nL/min over a 120 min HPLC run. Eluting peptides were ionized in a Nanospray Flex ion source using stainless-steel emitters and analyzed in a Quadrupole-Orbitrap "Fusion" mass spectrometer (Thermo Waltham, MA). The peptide ion stream was analyzed using a "top speed, high/low" MS/MS method that utilized the Orbitrap sector to detect peptide parent ions, the quadrupole sector for data-dependent ion selection for MS/MS, the ion routing multipole sector for MS/MS fragmentation by HCD, and the ion trap sector to analyze MS/MS fragments. Data were analyzed by using MaxQuant v1.6.10.43 (Cox and Mann, 2008) to search the instrument RAW files, concatenating four RAW files as "fractions" for each sample. Searches used the default MaxQuant parameters, supplemented with the variable modification Gln cyclization to pyro-glutamate, use of the match-between-runs feature to transfer MS/MS identifications between MS

peaks, and use of the label-free quantification (LFQ) algorithm (Cox et al., 2014) for protein quantitation. LFQ protein intensities were analyzed using Perseus**.**

2.5. Bioinformatics Analysis of the Differentially Abundant Proteins

The proteomics data were analyzed by several approaches. MaxQuant v1.5.2.8 (https://www.maxquant.org) was employed for protein identification and normalization of LFQ MS/MS data. MS/MS data were searched against a Uniprot reference proteome database of Sus scrofa using default MaxQuant settings with the addition of match between runs.

All statistical analyses, processing, and visualization were performed using Perseus (V1.6.2.1, https://omictools.com/perseus-tool) biostatistics software. ANOVA-based multi-sample *t*-test was performed using a cut-off of $P < 0.05$ of $\log_2$ transformed LFQ protein intensities to identify statistically significant differentially abundant proteins for 5 treatment comparisons (NC vs PC, NC vs LD, NC vs HD, LDR vs LD, HDR vs HD). Principal Component Analysis (PCA) plots and Hierarchical clustering of differentially abundant proteins were constructed and visualized using Perseus. Further, protein-protein interaction network analysis was done by querying the String database for known and predicted protein-protein interaction of the corresponding differentially abundant proteins up-and down-regulated in the serum samples with a confidence score of 0.8 and 40 addition interactors. The obtained string protein-protein interactions were visualized in Cytoscape (V.3.8.0; https://cytoscape.org). Functional characterization of differentially abundant proteins into biological processes, molecular functions, and cell localization was done using David functional annotation tool (https://david.ncifcrf.gov). Also, the proteins with limited description in the database were further explored via a literature search using Unipot (https://www.uniprot.org/uniprot/).

2.6. Statistical Analysis

Concentration of serum metabolites, plasma nitrogen containing compounds and body fat and lean content data were analyzed using the univariate ANOVA procedure of SPSS® (IMB SPSS Statistics version 23, Armonk, NY, USA). Means of dietary groups were separated by Tukey's post hoc analysis. $P \leq 0.05$ and $0.05 < P \leq 0.1$ were considered as statistical significance and trends, respectively. For proteomics data, pairwise student *t-test* was used to determine differently abundant proteins among specific groups of interest (PC vs NC, NC vs LD, NC vs HD, LD vs LDR and HD vs HDR) on perseus (V1.6.2.1, https://omictools.com/perseus-tool) with differences being considered significant at P value ≤ 0.05.

3. Results

3.1. Plasma Metabolites and Body composition

Pigs fed with HD had 119% higher plasma triglycerides than NC (Table 1). The same group of pigs tended to have 97% higher plasma triglycerides than those fed with LD diet. No differences were detected among groups on plasma concentration of glucose and cholesterol. Pigs fed with VLP diets (i.e. NC, LD, HD, LDR, and HDR) had lower body lean mass than PC. Relative to PC group, pigs in LD, HD, and HDR groups had lower body lean percent, but NC and LDR did not differ. Relative to PC, pigs fed with NC, HD, and LDR had a lower body fat mass, but LD and HDR did not change. Further, pigs fed with LD and HDR had a 38% and 43% higher fat mass than those fed with NC diet. Pigs fed with LD and HDR diets had a 20% and 23% higher body fat percent compared to those fed with PC diet.

Table 1. Plasma metabolites and body composition of nursery pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase

Items	Diets[1]						SEM[2]	*P*-value
	PC	**NC**	**LD**	**HD**	**LDR**	**HDR**		
Triglycerides, mg/dL	28.04±3.94^{ac}	15.75±3.69^{a}	17.65±4.67^{ab*}	34.64±3.94^{bc*}	26.26±3.69^{ab}	22.40±4.26^{ab}	1.83	0.02
Glucose, mg/dL	114.94±11.3	105.32±10.61	110.08±11.3	130.34±11.3	116.04±11.3	146.24±12.2	4.82	0.16
Cholesterol, mg/dL	74.97±25.1	103.61±23.5	128.60±23.5	91.01±25.1	162.46±23.5	158.94±23.5	10.46	0.07
Lean mass, kg	24.11±0.86^{a}	13.28±0.81^{b}	16.23±0.81^{b}	14.95±0.81^{b}	14.76±0.86^{b}	16.36±1.02^{b}	0.63	<0.01
Fat mass, kg	3.35±0.19^{ad}	2.03±0.18^{b}	2.82±0.18^{ac}	2.40±0.18^{bc}	2.40±0.19^{bc}	2.92±2.34^{cd}	0.10	<0.01
Lean, %	86.54±0.49^{a}	85.22±0.46^{ac}	83.58±0.46^{bc}	84.49±0.46^{bc}	84.93±0.49^{ac}	83.43±0.58^{bc}	0.24	<0.01
Fat, %	12.10±0.50^{a}	12.93±0.50^{ab}	14.50±0.46^{b}	13.56±0.46^{ab}	13.35±0.50^{ab}	14.87±0.59^{b}	0.23	<0.01

[1]**PC** (positive control): normal protein, adequate calcium (Ca) and available phosphorous (aP), no corn-expressed phytase (CEP) added; **NC** (negative control): low protein, adequate Ca and aP, no CEP added; **LD**: NC + CEP added at low dose, i.e. 2,000 one phytase unit (FTU)/kg of diet; **HD**: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; **LDR**: LD with reduced Ca and P; **HDR**: HD with reduced Ca and P. Values are means ± SE. *n*=8 for each dietary group.

[2]SEM: Standard error of mean

[a-d]Within a row, values without a common superscript letter differ ($P \leq 0.05$)

[*]Within a row, values with a common superscript symbol are tended to be different ($0.05 < P \leq 0.1$

3.2. Plasma Nitrogen-Containing Compounds

Pigs fed with VLP diets (i.e. NC, LD, HD, LDR, and HDR) (Table 2) had a lower plasma concentration of isoleucine, valine, histidine, arginine, tyrosine, and asparagine and higher plasma concentration of threonine, methionine, and lysine than PC. Compared to PC, the NC diet reduced the plasma leucine and phenylalanine concentrations; however, LD recovered their levels. Additionally, pigs fed with HD had a higher plasma alanine and glycine and tended to have a greater serine than PC. The rest of the plasma amino acids did not show significant or meaningful changes across groups.

Table 2. The composition (nmol/mL) of plasma nitrogen-containing compounds of nursery pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase

Item	PC	NC	LD	HD	LDR	HDR	SEM[2]	*P*-value
Valine	283.1±36.5[a]	84.33±30.8[b]	125.6±33.3[b]	58.08±30.8[b]	126.4±30.8[b]	81.12±30.8[b]	16.42	<0.01
Methionine	48.50±10.6[a*]	90.28±10.6[ab*]	111.3±10.6[b]	109.0±9.87[b]	106.7±9.87[b]	116.5±9.87[b]	5.35	<0.01
Threonine	586.7±236[a]	1704±219[b]	2243±236[b]	1986±219[b]	1766±219[b]	2212±236[b]	122.8	<0.01
Isoleucine	142.9±21.2[a]	32.42±17.9[b]	36.47±19.6[b]	35.10±17.9[b]	49.46±17.9[b]	35.94±17.9[b]	9.13	<0.01
Leucine	274.8±19.3[a]	183.0±16.3[b]	218.9±17.6[ab]	185.9±16.3[b]	183.6±16.3[b]	186.7±16.3[b]	8.13	<0.01
Phenylalanine	118.5±7.9[a]	85.50±6.7[bc]	100.0±7.2[ac]	84.89±6.7[bc]	96.82±6.7[bc]	87.27±6.7[bc]	3.20	0.02
Tryptophan	76.40±6.94	83.18±6.42	83.10±6.94	85.85±6.42	92.01±6.42	96.04±6.42	2.70	0.37
Lysine	212.9±71.5[a]	716.1±66.2[b]	847.1±71.5[b]	807.7±66.2[b]	779.7±66.2[b]	793.9±66.2[b]	42.26	<0.01
Histidine	120.7±6.12[a]	58.80±5.66[b]	65.71±6.12[b]	52.67±5.66[b]	56.86±5.66[b]	56.12±5.66[b]	4.27	<0.01
Arginine	243.1±16.8[a]	89.72±15.5[b]	85.50±16.8[b]	96.85±15.5[b]	67.77±15.5[b]	56.20±15.5[b]	11.40	<0.01
Aspartic acid	42.58±8.29	43.74±7.67	37.37±8.29	38.94±7.67	54.37±7.67	51.76±7.67	3.16	0.57
Serine	199.2±18.1[*]	227.5±16.7	241.7±18.1	268.5±16.7[*]	246.5±16.7	254.2±16.7	7.39	0.12
Glutamic acid	357.4±42.5	314.8±39.3	260.5±42.5	284.7±39.3	348.7±39.3	340.9±39.3	16.33	0.55
Glutamine	762.2±54.4	674.4±50.4	716.6±54.4	664.1±50.4	633.1±50.4	653.8±50.4	20.81	0.56
Glycine	1375±143[a#]	1556±133[a]	1941±143[ac#]	2137±133[c]	1832±133[ac]	1899±133[ac]	65.52	<0.01
Alanine	773.8±84.3[a#*]	1111±78.0[bc*]	1103±84.3[c#]	1242±78.0[bc]	1120±78.0[bc]	1067±78.0[ac]	37.73	0.01
Tyrosine	187.1±8.94[a]	76.80±8.27[b]	85.50±8.94[b]	86.49±8.27[b]	91.88±8.27[b]	91.26±8.27[b]	6.65	<0.01
Asparagine	160.0±11.0[a]	100.0±10.2[b]	110.0±11.0[b]	109.5±10.2[b]	101.4±10.2[b]	97.38±10.2[b]	5.19	<0.01
Proline	438.2±29.7	364.2±27.5	443.5±29.7	430.3±27.5	384.6±27.5	406.3±27.5	11.73	0.30
Cysteine	8.90±2.30	8.10±2.12	7.89±2.30	6.15±2.12	10.23±2.12	9.18±2.12	0.85	0.83
Ammonia	371.5±59.3	404.8±54.9	459.3±59.3	455.2±54.9	572.3±54.9	494.5±54.9	23.80	0.19
Creatinine	122.5±12.4	153.2±11.5	144.3±12.4	164.2±11.5	157.6±11.5	163.0±11.5	5.03	0.17
Taurine	156.3±42.8	173.3±39.6	136.5±42.8[$]	272.3±39.6	240.2±39.6	310.0±42.8[$]	18.65	0.03
Sarcosine	53.75±5.89[a]	65.64±5.45[ac]	65.75±5.89[ac]	54.92±5.45[a]	80.28±5.4[bc]	67.58±5.8[ac]	2.60	0.02
3,methylhistidine	9.50±0.79	9.50±0.73	8.16±0.79	8.92±0.79	8.92±0.73	8.75±0.79	0.30	0.83
1,methylhistidine	31.41±3.82	22.78±3.53	22.91±3.82	28.71±3.53	22.35±3.53	18.25±3.82	1.55	0.18
Ethanolamine	10.00±2.11	13.14±1.96	13.00±2.11	14.64±1.96	14.07±1.96	11.75±2.11	0.81	0.65
Carnosine	53.08±13.2	29.00±12.7	29.08±13.2	36.21±12.2	69.92±12.2	22.50±13.2	5.54	0.09
Hydroxyproline	140.2±12.7	147.3±11.7	143.0±12.7	156.5±11.7	145.9±11.7	153.9±11.7	4.68	0.92

Citrulline	66.65±7.2	88.46±6.7	80.80±7.2	82.43±6.7	80.14±6.7	65.58±6.75	2.96	0.14
Ornithine	213.0±28.4	130.9±26.2	129.9±28.4	121.7±26.2	168.8±26.2	131.5±26.2	11.42	0.18
α-amino butyric acid	30.25±5.95	43.85±5.51	41.25±5.96	30.78±5.51	42.35±5.51	46.25±5.95	2.40	0.24

[1]**PC** (positive control): normal protein, adequate calcium (Ca) and available phosphorous (aP), no corn-expressed phytase (CEP) added; **NC** (negative control): low protein, adequate Ca and aP, no CEP added; **LD**: NC + CEP added at low dose, i.e. 2,000 one phytase unit (FTU)/kg of diet; **HD**: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; **LDR**: LD with reduced Ca and P; **HDR**: HD with reduced Ca and P. Values are means ± SE. *n*=6 for each dietary group.

[2]SEM: Standard error of mean

[a-c]Within a row, values without a common superscript letter differ ($P \leq 0.05$)

[*#$]Within a row, values with a common superscript symbol are tended to be different ($0.05 < P \leq 0.1$).

3.3. Differentially Abundant Proteins in Serum of Pigs

Following serum proteomics analysis, 703 proteins were identified and quantified in the serum proteome of pigs. Out of these, 25 proteins showed differential expression ($P < 0.05$) among all treatments (Fig. 1A, and Table 3). When the NC group was compared to PC group, a total of 660 common proteins were present in both NC and PC groups (93.7%), of these, 23 (3.38%) proteins were upregulated in NC, and 20 (2.92%) proteins were upregulated in PC (Fig. 1B). When LD and NC groups were compared, a total of 680 common proteins were present in both groups (96.61%), of these, 10 (1.47%) proteins were upregulated in LD and 13 (1.91%) proteins were upregulated in NC (Fig. 1C). The comparison between HD and NC revealed a total of 679 common proteins (96.47%) being present in both groups, of these, 14 (2.06%) proteins were upregulated in HD and 10 (1.45%) proteins were upregulated in NC (Fig. 1D). While the comparison between LDR and LD groups showed a total of 677 common proteins in these groups (96.17%), 14 (2.06%) of proteins were upregulated in LDR and 12 (1.77%) proteins were upregulated in LD (Fig. 1E). Furthermore, when HDR vs HD groups were compared, a total of 680 common proteins were present in both HDR and HD groups (96.62%), of these 3 (0.44%) proteins were upregulated in HDR, and 20 (2.94%) proteins were upregulated in HD (Fig. 1F).

Table 3. Proteins identified with significantly differential abundance in serum of nursery pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase.

Protein ID	Protein description	Molecular function	Biological process	Gene name	*P*-value
P21753	Actin monomer binding	actin monomer binding	regulation of cell migration	TMSB10	0.004
P68137	Actin, alpha skeletal muscle	ATP binding	positive regulation of gene expression	ACTA1	0.000
Q95274	Thymosin beta 4 X-linked	actin monomer binding	regulation of cell migration	TMSB4	0.017
Q95JB4	Liver-expressed antimicrobial pept2	Defense response to bacterium	-	LEAP2	0.041
F1SQ51	Sus scrofa basic proline-rich protein	-	-	TP23	0.044
Q9GKQ6	Uncharacterized protein	-	-	BGN	0.043
Q9TV62	Myosin, heavy chain 2, skeletal muscle	Muscle contraction	actin filament binding	MYH4	0.001
A0A287A391	Eukaryotic translation elongation factor 1 alpha 1	GTPase activity	-	EEF1A1	0.031
A0A286ZYQ7	Ig-like domain-containing protein	-	-	IGKV2D-40	0.007
A0A287BLH9	Uncharacterized protein	-	immune response	IGLV3-1	0.023
A0A287A6F0	Myosin light chain, phosphorylatable, fast skeletal muscle.	calcium ion binding	-	MYLPF	0.026
A0A287AAU1	Cellular communication network factor 3	insulin-like growth factor binding	signal transduction	CCN3	0.000
A0A287BAK1	Structural constituent of cytoskeleton	actin filament binding	cell adhesion	TLN1	0.024
A0A287B7U0	Ribosomal protein lateral stalk subunit P2	structural constituent of ribosome	cytoplasmic translational elongation	RPLP2	0.041
A0A5G2QYD1	Hyaluronic acid binding	carbohydrate binding	cell adhesion	ACAN	0.048
A0A5G2Q8S2	Peroxidasin	peroxidase activity	response to oxidative stress	PXDN	0.012
K7GRU7	Angiotensin-converting enzyme	carboxypeptidase activity	-	ACE	0.026
F1RVS9	Peptidase inhibitor 16	-	regulation of cell growth involved in cardiac muscle	PI16	0.000
F1SC51	Muscle alpha-actinin binding	actin binding	muscle structure development	PDLIM1	0.029
F1SFA7	Collagen type I alpha 2 chain	platelet-derived growth factor binding	bone mineralization	COL1A2	0.040
F1SKG1	Inhibin subunit beta E	cytokine activity	SMAD protein signal transduction	INHBE	0.046

I3L5L0	Tumor necrosis factor receptor superfamily member 10A-like	TRAIL binding	apoptotic process	LOC100737977	0.007
I3LDS3	keratin	protein heterodimerization activity	keratinocyte differentiation	KRT10	0.041
I3LM99	Grancalcin	calcium ion binding		GCA	0.001
T1UNN8	Angiopoietin like 8	-	positive regulation of protein processing	ANGPTL8	0.000

(-) not available in sus scrofa (pig) database (https://www.uniprot.org/)

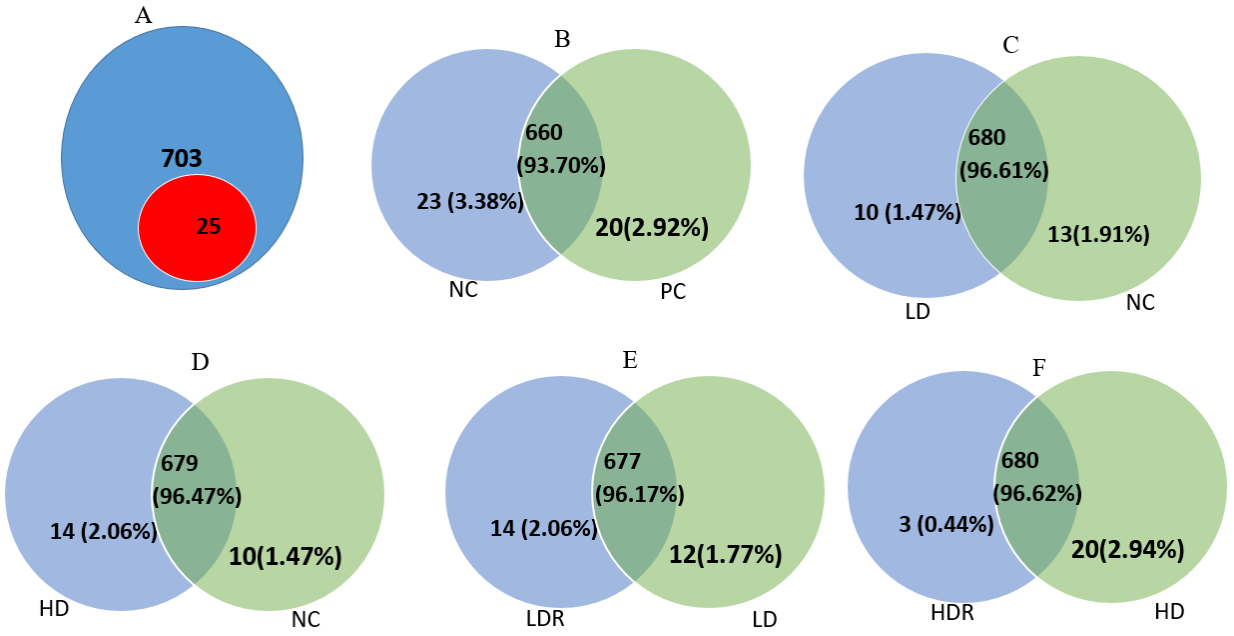

Figure 1. Venn diagrams of total identified proteins in serum of pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase. (A) the number of total identified proteins across biological replicates (703 proteins) with 25 proteins identified with significantly differential abundance across dietary groups, (B) NC vs PC, (C) LD vs NC, (D) HD vs NC, (E) LDR vs LD, (F) HDR vs HD.

PC (positive control): normal protein, adequate calcium (Ca) and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 one phytase unit (FTU)/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced Ca and P; HDR: HD with reduced Ca and P. n = 7-8 for each dietary group.

To better assess how phytase supplementation might influence changes in growth performance and serum protein expression profiles, a pairwise comparison was employed. The principal component analysis (PCA) showed distinctive separation between different dietary treatments (Fig 2A-F), suggesting that each treatment group exhibits differential protein expression profiles when compared to their control groups. Furthermore, hierarchical clustering analysis also showed distinctive clusters of up-regulated and down-regulated proteins differentially abundant among treatment groups (Fig 3A-F). Thus, this data shows that protein expression profiles were unique within each treatment group.

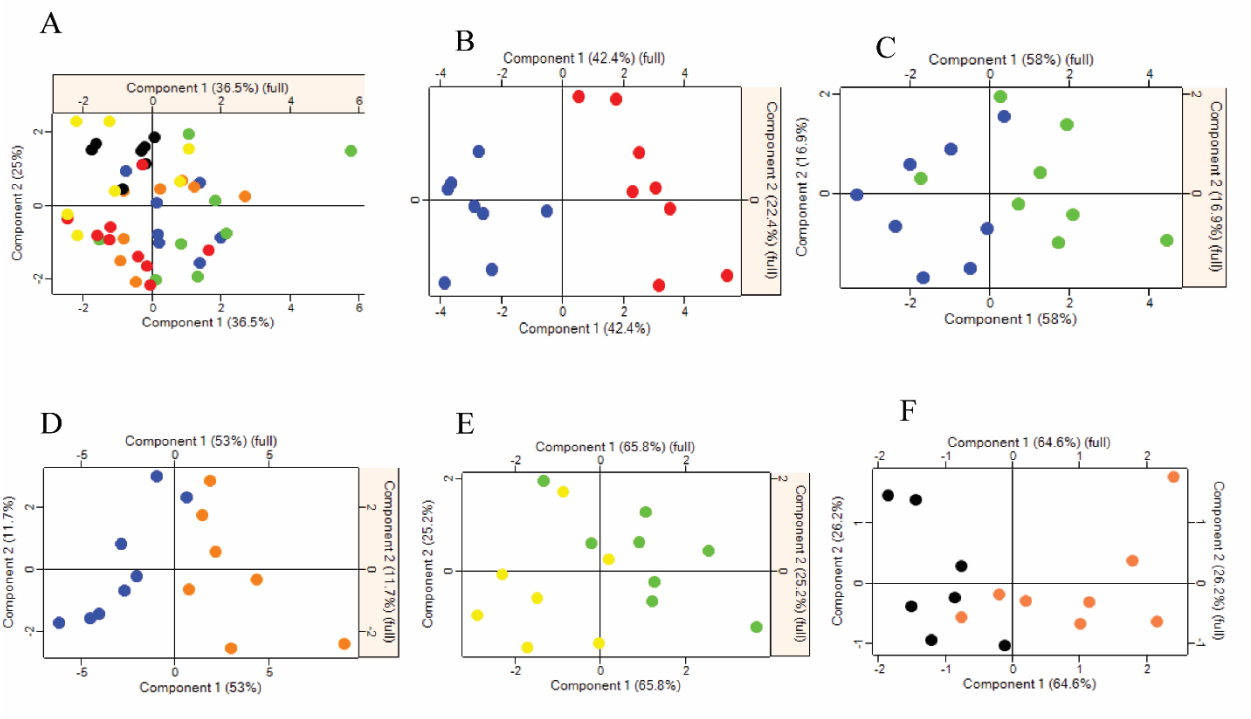

Figure 2. Principal Component Analysis (PCA) score plots of differentially expressed protein in serum of pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase. (A) overall, (B) NC vs. PC, (C) LD vs. NC, (D) HD vs. NC, (E) LDR vs. LD, (F) HDR vs. HD. Each circle represents an individual pig and the red, blue, green, orange, yellow and black circles are representative of PC, NC, LD, HD, LDR and HDR groups, respectively. PC (positive control): normal protein, adequate calcium (Ca) and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 one phytase unit (FTU)/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced Ca and P; HDR: HD with reduced Ca and P. n = 7-8 for each.

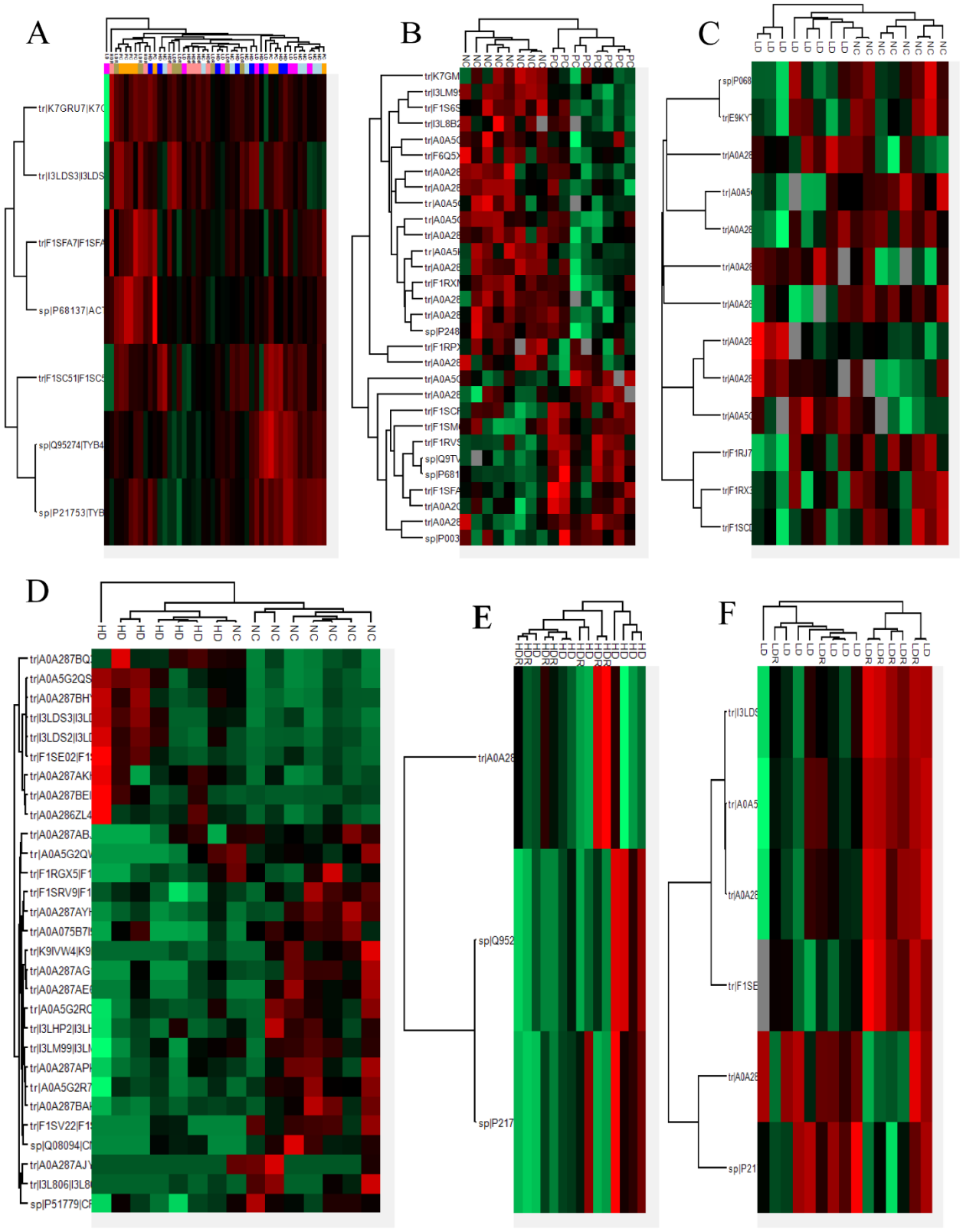
A
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Figure 3. Hierarchical clustering of differentially expressed protein in serum of pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase. (A) overall, (B) NC vs PC, (C) LD vs NC, (D) HD vs NC, (E) LDR vs LD, (F) HDR vs HD. The red color indicates the high abundance and green color indicates the low abundance. PC (positive control): normal protein, adequate calcium (Ca) and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 one phytase unit (FTU)/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced Ca and P; HDR: HD with reduced Ca and P. n = 7-8 for each dietary group.

To further understand the biological processes and molecular functions of the up-regulated and down-regulated proteins among different groups, a library search was done using uniport (https://www.uniprot.org/) and data are shown in Table 4. Compared to PC, NC upregulated a group of proteins such as ameloblastin (AMBN) and insulin-like growth factor-binding protein 2 (IGFBP2) that were involved in the regulation of growth factor activity and IGF-1 binding molecular functions, respectively. Relative to PC, NC downregulated a group of proteins including collagen type I alpha 2 chain (COL1A2), IGF-1, and peptidase inhibitor 16 (PI16) that were involved in bone mineralization, growth regulation and regulation of cell growth involved in cardiac muscle cell development, respectively. Compared to NC, LD upregulated proteins such as selenoprotein P (SELENOP) and collagen type V alpha 1 (COL5A1) that were involved in regulation of growth and blood vessel development, respectively. Relative to NC, LD down-regulated proteins such as IGFBP4 and biglycans (BGN) that were involved in growth regulation by IGFs and collagen fiber assembly, respectively. In the HD vs. NC comparison, pigs fed with HD diets upregulated proteins such as IGFBP3 and secretogranin V (SCG5) that are involved in regulation of IGF receptor signaling pathway and regulation of hormone secretion, respectively, while they downregulated proteins such as GC vitamin D binding protein (GC), and vasorin

(VASN) that are involved on vitamin D metabolic process and cellular response to redox state, respectively (Table 4). In the LDR vs LD comparison, some of the upregulated proteins in LDR group were TMF1 regulated nuclear protein 1 (TRNP1), arginase 1 (ARG1), heat shock protein family E (HSPE1) and biglycans (BGN) that are important for regulation of cell cycle, involved in urea cycle, protein folding and collagen fiber assembly, respectively. Relative to LD, LDR down-regulated proteins included grancalcin (GCA), lipocalin 15 (LCN15) and lecithin-cholesterol acyltransferas (LCAT) that play and important role in calcium ion binding, retinol binding and cholesterol metabolic process, respectively. Compared to HD, HDR upregulated proteins such as myristoylated alanine rich protein kinase C substrate (MARCKS) involved in actin crosslink formation, while downregulated proteins such as thymosin beta 4 X-linked (TMSB4) and calpain small subunit 1 (CAPNS) involved in regulation of cell migration and proteolysis, respectively.

Table 4. Paired-wise comparison of proteins identified with significantly differential abundance in serum of nursery pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase.

Protein ID	Protein description	Molecular function	Biological process	Gene name	*P*-value	Fold change
NC vs PC[1]						
T1UNN8	Angiopoietin like 8	structural constituent of cytoskeleton	cell adhesion	ANGPTL8	0.001	17.97
F6Q5X1	Serum amyloid A protein	Apolipoprotein of the HDL complex.	acute-phase response	SAA2	0.028	3.97
A0A287B0B8	Pulmonary surfactant-associated protein B	-	lipid metabolic process	SFTPB	0.003	2.91
A0A5K1TWC1	Apolipoprotein D	lipid binding	lipid transport	APOD	0.001	2.63
A0A5G2R684	Uncharacterized protein	-	-	MARCOL	0.018	2.54
Q28989	Ameloblastin	growth factor activity	biomineral tissue development	AMBN	0.033	2.52
A0A287ALJ6	Ig-like domain-containing protein	-	immune response	IGKV2D-29	0.011	2.37
I3LM99	Uncharacterized protein			GCA	0.000	2.24
A0A287AJ94	Microsemino protein, prostate associated	Calcium ion binding	protein heterodimerization	MSMP	0.025	2.14
K7GM40	Apolipoprotein A1	cholesterol transfer activity	adrenal gland development	APOA1	0.031	2.08
F1RPX3	Secretoglobin family 1A member 1	-	signal transduction	SCGB1A1	0.042	1.97
F1RXM6	Serpin family A member 7	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity	SERPINA7	0.018	1.83
A0A287AEV6	Uncharacterized protein	-	positive regulation of neutrophil extravasation	CD99	0.015	1.83
A0A287AP28	Ig-like domain-containing protein	-	Glycoprotein	IGHV3-23	0.035	1.80

A0A5G2R113	Uncharacterized protein	dermokine	cornified envelope assembly	DMKN	0.026	1.73
I3L8B2	Uncharacterized protein	extracellular matrix structural constituent	extracellular matrix organization	COL9A2	0.040	1.70
A0A287APK0	6-phosphogluconolactonase (Pentose phosphate pathway)	6-phosphogluconolactonase activity	carbohydrate metabolic process	PGLS	0.050	1.61
P24853	Insulin like growth factor binding protein 2	insulin-like growth factor I binding	positive regulation of growth	IGFBP2	0.015	1.59
A0A5G2QE21	Uncharacterized protein	Serine peptidase inhibitor Kazal type 5	-	SPINK5	0.032	1.47
F1S6S9	Peptidase S1 domain-containing protein	Serine-type endopeptidase activity	negative regulation of phagocytosis	PRTN3	0.002	1.44
A0A5G2R4W0	LAM_G_DOMAIN domain-containing protein	Collagen type XVIII alpha 1 chain	-	COL18A1	0.038	1.37
A0A5G2QYD1	Aggrecan core protein	Hyaluronic acid binding	cell adhesion	ACAN	0.047	1.37
A0A287ASA4	Aa_trans domain-containing protein	Solute carrier family 38 member 10	-	SLC38A10	0.021	1.36
F1SFA7	Fibrillar collagen NC1 domain-containing protein	Platelet-derived growth factor binding	bone mineralization	COL1A2	0.033	-1.33
A0A2C9F3F9	Insulin like growth factor 1	growth factor activity	-	IGF1	0.017	-1.35
F1SM61	Fibulin 1	peptidase activator activity	extracellular matrix organization	FBLN1	0.043	-1.49
A0A5G2QJN1	LFNG O-fucosylpeptide 3-beta-N-Acetylglucosaminyltransferase.	metal ion binding	pattern specification process	LFNG	0.020	-1.62
F1RM86	Uncharacterized protein	Metalloendopeptidase activity	-	ADAMDEC1	0.030	-1.72
A0A287A391	Eukaryotic translation elongation factor 1 alpha 1	GTPase activity	-	EEF1A1	0.013	-2.00
A6P7L6	Calcitonin-related polypeptide beta	calcitonin receptor binding	regulation of cytosolic calcium ion concentration	CALCB	0.027	-2.42
I3L5L0	Uncharacterized protein	TRAIL binding	signal transduction	LOC100737977	0.037	-2.44

F1SCF1	SERPIN domain-containing protein	Serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity	SERPINA6	0.029	-2.71
P00355	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	peptidyl-cysteine S-nitrosylase activity	glucose metabolic process	GAPDH	0.022	-3.02
P68137	ATP binding (Actin)			ACTA1	0.001	-3.13
A0A287AAU1	Cellular communication network factor 3	insulin-like growth factor binding	-	CCN3	0.017	-3.19
F1RVS9	SCP domain-containing protein	Peptidase inhibitor 16	negative regulation of cell growth involved in cardiac muscle cell development	PI16	0.001	-3.60
A0A287BEI1	Keratin 77	-	-	KRT77	0.005	-4.38
F1RFS7	Matrix metallopeptidase 15	Cofactor for Ca and Zn	metalloendopeptidase activity	MMP15	0.032	-4.73
F1S8P9	Aldolase_II domain-containing protein	-	-	ADD1	0.042	-4.86
A0A5G2R4A8	Heterogeneous nuclear ribonucleoprotein A3	RNA binding	-	HNRNPA3	0.043	-5.18
A0A287ASI0	Keratin 7	-	-	KRT7	0.029	-5.55
A0A287A6F0	Uncharacterized protein	calcium ion binding	-	MYLPF	0.022	-10.86
Q9TV62	Myosin, heavy chain 2, skeletal muscle	actin filament binding	muscle contraction	MYH4	0.000	-14.40
LD vs. NC[1]						
I3LJP2	Selenoprotein P	selenium binding	regulation of growth	SELENOP	0.033	5.21
A0A5G2QPS7	Aldolase_II domain-containing protein	-	-	ADD2	0.050	4.33
A0A287BLH9	Uncharacterized protein	immunoglobulin production	-	IGLV3-1	0.022	3.63
A0A286ZTG2	Uncharacterized protein	metal ion binding	- Muscleblind like splicing regulator 1	MBNL1	0.015	2.32

F2Z5F7	Protein dpy-30 homolog isoform 1	protein homodimerization activity	endosomal transport	DPY30	0.026	2.29
A0A287B7U0	Ribosomal protein lateral stalk subunit P2	structural constituent of ribosome	translational elongation	RPLP2	0.022	2.23
F1S021	Collagen type V alpha 1 chain	platelet-derived growth factor binding	blood vessel development	COL5A1	0.024	1.83
A0A5G2QJN1	Beta-1,3-N-acetylglucosaminyltransferase	metal ion binding	pattern specification process	LFNG	0.007	1.66
A0A286ZL45	Dedicator of cytokinesis 2	guanyl-nucleotide exchange factor activity	actin cytoskeleton organization	DOCK2	0.048	1.41
A0A287ARV5	Ubiquitin related modifier 1	sulfur carrier	protein urmylation	URM1	0.024	1.34
A0A287AMT2	Uncharacterized protein	Laminin subunit beta 2	-	LAMB2	0.043	-1.33
Q9GKQ6	Biglycan	collagen fiber assembly	-	BGN	0.005	-1.49
F1RX36	Fibrinogen alpha chain	signaling receptor binding	plasminogen activation	FGA	0.037	-1.60
A0A287BGE8	Uncharacterized protein	-	-	GRN	0.038	-1.65
F1RJ72	BRICHOS domain-containing protein	-	endothelial cell morphogenesis	CNMD	0.045	-1.66
E9KYT3	Insulin like growth factor binding protein 4	insulin-like growth factor I binding	Either inhibit or stimulate the growth promoting effects of the IGFs	IGFBP4	0.039	-1.70
F1SCD0	SERPIN domain-containing protein	-	-	SERPINA3-2	0.017	-1.81
P06867	Plasminogen	serine-type endopeptidase activity	blood coagulation	PLG	0.035	-2.00
A0A5G2QWT2	Ig-like domain-containing protein	Glycoprotein	-	IGLV3-21	0.002	-2.06
A0A286ZYQ7	Ig-like domain-containing protein	Glycoprotein	-	IGKV2D-28	0.006	-2.80

A0A287ADT3	BCL2 like 15	-	regulation of apoptotic process	BCL2L15	0.041	-2.80
Q95JC8	Arginase 1, Catalytic activity	-	Involved in urea cycle	ARG1	0.048	-3.31
F1SCC7	SERPIN domain-containing protein	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity	LOC396684	0.004	-5.39
HD vs. NC[1]						
Q9TV62	Myosin, heavy chain 2, skeletal muscle	actin filament binding	muscle contraction	MYH1	0.004	20.38
F1SQ51	Uncharacterized protein	Basic proline-rich protein	-	TP23	0.015	17.30
A0A287B409	Uncharacterized protein	proton transmembrane transporter activity	transmembrane transport	IGKV2D-29	0.017	7.15
A0A287BEI1	IF rod domain-containing protein	-	-	KRT77	0.017	4.46
A0A5G2RFC8	Uncharacterized protein	-	-	UMAD1	0.043	3.83
F1RFS7	ZnMc domain-containing protein	zinc ion binding	-	MMP15	0.030	3.51
A0A5G2QW63	Uncharacterized protein	-	-	CCDC117	0.046	2.61
F1SR80	Tubulin alpha chain	GTPase activity	microtubule-based process	LOC100158003	0.043	2.01
A0A5G2RGV2	Integral membrane protein 2B	-	-	ITM2B	0.013	1.94
A0A5G2QJN1	Beta-1,3-N-acetylglucosaminyltransferase	O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase activity	pattern specification process	LFNG	0.036	1.87
A0A286ZL45	Uncharacterized protein	guanyl-nucleotide exchange factor activity	actin cytoskeleton organization	DOCK2	0.013	1.74
P16611	Insulin-like growth factor-binding protein 3	fibronectin-binding	regulation of insulin-like growth factor receptor signaling pathway	IGFBP3	0.038	1.65
F1RM86	ADAM like decysin 1	metalloendopeptidase activity	-	ADAMDEC1	0.041	1.55
P01165	Neuroendocrine protein 7B2	unfolded protein binding	regulation of hormone secretion	SCG5	0.013	1.14
A0A287BGE8	Uncharacterized protein	-	-	GRN	0.026	-1.31

I3L5L0	Uncharacterized protein	TRAIL binding	signal transduction	LOC100624226	0.045	-1.63
I3LN42	Gc-globulin	calcidiol binding	vitamin D metabolic process	GC	0.017	-1.88
A0A287ALJ6	Ig-like domain-containing protein	immune response	-	IGKV2D-29	0.029	-2.12
F1SEC5	Cell growth regulator with EF-hand domain 1	calcium ion binding	-	CGREF1	0.003	-2.31
I3LS87	Transforming growth factor beta binding	transforming growth factor beta binding	cellular response to redox state	VASN	0.009	-2.49
A0A5G2QE94	Immunoglobulin superfamily containing leucine	-	-	ISLR	0.008	-2.61
A0A287B5C1	Uncharacterized protein	-	-	IGHM	0.008	-2.62
A0A287A0Y1	Uncharacterized protein	-	-	PFDN5	0.005	-4.40
A0A5G2RLN3	Calcium voltage-gated channel subunit alpha1 E	calcium ion binding	regulation of ion transmembrane transport	CACNA1E	0.036	-19.7
LDR vs. LD[1]						
I3LDS2	TMF1 regulated nuclear protein 1	DNA binding	regulation of cell cycle	TRNP1	0.014	11.68
A0A287ADT3	BCL2 like 15	regulation of apoptotic process	-	BCL2L15	0.022	6.43
Q95JC8		Catalytic activity	involved in urea cycle	ARG1	0.028	6.11
I3LGN8	Plakophilin 1	cadherin binding	cell-cell junction assembly	PKP1	0.009	5.18
A0A287BHY5	keratin 2	cytoskeletal protein binding	intermediate filament organization	KRT2	0.024	5.02
I3LDS3	IF rod domain-containing protein	structural constituent of skin epidermis	peptide cross-linking	KRT10	0.016	4.91
A0A5G2QSE8	keratin 3, IF rod domain-containing protein	-	-	KRT3	0.026	4.42
F1SE02	Uncharacterized protein	-	-	CEP128	0.014	4.31
A0A287B8U7	Plexin domain containing 2	-	-	PLXDC2	0.046	3.22
A0A5G2RCI8	Coagulation factor XII	serine-type endopeptidase activity	-	F12	0.021	2.25

F1SHM0	Pyruvate kinase, muscle	pyruvate kinase activity	-	PKM	0.024	2.22
A0A286ZX29	serpin family B member 12	serine-type endopeptidase inhibitor activity	hematopoietic progenitor cell differentiation	SERPINB12	0.041	2.01
A0A287A1U5	Heat shock protein family E	ATP binding	protein folding	HSPE1	0.005	1.52
Q9GKQ6	Biglycan	Involved in collagen fiber assembly		BGN	0.025	1.40
I3LM99	Grancalcin	calcium ion binding	-	GCA	0.044	-1.41
P04119	Beta-lactoglobulin-1A/1C	retinol binding	-	LCN15	0.049	-1.50
A0A286ZTG2	Uncharacterized protein	Muscle blind like splicing regulator 1	-	MBNL1	0.005	-1.53
A0A287BRL8	Myristoylated alanine rich protein kinase C substrate	actin filament binding	actin filament bundle assembly	MARCKS	0.028	-1.79
I3LHP2	Acyl-protein thioesterase 2 isoform X3	lysophospholipase activity	protein depalmitoylation	LYPLA2	0.043	-1.83
A0A287B7U0	Ribosomal protein lateral stalk subunit P2	structural constituent of ribosome	translational elongation	RPLP2	0.024	-1.88
F1S862	Sulfiredoxin 1	sulfiredoxin activity	cellular response to oxidative stress	SRXN1	0.033	-2.02
I3LK29	Phosphatidylcholine-sterol acyltransferase	apolipoprotein A-I binding	cholesterol metabolic process	LCAT	0.018	-2.11
I3LEC2	Uncharacterized protein	mRNA binding	regulation of gene expression	PCBP1	0.021	-2.14
A0A5G2Q8S2	Peroxidasin	heme binding	response to oxidative stress	PXDN	0.014	-2.35
P21753	Thymosin beta-10	actin monomer binding	regulation of cell migration	TMSB10	0.013	-2.99
A0A287AAU1	Uncharacterized protein	insulin-like growth factor binding	-	CCN3	0.000	-3.19
HDR vs. HD[1]						
A0A5G2QXT5	Uncharacterized protein	-	-	IGHG4	0.020	2.36
F1SLW6	Uncharacterized protein	-	-	MFAP5	0.048	2.16

A0A287BRL8	Myristoylated alanine rich protein kinase C substrate	kinase activity	actin crosslink formation	MARCKS	0.037	1.56
A0A287BAK1	Talin 1	actin filament binding	cell adhesion	TLN1	0.049	-1.58
Q95274	Thymosin beta 4 X-linked	actin monomer binding	regulation of cell migration	TMSB4	0.004	-1.64
A0A287ACX2	Uncharacterized protein	centrosome localization	-	NIN	0.043	-1.66
A0A5G2R7T5	Uncharacterized protein	-	-	TMEM40	0.041	-1.80
P04574	Calpain small subunit 1	calcium ion binding	proteolysis	CAPNS1	0.021	-1.99
Q29290	Cystatin B	protease binding	adult locomotory behavior	CSTB	0.016	-1.99
A0A287ALA0	Brain abundant membrane attached signal protein 1	protein domain specific binding	glomerular visceral epithelial cell differentiation	BASP1	0.041	-2.15
P21753	Thymosin beta-10 (regulation of cell migration)	actin monomer binding	regulation of cell migration	TMSB10	0.014	-2.53
M3VJZ7	LIM and SH3 protein 1	actin filament binding	-	LASP1	0.000	-2.55
A0A5G2QRL2	PDZ domain-containing protein	-	-	PDLIM1	0.013	-2.62
K7GLE1	Annexin A1	phospholipase inhibitor activity	-	ANXA1	0.029	-2.71
F1RLQ3	Semaphorin 4A	semaphorin receptor binding	-	SEMA4A	0.027	-2.72
A0A287AKM8	Corneodesmosin	Protein homodimerization activity	skin morphogenesis	CDSN	0.047	-2.78
A0A287BEC7	Vesicle associated membrane protein 3	SNAP receptor activity	vesicle fusion	VAMP3	0.029	-3.10
A0A480PPD4	Eukaryotic translation initiation factor 4H	translation initiation factor activity	-	EIF4H	0.014	-3.33
A0A5G2QR43	Uncharacterized protein	-	-	GOLGB1	0.045	-3.39
P81693	Uncharacterized protein	-	-	ACP1	0.037	-3.77
A0A287BQI8	keratin, type II cytoskeletal 3-like	-	-	LOC100155249	0.030	-4.23
M3UZ42	IF rod domain-containing protein	brain development	Negative regulation of protein import into nucleus	UFM1	0.049	-7.38
A0A287B409	Uncharacterized protein	-	-	ATP6V0E1	0.004	-7.60

[1]PC (positive control): normal protein, adequate calcium (Ca) and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP added at low dose, i.e. 2,000 one phytase unit (FTU)/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced Ca and P; HDR: HD with reduced Ca and P. n = 8 for each dietary group

[(-)] not available in sus scrofa (pig) database (https://www.uniprot.org/)

To further understand the potential protein-protein interaction between the up-regulated and down-regulated proteins among the different treatments group, a network analysis was employed using the String database plugin in Cytoscape (Version 3.8.0). When comparing the differentially abundant proteins between PC and NC groups (Fig. 4A), few connections were found between the proteins regulating growth (i.e. IGF-1, IGFBP2). When LD vs NC groups were considered (Fig. 4B) network analysis revealed an interaction between the up-regulated proteins (DOCK2), and the downregulated proteins (ARG1), and between the downregulated proteins such as LAMB2 and BGN, and IGFBP4, FGA, and PLG. These proteins are involved in platelet-derived growth factor binding, guanyl-nucleotide exchange factor activity, IGF-I binding, and urea cycle molecular functions. Comparing HD vs NC (Fig. 4C), the upregulated proteins (IGFBP3, DOCK2), showed interactions with the downregulated proteins (DRN, PFDN5, GC, CACNA1E) The LDR vs LD comparison (Fig.4D) showed only connections between three upregulated proteins (i.e. KRT10, KRT3 and KRT2).

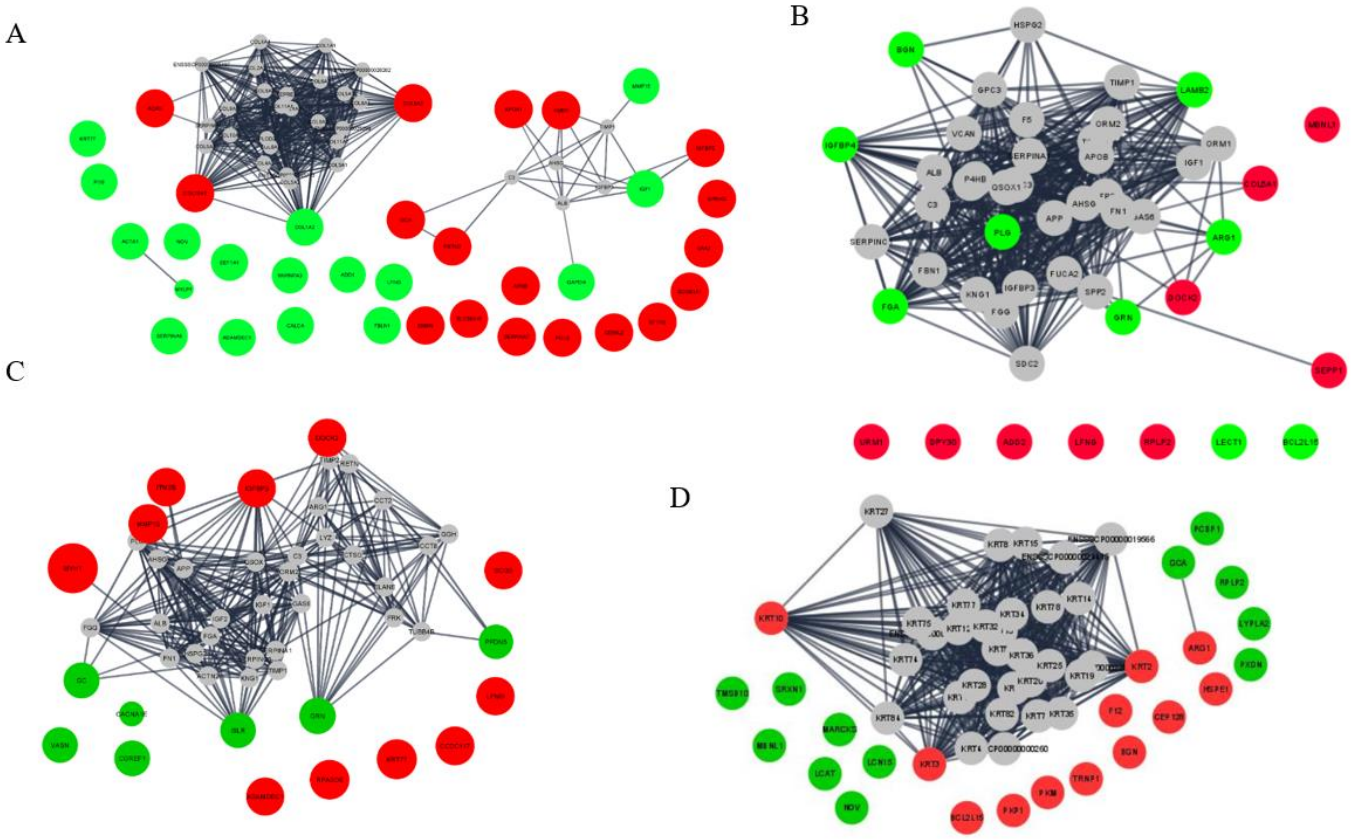

Figure 4. Network analysis of differentially expressed proteins in serum of nursery pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase.

Network of interactions of differentially expressed proteins for (A) NC vs PC, (B) LD vs NC, (C) HD vs NC, (D) LDR vs LD. No protein interactions were detected for HDR vs. HD. The red and green colors are representative of upregulated and downregulated proteins respectively, and the grey color represent the 40% of additional interactors. PC (positive control): normal protein, adequate calcium (Ca) and available phosphorous (aP), no corn-expressed phytase (CEP) added; NC (negative control): low protein, adequate Ca and aP, no CEP added; LD: NC + CEP

added at low dose, i.e. 2,000 one phytase unit (FTU)/kg of diet; HD: NC + CEP added at high dose, i.e. 4,000 FTU/kg of diet; LDR: LD with reduced Ca and P; HDR: HD with reduced Ca and P. *n* = 7-8 for each dietary group.

To examine the relationship between serum proteomics profile and growth performance of pigs supplemented with CEP from our previously published data (Shili et al., 2020), the Pearson correlation analysis was employed (Fig. 5). The results showed that there is a significant positive correlation between feed intake (FI), ADG, final body weight (FBW), G:F ratio, bone mineral density (BMD), bone mineral content (BMC), body lean and fat mass with changes in expression of proteins such as P68137 (ACTA1), A0A287A6F0 (MYLPF), A0A287A391 (EEF1A1), A0A287AAU1 (CCN3), A0A287B7U0 (RPLP2), A0A5G2Q8S2 (PXDN), and K7GRU7 (COL1A2). Also, there was a negative correlation between FBW, G:F ratio, BMD, BMC, body lean and fat mass with the changes in expression of proteins such as Q95274 (TMSB4), Q95JB4 (LEAP2), A0A286ZYQ7 (IGKV2D-40), A0A287BAK1 (TLN1), F1SKG1 (INHBE), I3LM99 (GCA), and T1UNN8 (ANGPTL8).

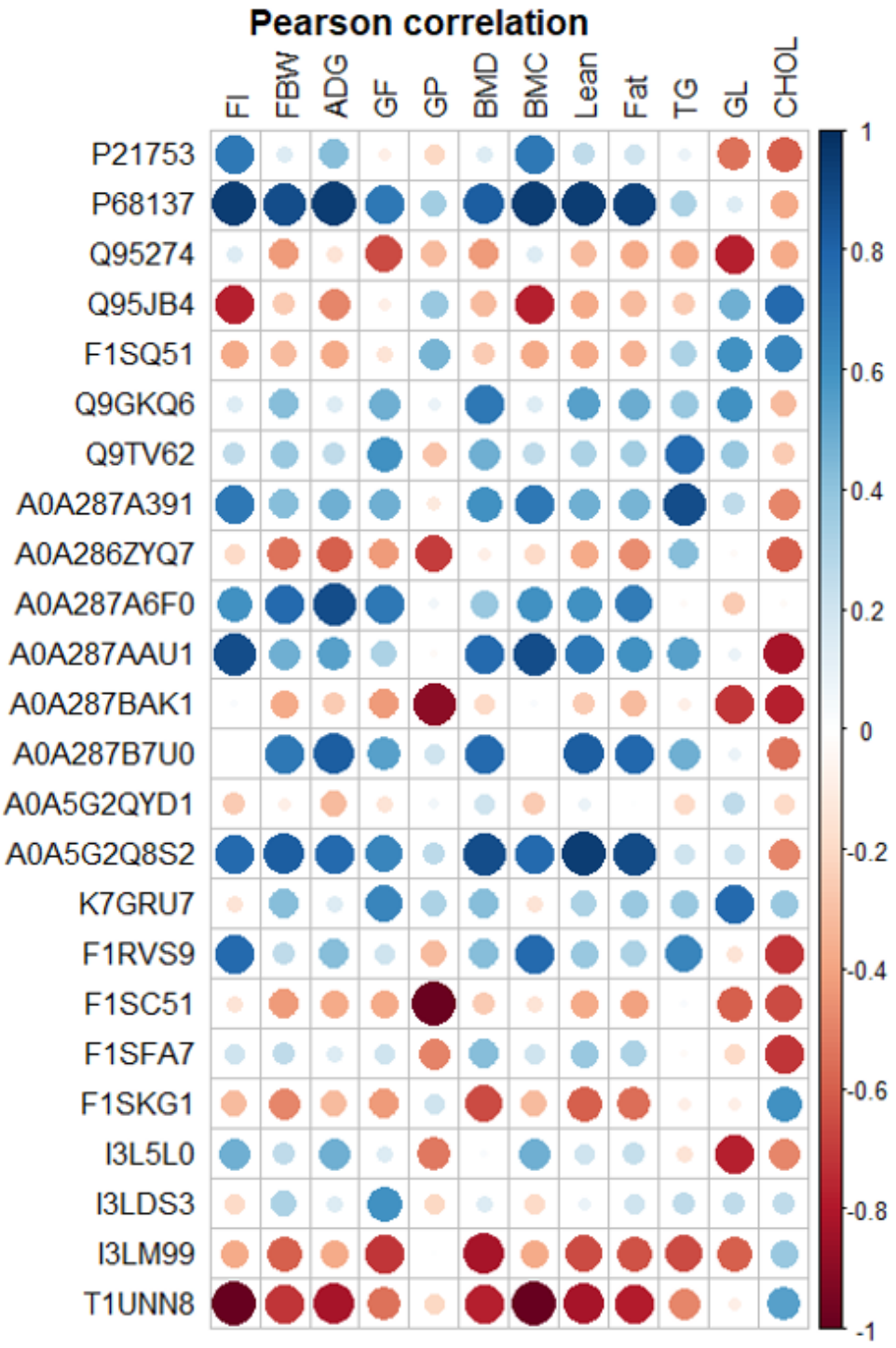

Figure 5. Pearson correlation analysis of overall differentially expressed proteins (protein ID) in serum of pigs fed with low protein diets with or without reduced calcium and phosphorous supplemented with a corn-expressed phytase identified by proteomics approach and growth performance related measurements. The color of circles is based on

the Pearson coefficient distribution: red represents a negative correlation ($P < 0.05$), blue represents a positive correlation ($P < 0.05$), and white is indicative of non-significant correlation ($P > 0.05$) with larger size circles having higher correlation coefficient. Feed intake (FI), final body weight (FBW), average daily gain (ADG), gain:feed ratio (G:F), gain:protein ratio (GP),bone mineral density (BMD), bone mineral content (BMC), triglyceride (TG), glucose (GL), cholesterol (CHOL).

4. Discussion

Very low-protein, Ca and P reduced diets could be potentially used to reduce the environmental concerns associated with excretion of nutrients, postweaning diarrhea, and feed cost in pigs (Yue & Qiao, 2008; Lordelo et al., 2008; Manjarin et al., 2012). However, these diets have a negative influence on the growth performance and health of pigs (Deng et al., 2007b; Yue and Qiao, 2008; Peng et al., 2016). The objective of the present study was to investigate whether supplementation of very low-protein, -Ca and –P diets with a CEP could alter the blood metabolites, nitrogen-containing compounds, and proteomics profiles in nursery pigs. Our study revealed several important findings: (1) very low protein diet reduced the plasma leucine and phenylalanine concentrations, but LD diet recovered the concentration of these two amino acids. Pigs fed with HD had a higher plasma alanine, glycine, and serine than PC, (2) LD changed the expression of serum proteins such as SELENOP, COL5A1, IGFBP4 and BGN that were involved in regulation of growth and blood vessel development, while HD altered the expression of serum proteins such as IGFBP3, SCG5, GC and VASN that are involved in regulation of IGF receptor signaling pathway, hormone secretion, vitamin D metabolic process and cellular response to redox state, (3) A positive correlation was found between growth performance parameters, BMD, BMC, and body composition with changes in expression of proteins such as ACTA1, MYLPF, EEF1A1, CCN3, RPLP2, PXDN, and COL1A2, but there was a negative correlation between these parameters with the changes in expression of proteins such as TMSB4, LEAP2, IGKV2D-40, TLN1, INHBE, GCA,

and ANGPTL8. Overall, CEP improved the blood profile of some essential AA and affected the expression of proteins involved in the regulation of growth and hormone secretion and metabolic process in pigs fed with VLP diets.

A very low protein diet reduced the plasma glycine but feeding pigs with HD diets recovered their levels. This might suggest that CEP improved the plasma concentration of some AA. It has been reported that supplementing diets with phytase enhances the AA digestibility in the intestine of pigs fed with standard protein diets (Johnston et al., 2004; Rutherfurd et al., 2012; Zeng et al., 2014). Similarly, Gagne et al (2002), observed that feeding pigs with standard protein diets containing 647 and 522 FTU/kg phytase raised the plasma concentrations of nitrogen-containing compounds few hours following a meal and in finishing pigs. Authors indicated that the addition of phytase improved the absorption of AA (Gagné et al., 2002). Also, Lala et al., (2020) reported an improvement in ileal digestibility of lysine, threonine, and tryptophan in growing pigs fed with a low protein (14% CP) diet when phytase was supplemented (Lala et al., 2020). This improvement in the profile of amino acids is linked with enhanced growth performance in pigs (Thongsong et al., 2019).

The utilization of proteomics in swine production is developed and its applications are mostly focused on identifying biomarkers of muscle development and indicators of stress and poor welfare at transport and slaughter (de Almeida and Bendixen, 2012). Although, serum proteome profile of pigs has been previously reported (Arrigo and Simon, 2010; Keshan Zhang, 2012), no data is available on alterations in serum proteomics profile when CEP is supplemented in very low protein diets in nursery pigs. Following proteomics analysis, 703 proteins were identified and quantified in the serum proteome of pigs. Out of these, 25 proteins showed differential abundance among all treatments. Others identified 542 proteins in the skeletal muscle with 19 of them showing differential expression between both barrows and gilts fed with standard protein diets without phytase supplementation (Hakimov et al., 2009). The PCA showed distinctive separation between

different dietary treatments, suggesting that each treatment group exhibit differential protein expression profiles when compared to control groups. Furthermore, hierarchical clustering analysis also showed distinctive clusters of upregulated and downregulated proteins differentially abundant among groups. Our data show that protein expression profiles were unique within each treatment group especially in pigs supplemented with CEP.

Here for the first time, we showed that supplementing VLP diets with CEP regardless of the doses used, changed the profile of serum proteins such as IGFBP3 and IGFBP4 involved in the regulation of growth suggesting an important role of these proteins in mediating the positive effects of CEP on growth performance. The IGFBPs act to transport IGFs in the circulation and deliver them to specific tissues, thereby modulating the bioavailability of IGFs to their receptors (Li et al. 2013). Having an important role in the GH-IGF-1 axis, the IGF-1 promotes skeletal growth through regulation of cellular differentiation and proliferation (Ahmed and Farquharson, 2010). Additionally, IGF-1 facilitates protein synthesis in muscle by activating mTOR signaling pathways (Sandri et al., 2013).

A positive correlation was found between growth performance parameters, BMD, BMC, and body composition of pigs fed with VLP diets supplemented with CEP and changes in expression of proteins such as ACTA1, MYLPF, EEF1A1, CCN3, RPLP2, PXDN, and COL1A2, but there was a negative correlation between these parameters with the changes in expression of proteins such as TMSB4, LEAP2, IGKV2D-40, TLN1, INHBE, GCA, and ANGPTL8. Some of these proteins are involved in bone mineralization and muscle structure development.

5. Conclusion

Supplementing VLP diets with CEP improved the blood profile of some essential amino acids and changed the abundance of serum proteins involved in the regulation of growth suggesting an important role of these proteins in mediating the effects of CEP on growth performance. Further, alterations in the expression of serum proteins that are important for bone mineralization and muscle structure development likely contributed to the beneficial effects of CEP on growth, body composition, and bone measurements in pigs fed with VLP diets.

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CHAPTER V

EFFECT OF A PHYTOGENIC WATER ADDITIVE ON GROWTH PERFORMANCE, BLOOD METABOLITES AND GENE EXPRESSION OF AMINO ACID TRANSPORTERS IN NURSERY PIGS FED WITH LOW-PROTEIN/HIGH-CARBOHYDRATE DIETS

This chapter is based on: *Shili CN, Habibi M, Sutton J, Barnes J, Burch-Konda J, Pezeshki A. Effect of a Phytogenic Water Additive on Growth Performance, Blood Metabolites and Gene Expression of Amino Acid Transporters in Nursery Pigs Fed with Low-Protein/High-Carbohydrate Diets. Animals (Basel). 2021 Feb 20;11(2):555. doi: 10.3390/ani11020555. PMID: 33672517; PMCID: PMC7923792.*

Abstract

The objective of this study was to investigate the effect of a phytogenic water additive (PWA) on growth performance and underlying factors involved in pigs fed with low-protein (LP)/high-carbohydrate diets. Forty-eight weaned barrows were allotted to six treatments for 4 weeks: CON-NS, control (CON) diet-no PWA; CON-LS, CON diet-low dose PWA (4 mL/L); CON-HS, CON diet-high dose PWA (8 mL/L); LP-NS, LP diet-no PWA; LP-LS, LP diet-low dose PWA; LP-HS, LP diet-high dose PWA. Relative to CON-NS, pigs fed with CON-HS had increased average daily gain, body weight and serum calcium (Ca) and phosphorous (P) and had decreased mRNA abundance of solute carrier family 7 member 11 and solute carrier family 6 member 19 in jejunum. Compared to LP-NS, pigs fed with LP-HS had increased muscle lean%, decreased muscle fat%, decreased serum Ca and increased serum P.

Compared to their NS counterparts, CON-LS, CON-HS, and LP-LS increased the concentration of plasma essential AA and those fed with CON-HS and LP-HS tended to reduce the abundance of the solute carrier family 7 member 1 transcript in skeletal muscle. Thus, PWA improved the performance of weaned pigs fed with protein-adequate diets likely through increased blood essential AA and affected the muscle composition when dietary protein was deficient.

Keywords: low-protein diets; phytogenic additive; growth performance; nutrient digestibility; metabolites; amino acids; amino acid transporters.

1. Introduction

The intensive swine production in developed countries has raised environmental and sustainable agriculture concerns associated with the excretion of large amounts of waste including nitrogen (N) or nitrogen-containing compounds [1,2]. Therefore, new strategies need to be developed to reduce N excretion from the swine industry to the environment. Low-protein (LP) diets are used to reduce N excretion and other toxic nitrogenous compounds in the swine industry [3–5]. These diets have also been reported to reduce feed cost and alleviate the incidence of diarrhea in early weaning pigs [6,7]. A slight reduction in dietary crude protein (CP), i.e., <25%, along with supplementing limiting amino acids (AA, i.e., lysine, methionine, tryptophan, and threonine) do not appear to have a negative impact on the performance of pigs [3,5,8,9]. However, in order to justify the use of LP diets at larger scales in commercial swine production, additional research is needed to further improve the growth performance of pigs fed with LP diets.

Phytogenic additives are substances originated from plants. These compounds have received increasing attention lately for use in swine and poultry production due to their positive effects on animal production and health [10–15].

Improvement in growth performance was observed when adequate-protein diets were supplemented with phytogenic additives such as Chinese herbal powder in early weaned piglets [16] and herb extract mixture (buckwheat, thyme, curcuma, black pepper, and ginger) in growing pigs [17].

Little is known about whether supplemental phytogenic additives have beneficial effects on the growth performance of pigs fed with LP diets. Manzanilla et al. (2009) showed that supplementing a plant extract mixture to a LP diet (18% CP) in weaned pigs had differential positive or negative effects on various variables measured [18]. In turn, Abousekken et al. (2015) reported that adding *Moringa oleifera* leaf extract to drinking water of broilers fed with LP diet (100 mL/L water) improved growth performance parameters such as weight gain and feed conversion ratio [19]. However, others showed that supplementing a plant extract YGF251 to a diet with very low protein content (14% and 12.5% CP) did not affect the growth performance of growing pigs [20]. Given the very low protein content of the diet used in the former study by Lei et al. (2019) [20] and the marginal effects of feed additives, it appears that the beneficial effects of the plant extract used were not fully explored in that study.

The improved performance in pigs fed with standard protein diets supplemented with phytogenic additives has been linked with improved feed intake and palatability [17,21], immune function [22,23] and blood total antioxidant capacity [24]. Further, phytogenic additives have been reported to enhance intestinal development, ecosystem and microbiota, and nutrient digestibility in pigs fed with standard protein diets [17,23–26,27]. Data on the mechanisms by which phytogenic additives may influence the growth of pigs fed with LP diets are scarce. Little is known about the effect of phytogenic additives on nutrient digestibility when pigs are offered LP diets. This is particularly important as LP diets have been shown to induce a negative effect on the digestibility of nutrients such as phosphorous (P) in monogastrics [28,29]. Xue et al. (2017) showed that ileal-digested P was decreased when the dietary protein content was reduced in growing pigs [28]. In a different study, Xue et al. (2016) reported a reduced total tract retention of P in broiler chickens fed with

diets with low protein content [29]. Further, we previously showed that the concentrations of important blood essential AA such as isoleucine, valine, phenylalanine, tyrosine, and tryptophan were decreased in pigs fed with LP diets [30,31], which can influence animal health and growth performance.

There is evidence that nutrient composition can alter intestinal AA transporter expression in pigs fed with LP diets [32]. Reyer et al. (2017) reported that incubating jejunal cells with a combination of essential oils and saponins at medium and high doses stimulated solute carrier family 15 member 1 (SLC15A1) recruitment to the cytoplasmic membrane in growing broilers [33]. Little is understood about whether phytogenic additives can change the concentration of blood AA as well as the expression of AA transporters in the gut and skeletal muscle of pigs fed with LP diets. We hypothesized that supplementing LP diets with a phytogenic additive would improve the growth performance of pigs through alterations in nutrient digestibility, blood metabolites and AA profile, the expression of intestinal and skeletal muscle AA transporters, and blood antioxidant capacity. Therefore, the objective of this study was to investigate the effect of a phytogenic water additive (PWA) on growth performance, nutrient digestibility, blood metabolites, AA profile and total antioxidant capacity, and gene expression of AA transporters in the gut and skeletal muscle of nursery pigs fed with low-protein/high-carbohydrate diets.

2. Materials and Methods

2.1. Animals, Housing, and Diets

All experimental procedures were performed in accordance with the Oklahoma State University Animal Care and Use Committee and were approved by this committee (Animal Care and Use Protocol # AG-18-6). Forty-eight weanling (three weeks old; 6.3 ± 1.2 kg body weight) crossbred barrows (Duroc sire line and Large White X Landrace dam) were used in this study (Seaboard, Hennessey, OK, USA). Upon arrival, pigs were group-housed and acclimated to the environment

in a facility with controlled temperature, lighting, and ventilation. The animal facility's temperature was set at 31 °C in the first week and then was reduced weekly by 1 °C.

Following 2 weeks of adaptation, all pigs (total n = 48) were weight matched (9.02 ± 0.17 kg), individually housed, and randomly allotted to one of the 6 dietary treatments (n = 8/treatment) in a completely randomized design with 3 × 2 factorial arrangements with the factors of PWA supplement (0, 4 and 8 mL/L of water) and dietary protein content (control and LP) for 4 weeks. Treatments included (1) control (CON) diet with no PWA supplement (CON-NS), (2) CON diet with a low dose of PWA supplement (4 mL/L of water) (CON-LS), (3) CON diet with a high dose of PWA supplement (8 mL/L of water) (CON-HS), (4) LP diet with no PWA supplement (LP-NS), (5) LP diet with a low dose of PWA supplement (4 mL/L of water) (LP-LS) and (6) LP diet with a high dose of PWA supplement (8 mL/L of water) (LP-HS). The used doses for PWA (Herbanimals®, Oklahoma City, OK, USA) were based on guidelines provided by Herbanimals Supplement LLC (Oklahoma City, OK, USA) and the route of administration was based on previous study [34]. The ingredients of PWA (Herbanimals®) used in the current study include 4.83% *Pandanus amaryllifolius Roxb*, 24.15% *Phyllanthus niruri*, 4.83% *Amomum cardamomum*, 13.04% *Zingiber zerumbet*, 14.49% *Apium Graveolens*, 14.49% *Anethum Graveolens*, 4.83% *Ocimum americanum*, 4.83% *Cinnamomum burmannii Blume*, 4.83% *Myristica fragrans Houtt* and 9.66% *Zingiber officinale roscoe*. The ingredients and analyzed composition of Herbanimals® for vitamins and minerals are given in Table S1.

Phase feeding was applied according to the recommendations of the nutrient requirement of swine [35]. Nursery phase 1 (N1) diet was fed for 1 week (days 1–7 of study), nursery phase 2 (N2) diet was fed for 2 weeks (days 8–21 of study) and nursery phase 3 (N3) diet was offered for 3 weeks (days 22–42 of study) (Table 1). To achieve the desired CP levels and maintain the energy content consistent between the CON and LP diets, the amount of soybean meal as the primary source of protein was decreased, and corn as the major source of carbohydrate was increased in LP diets. Therefore, LP diets, as expected, had a higher carbohydrate content than CON diets (Table 1). LP

diets were supplemented with limiting AA (i.e., lysine, methionine, threonine, and tryptophan) at levels equal to the CON diet. All diets contained 0.5% chromium oxide as an indigestible marker for apparent total tract digestibility tests. All pigs had free access to feed and water throughout this study.

Table 1. Ingredients and chemical composition of experimental diets (as-fed basis).

	Diets [1]				
	N1	N2		N3	
		CON	LP	CON	LP
Ingredients, %					
Corn, yellow dent [2]	32.21	45.04	64.72	57.06	73.49
Soybean meal, 47.5% CP [2]	15.00	36.22	13.94	38.39	19.95
Fish meal, menhaden [2]	6.00	4.90	4.98	2.07	2.11
Whey, dried [2]	25.00	5.87	5.97	-	-
Lactose [2]	7.00	-	-	-	-
Corn starch [2]	-	5.87	5.97	-	-
Plasma spray-dried [2]	6.00	-	-	-	-
Soy protein concentrate [2]	2.20	-	-	-	-
Soybean oil [2]	4.00	-	-	-	-
Dicalcium phosphate [2]	0.67	0.76	0.85	0.99	1.15
Limestone [2]	0.45	0.54	0.67	0.62	0.68
Nursery vitamin premix [3]	0.05	0.19	0.19	0.18	0.20
Salt [2]	0.50	0.49	0.50	0.57	0.52
Chromium oxide [2]	-	0.50	0.50	0.50	0.52
Trace mineral premix [4]	0.06	0.07	0.06	0.07	0.08
Selplex [2]	0.05	-	-	-	-
Choline Cl [2]	0.03	-	-	-	-
Zinc oxide, 72% Zn [2]	0.35	-	-	-	-
L-Lysine, sulfate [2]	0.17	-	1.12	-	0.88
DL-Methionine [2]	0.18	-	0.14	-	0.08
L-Threonine [2]	0.07	-	0.28	-	0.22
L-Tryptophan [2]	-	-	0.13	-	0.10
Calculated Chemical Composition [5]					
Dry matter, %	92.34	90.72	90.72	89.66	89.60
ME, Mcal/kg	3.52	3.40	3.40	3.30	3.32
Crude protein, %	22.99	24.71	17.03	24.26	17.83
Crude fiber, %	1.39	2.31	2.01	2.64	2.39
Crude fat, %	6.68	3.36	3.46	3.57	3.66
Nitrogen, %	3.65	3.95	2.72	3.88	2.85
Calcium, %	0.89	0.80	0.80	0.70	0.70
Phosphorous, %	0.79	0.71	0.63	0.68	0.62
Available phosphorous, %	0.59	0.40	0.40	0.32	0.33
SID Lysine, %	1.54	1.35	1.35	1.24	1.23
SID Threonine, %	0.97	0.84	0.80	0.80	0.76
SID Methionine, %	0.51	0.38	0.41	0.36	0.35
SID Tryptophan, %	0.27	0.27	0.28	0.27	0.27
Analyzed Chemical Composition [6]					
Dry matter, %	90.10	89.00	87.00	91.30	87.40
Crude protein, %	22.70	24.70	17.30	23.00	17.70
Carbohydrate, % kcal	45.33	70.21	79.98	77.79	86.10
Crude fiber, %	1.30	2.00	1.60	3.50	2.10
Calcium, %	0.85	0.81	0.82	0.80	0.85
Phosphorus, %	0.75	0.65	0.56	0.70	0.61

Nitrogen, %	3.60	3.90	2.80	3.68	2.80

[1] CON: standard protein diet; LP: low-protein diet, N1: nursery phase 1, fed for one week of study (from d 1 to 7); N2: nursery phase 2, fed for two weeks of study (from day 8 to 21; 7–11 kg body weight); N3: nursery phase 3, fed for three weeks of study (from day 22 to 42; 11–25 kg body weight). [2] Corn, fish meal, soybean meal, whey, lactose, corn starch, plasma spray-dried, soy protein concentrate, soybean oil, dicalcium phosphate, limestone, salt, and choline chloride (Cl) were obtained from Nutra Blend, LLC (Neosho, MO). DL-methionine (99%) (MetAMINO®) and L-lysine, sulfate (Biolys®) were obtained from Evonik (Kennesaw, GA). L-threonine (98.5%) and L-tryptophan (98%) were obtained from Ajinomoto (Overland Park, KS). Chromium oxide was purchased from Fisher Scientific (Bartlesville, OK). Selplex was obtained from Alltech (Lexington, Kentucky). [3] Vitamins premix was purchased from Nutra Blend, LLC (Neosho, MO). Vitamins premix contained: vitamin A, 1,650,000 IU/kg; vitamin D3, 660,000 IU/kg; vitamin E, 17,600 IU/kg; vitamin B12, 13.2 mg/kg; vitamin K (menadione), 1,320 mg/kg; niacin, 19,800 mg/kg; D-pantothenic acid, 11,000 mg/kg; riboflavin, 3,300 mg/kg; phytase, 300,000 FYT/kg. [4] Trace minerals premix was purchased from Nutra Blend, LLC (Neosho, MO). Trace minerals premix contained: iron, 73,000 ppm; zinc, 73,000 ppm; manganese, 22,000 ppm; copper, 11,000 ppm; iodine, 198 ppm; selenium, 198 ppm. [5] Values were calculated using National Swine Nutrition Guide (NSNG; V 2.0). [6] Diets were analyzed by ServiTech (Dodge City, KS). The carbohydrate (% kcal of gross energy) was calculated from the estimated caloric value of carbohydrates at 4 kcal/g.

2.2. Growth Performance

The individual feed and water intakes were recorded daily and body weight was measured weekly. The average daily gain (ADG), average daily feed intake (ADFI), cumulative feed intake (CFI), weight gain to feed intake ratio (G:F), and weight gain to protein intake ratio (G:P) were computed by using the individual feed intake and body weight records and analyzed dietary protein concentration. The weekly body weight gain (BWG), cumulative feed intake (CFI), cumulative protein intake (CPI), and G:F and G:P ratios were calculated for 4 weeks of data collected.

2.3. Feed and Fecal Samples Collection

Approximately 500 g feed samples were collected after mixing each diet and stored at −20 °C until analysis. Fecal samples were collected at week 6 of this study following transferring the pigs to metabolic crates for 24 h, where they had free access to feed and water. Following collection, the fecal samples were stored at −20 °C until analysis.

2.4. Blood and Tissue Samples Collection

At the end of this study, blood samples were drawn from the jugular vein of all pigs in the supine position in 10 mL sterile dry and 3 mL sterile sodium heparin-coated tubes (BD, Franklin Lakes, NJ, USA) for collection of serum and plasma, respectively. Blood samples were placed on ice after collection, transferred to the laboratory, and centrifuged at 4 °C for 10 min and at 2000 × *g* to collect the serum or plasma. The collected serum and plasma were stored at −80 °C until further analysis. After blood collection, all pigs were euthanized via CO2 asphyxiation, and skeletal muscle (i.e., Biceps femoris) and jejunum samples were collected. Tissue samples were rinsed with distilled water after dissection, snap-frozen in liquid nitrogen, and stored at −80 °C until later processing. Separate muscle samples were collected (~150 g) and stored at −20 °C for lean and fat content analysis.

2.5. Feed and Fecal Samples Composition Analysis

As we previously described [36], feed samples were analyzed by ServiTech Laboratories (Dodge City, KS, USA) for dry matter, crude fiber, calcium (Ca), P, N, and chromium using official methods of analysis of AOAC [37]. Fecal samples were analyzed by ServiTech Laboratories (Dodge City, KS, USA) for Ca, P, N, and chromium using the methods indicated above for analysis of feed samples.

2.6. Plasma Nitrogen-Containing Compounds Analysis

Plasma samples were analyzed for nitrogen-containing compounds using a LI-BASED Hitachi 8900 (Hitachi High-Technologies Corporation, Tokyo, Japan) as previously described [38] at Molecular Structure Facility, Proteomics Core (UC Davis Genome Center, Davis, CA). Briefly, plasma samples were thawed at room temperature, acidified to 2% sulfosalicylic acid and incubated for 15 min at room temperature. Samples were then stored at −20 °C overnight. The following day, the samples were thawed and diluted with Li sample diluent (Pickering Labs, Mountain View, CA, USA) containing 100 nmol/mL AE-Cys, before injection (50 uL). The ion-exchange chromatography using a HITACHI L-8900 Amino Acid Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan) with a post-column ninhydrin reaction was used to separate free AA. Column and buffers were provided by Hitachi (Tarrytown, NY, USA), and ninhydrin was provided by Wako Chemicals (Richmond, CA, USA). The AA standards (Sigma-Aldrich, St. Louis, MO, USA) were used for the calibration of the Amino Acid Analyzer. Absorbances were measured at both 570 and 440 nm after the reaction with ninhydrin to regulate the response factor for each AA and to quantify AA concentrations relative to the known AA and related compound standards (0.5 μmole/mL in 0.2 N lithium citrate, pH 2.2 containing 0.1% phenol and 2% thiodiglycol). The internal standard (AE-Cys, Sigma #A2636) was included due to variations in injection volume that might be caused by the autosampler.

2.7. Muscle Composition Analysis

Muscle samples collected from each pig were scanned by dual-energy X-ray absorptiometry (DEXA; Hologic, Discovery QDR Series, Bedford, MA, USA) to determine the lean and fat content using the rodent's calibration feature.

2.8. Serum Metabolites Analysis

The concentrations of Ca, P, alkaline phosphatase (ALP), and blood urea nitrogen (BUN) were measured in serum samples (>300 µL) using an automated chemistry analyzer system (CLC 480/BioLis24i, Carolina Liquid Chemistries Corp., Brea, CA, USA). After calibration of the equipment (Catalogue # BL-442600, Multi-Analyte calibrator for Synchron CX/LX), the reagents of Ca (Product #BL251), P (Product #BL218), ALP (Product #BL206), and BUN (Product #BL252) were used to quantify the concentration of these parameters in serum. The absorbances of Ca and P were recorded at 660 and 340 nm, respectively. The absorbances of BUN and ALP were measured at 405 nm.

2.9. RNA Isolation and RT-qPCR Analysis

Total RNA extraction, reverse transcription, and quantitative PCR were performed as we described previously [39]. Approximately 300 mg of skeletal muscle and jejunum tissues were grounded using a mortar and pestle and liquid nitrogen. The ground samples then were homogenized with 1 mL Qiazol (Qiagen, Catalogue #74106, Germantown, MD, USA). The RNA was isolated using RNeasy® mini kit (Qiagen, Catalogue #74106, Germantown, MD). The isolated RNA concentration was quantified and the ratio of absorbance at 260 nm and 280 nm was recorded using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher, Waltham, MA, USA). The 260/280 nm absorption ratios for all RNA samples were 1.7–2.0. RNA samples were DNAse treated (Thermo Fisher, Waltham, MA, USA) and used for complementary DNA (cDNA) synthesis. cDNA was synthesized in a 20 µL reaction volume using 5 µL of DNase-treated RNA (1.25 µg), 4 µL of 5x first-strand buffer, 2 µL of random primers (3 µg/µL), 2 µL of deoxyribonucleotide triphosphate (dNTP) mix [2.5 mM], 2 µL of dithiothreitol (DTT) (0.1 M), 1 µL of RNaseOUT (40 U/µL), 1 µL of superscript II reverse transcriptase (200 U/µL), and 3 µL of RNase/DNase-free water (Thermo Fisher Scientific Waltham, MA) using the following program: 22 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and terminated at 4 °C in a thermocycler (T100™

Thermal Cycler, Bio-Rad, Hercules, CA, USA). The primers' sequences were obtained from previous publications as listed in Table 2 [40–43]. Using a 96-well plate, the real-time quantitative PCR (qPCR) was performed using a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The PCR was performed in a 25 μL total volume reaction, with 2 μL of the first-strand cDNA, 12.5 μL of SYBR Green master mix (Applied Biosystems Inc. Waltham, MA, USA), 0.2 μL of F, 0.2 μL of R sequences of each primer (100 mM), and 10.1 μL of RNase/DNase-free water (Thermo Fisher Scientific Waltham, MA, USA). The qPCR program used was: denaturation at 50 °C for 2 min and 95 °C for 10 min, 40 cycles amplification at 95 °C for 15 s and 60 °C for 1 min; then a melt curve program: 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Finally, the $2^{-\Delta\Delta CT}$ method [44] was used for the calculation of the mRNA abundance of target genes that were normalized to β-actin mRNA abundance as a housekeeping gene.

Table 2. Primer sequences, forward (F) and reverse (R), location on the template, amplicon size (bp), and GenBank accession numbers for both target and reference genes for reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR).

Genes [1]	Sequence (5′ → 3′)	Location on Template	Amplicon Length (bp)	GenBank Accession Number
SLC15A1	F: AGCATCTTCTTCATCGTGGTCAA	43-65	206	NM_214347.1
	R: GTCTTGAACTTCCCCAGCCA	229-248		
SLC7A9	F: ATCGGTCTGGCGTTTTAT	816-833	145	XM_021093176.1
	R: GGATCTAGCACCCTGTCA	943-960		
SLC7A8	F: TTTCCAGGAACCTGACATCG	576-595	200	XM_003128550.6
	R: ACATTGCAGTGACATAAGCG	756-775		
SLC6A19	F: CACAACAACTGCGAGAAGGA	1101-1120	155	XM_003359855.4
	R: CCGTTGATAAGCGTCAGGAT	1236-1255		
SLC3A1	F: TTTCCGCAATCCTGATGTTC	1107-1126	146	NM_001123042.1
	R: GGGTCTTATTCACTTGGGTC	1233-1252		
SLC7A11	F: CGGCTCCTGGGAAATTTCTC	1297-1316	72	XM_021101587.1
	R: ACCATTCATGGAGCCAAAGC	1349-1368		
SLC7A1	F: TCTCATCCTAACGGGACTTTTAACTC	2525-2550	85	XM_021065165.1
	R: GACCAGAACGTTGATACACGTGAA	2586-2609		
β-Actin	F: CTGCGGCATCCACGAAACT	944-962	147	XM_021086047.1
	R: AGGGCCGTGATCTCCTTCTG	1071-1090		

[1] *SLC15A1*: solute carrier family 15 member 1 [40], *SLC7A9*: solute carrier family 7 member 9 [40], *SLC7A8:* solute carrier family 7 member 8 [40], *SLC6A19:* solute carrier family 6 member 19 [41], *SLC3A1:* solute carrier family 3 member 1 [41], *SLC7A11:* solute carrier family 7 member 11 [41], *SLC7A1:* solute carrier family 7 member 1 [42], *and β-actin:* beta-actin [43]. F: forward (sense) primer; R: reverse (antisense) primer.

2.10. Total Antioxidant Capacity

The total antioxidant capacity (TAC) of serum was analyzed using a TAC kit (ab65329, Abcam, Cambridge, MA, USA) following the manufacturer's instructions. Briefly, after preparing all the reagents to work concentrations, 100 µL of standards solution, 5 µL of serum samples, 5 µL of protein masks, and 90 µL of water were added to each well of a 96-well plate in duplicate. The plate was placed on a plate shaker in the dark for 1.5 h at room temperature to reduce the added 100 µL of Cu^{2+}. After the reduction of Cu^{2+}, the solution was chelated with a colorimetric probe, and absorbance was measured at 570 nm using an Epoch microplate reader spectrophotometer (BioTek® instrument, Inc. Winooski, VT, USA). The intra-assay coefficient of variation (CV) was 6.55%.

2.11. Apparent Fecal Digestibility of Nutrients

As we previously described [36], apparent fecal digestibility (AFD) of Ca, P, and N was calculated using the index method with chromium as an external marker. The following equation was used to calculate the AFD of nutrients: 100 − (100 × (marker concentration in feed/marker concentration in feces) × (nutrient concentration in feces/nutrient concentration in feed).

2.12. Statistical Analysis

As we previously described [36] using Al-Therapy Statistics (https://www.ai-therapy.com/psychology-statistics/sample-size-calculator (accessed on 3/7/2018) and data from our previous study [30], we measured the sample size before starting this study. Our power analysis showed that when 8 pigs/dietary group is used, a 7.90 kg difference in body weight between two groups (SD: control diet = 4.27 kg, low-protein diet = 4.26 kg) can be detected with 93% power ($\alpha = 0.05$; effect size = 1.852). The ADG, ADFI, final body weight, average daily water intake (ADWI), average daily protein intake (ADPI), G:F and G:P ratios, AFD of nutrients, blood metabolites, DEXA, TAC, and gene expression data were analyzed using two-way ANOVA including the main effects of protein, PWA and protein × PWA in the model (SPSS ®, IMB SPSS Statistics version 23, Armonk, NY, USA). The difference between the means

of treatments for all parameters was separated using pairwise Student's t-test corrected by the Benjamini–Hochberg procedure [45] with 0.1 false discovery for 5 comparisons, i.e., CON-NS vs. CON-LS, CON-NS vs. CON-HS, CON-NS vs. LP-NS, LP-NS vs. LP-LS, LP-NS vs. LP-HS. The daily feed intake, weekly body weight, and weekly growth performance data were analyzed with a linear mixed model of SPSS ® (IMB SPSS Statistics version 23, Armonk, NY, USA), with the effect of protein, PWA, time, protein × PWA, protein × time, PWA × time and protein × PWA × time as fixed and the pig as a random variable included in the model. Based on the smallest values of fit statistics for the corrected Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC), the covariance structure of the repeated measurements model for daily feed intake, and weekly body weight and CPI were autoregressive and for weekly BWG, CFI, CWI, G:F and G:P were heterogeneous autoregressive. $p \leq 0.05$ and $0.05 < p \leq 0.1$ were considered to declare significant difference and trends, respectively.

3. Results

3.1. Growth Performance

The initial BW was not different among groups (9.02 ± 0.17 kg; Table 3). There was a significant effect of dietary protein ($p < 0.05$) on final BW and ADG, with LP pigs having lower final BW (24.37 vs. 26.83 kg) and ADG (0.55 vs. 0.63 kg) than CON pigs (Table 3). However, there were no differences between LP-NS and CON-NS pigs on final BW and ADG. The effect of protein × PWA on final BW and ADG tended to be significant ($p < 0.1$; Table 3). While there were no differences in ADG and final BW of pigs fed with LP-NS, LP-LS, and LP-HS, pigs in CON-HS had 19% higher ADG and tended to have 15% higher final BW compared to those fed CON-NS (Table 3; Figure S1A). When the data were analyzed on a weekly basis, there was a significant protein effect on BWG, with LP pigs having lower BWG than CON counterparts during the first (3.60 vs. 4.10 kg) and second week (3.53 vs. 4.13 kg) of the study ($p < 0.01$; Table 4). No differences in BWG were detected among treatments during the first three weeks, but pigs fed CON-HS had 34% higher ($p < 0.05$) BWG than those fed with CON-NS at week 4 (Table 4).

Table 3. Growth performance and muscle fat and lean content of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive.

	Diets [1]							*p*-Value		
Parameters	**CON-NS**	**CON-LS**	**CON-HS**	**LP-NS**	**LP-LS**	**LP-HS**	**SEM [2]**	**Protein**	**PWA [3]**	**Protein × PWA**
Initial BW [4], kg	8.82	9.10	9.63	9.05	8.76	8.76	0.17	0.36	0.78	0.46
Final BW [4], kg	25.34 *	26.08	29.09 *	24.89	25.00	23.25	0.56	0.02	0.72	0.09
ADG [4], kg/d	0.59	0.60	0.70 [b]	0.57	0.58	0.52	0.01	0.01	0.75	0.07
ADFI [4], kg/d	0.86	0.95	0.97	0.84	0.91	0.86	0.02	0.23	0.30	0.69
ADWI [4], L/d	3.09	2.98	4.17	2.78	3.54 [d]	2.98	0.17	0.35	0.31	0.12
G:F [4], kg/kg	0.69	0.63	0.72	0.67	0.62	0.61	0.01	0.01	0.97	0.72
G:P [4], kg/kg	2.97	2.83	3.09	3.83 [c]	3.58	3.38	0.09	0.01	0.33	0.12
Muscle lean%	82.83	83.35	86.00	78.58	80.32	88.7 [e]	0.86	0.01	0.01	0.01
Muscle fat%	16.62	16.06	13.51	20.85	13.33	9.41 [e]	0.82	0.01	0.01	0.01

[1] CON-NS: control diet with no phytogenic water additive (PWA) supplemented, CON-LS: control diet with a low dose of PWA (4 mL/L of water) supplemented, CON-HS: control diet with a high dose of PWA (8 mL/L of water) supplemented, LP-NS: low-protein diet with no PWA added, LP-LS: low-protein diet with a low dose of PWA (4 mL/L of water) added and LP-HS: low-protein diet with a high dose of PWA (8 mL/L of water) added. Values are the means. n = 8/diet. [2] SEM: standard error of the mean. [3] PWA: phytogenic water additive. [4] BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; ADWI: average daily water intake; G:F: gain:feed ratio; G:P: gain:protein ratio. [b] $p \leq 0.05$ CON-NS vs. CON-HS, [c] $p \leq 0.05$ CON-NS

vs. LP-NS, [d] $p \leq 0.05$ LP-NS vs. LP-LS, and [e] $p \leq 0.05$ LP-NS vs. LP-HS. * Within a row, values with a common superscript symbol tend to be different ($0.05 < p \leq 0.1$).

The effects of protein, PWA, and protein × PWA on ADFI and ADWI were not significant (Table 3). There was an overall significant effect of time ($p < 0.01$) on FI, with pigs fed with CON-HS tending to have a 70% higher feed intake compared to those fed with CON-NS on day 5 (Figure S1B). Cumulative feed intake did not change among groups during the entire study (Table 4). When CPI was assessed on a weekly basis, there was a significant effect of protein during all four weeks of this study, with LP pigs having a lower CPI than CON pigs (0.57 vs. 0.88, 0.92 vs. 1.31, 1.26 vs. 1.71, 1.53 vs. 2.11 kg for weeks 1 to 4, respectively). Additionally, the effect of PWA on CPI was significant in the second week of study with a dose-dependent increase in CPI (0.99, 1.42, and 1.22 kg for NS, LS, and HS, respectively). There was a significant effect of protein × PWA on CPI during week 4 of this study, suggesting that PWA improved the CPI only with a standard level of dietary protein (Table 4). During weeks 2 and 3, pigs fed LP-NS tended to have 30% and 28% less CPI than those fed with CON-NS, respectively, while pigs from the CON-HS group tended to have a 20% higher CPI during the second week compared to CON-NS pigs. Additionally, pigs fed LP-HS tended to have a 26% higher CPI than those fed with LN-NS during the second week of this study. Pigs in the LP-LS group had 27% higher ADWI than pigs fed with LP-NS (Table 3). When water intake was analyzed weekly, there was a significant effect of protein on CWI during week 2 of this study with LP pigs having a lower water intake than CON (18.30 vs. 24.13 L) (Table 4). Pigs fed with LP-NS consumed 48% less water during the second week compared to those fed with CON-NS. Pigs fed with LP-HS consumed 83% more water compared to animals fed with LP-NS during the second week of this study. On week 4, LP-LS pigs tended to consume 47% more water than LP-NS.

There was a significant effect of protein ($p < 0.05$) on G:F and G:P ratios (Table 3). Relative to CON, LP pigs had lower G:F (0.62 vs. 0.70) and greater G:P ratios (3.59 vs. 2.96). However, no differences in G:F ratio were detected when CON-NS and LP-NS pigs were compared. Pigs fed with LP-NS diet had a 29% higher G:P than the CON-NS group. There was a significant protein effect on G:F ratio ($p < 0.05$) on week 1, with LP pigs having a lower G:F ratio compared to CON

pigs (0.51 vs. 0.78). Additionally, the effect of PWA on G:F ratio was significant ($p < 0.05$) in week 1 and 2, with a dose-dependent increase on G:F ratio during the first week (0.50, 0.59, and 0.83) and a decrease in G:F ratio during the second week (0.77, 0.76 and 0.62) for NS, LS, and HS, respectively (Table 4). A significant protein effect ($p < 0.05$) was detected on weekly G:P ratio during weeks 2, 3, and 4, with a higher G:P ratio in pigs fed with LP diets than those fed with CON diets (3.88 vs. 3.31, 3.63 vs. 2.94 and 3.60 vs. 3.01, for weeks 2, 3 and 4, respectively). The effect of PWA on the G:P ratio in weeks 1 and 2 was significant. During week 1, increasing the levels of PWA, increased the G:P ratio (2.40, 2.76, and 3.72 for NS, LS, and HS, respectively). Pigs fed with LP-HS had a 57% increase and a 22% decrease in the G:P ratio compared to those fed with LP-NS, during the first and third week of this study, respectively. Pigs fed with LP-NS had a 43% and 32% greater G:P ratio than those fed with CON-NS during the third and fourth weeks of study, respectively (Table 4).

Table 4. Weekly growth performance of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive.

	Diets [1]						SEM [2]	*p*-Value		
Parameters	**CON-NS**	**CON-LS**	**CON-HS**	**LP-NS**	**LP-LS**	**LP-HS**		**Protein**	**PWA [3]**	**Protein × PWA**
BWG [4], kg										
Wk 1	1.98	2.32	2.97	1.36	1.24	2.77	0.12	0.04	0.47	0.18
Wk 2	4.05	4.24	4.20	3.33	4.13	3.22	0.13	0.03	0.19	0.41
Wk 3	5.01	4.80	4.91	4.96	5.09	3.90	0.18	0.39	0.47	0.20
Wk 4	5.48	5.88	7.37[b]	6.22	5.77	4.75	0.21	0.14	0.77	0.03
CFI [4], kg										
Wk1	3.35	3.63	3.76	2.97	3.10	3.82	0.14	0.32	0.19	0.69
Wk 2	5.11	5.83	6.17	4.61	5.33	5.80	0.19	0.21	0.04	0.98
Wk 3	7.30	7.72	7.38	6.79	7.83	6.85	0.22	0.49	0.34	0.80
Wk 4	8.28	8.92	9.67	9.33	9.49	7.86	0.25	0.89	0.72	0.05
CPI [4], kg										
Wk1	0.83	0.90	0.93	0.51	0.54	0.66	0.03	0.01	0.26	0.84
Wk 2	1.17*[#]	1.34	1.41[#]	0.81*[$]	0.94	1.02[$]	0.04	0.01	0.04	0.97
Wk 3	1.67*	1.77	1.69	1.20*	1.38	1.21	0.05	0.01	0.38	0.89
Wk 4	1.89	2.18	2.25	1.62	1.64	1.33	0.06	0.01	0.42	0.03
CWI [4], L										
Wk 1	12.31	15.95	14.45	12.42	10.90	13.95	0.92	0.34	0.73	0.46
Wk 2	24.19	23.18	25.02	12.54[c]	19.52	22.93[e]	1.50	0.05	0.30	0.36
Wk 3	25.51	22.90	30.71	26.09	29.98	23.65	1.55	0.96	0.93	0.20
Wk 4	30.04	26.43	34.94	26.13*	38.39*	24.72	1.85	0.81	0.63	0.04
G:F [4], kg/kg										
Wk 1	0.59	0.64	0.79	0.46	0.40	0.73	0.05	0.01	0.03	0.60
Wk 2	0.79	0.72	0.68	0.73	0.77	0.57	0.02	0.55	0.01	0.31
Wk 3	0.69	0.62	0.66	0.73	0.66	0.57	0.02	0.54	0.13	0.15
Wk 4	0.66	0.63	0.76	0.67	0.61	0.60	0.01	0.16	0.54	0.38
G:P [4], kg/kg										
Wk 1	2.39	2.58	3.19	2.67	2.30	4.20 [e]	0.20	0.94	0.03	0.14

Wk 2	3.46	3.16	2.98	4.11	4.39	3.16	0.10	0.02	0.01	0.22
Wk 3	3.00	2.71	2.91	4.13 [c]	3.69	3.22 [e]	0.09	0.01	0.06	0.16
Wk 4	2.90	2.70	3.28	3.84 [c]	3.52	3.57	0.07	0.01	0.56	0.27

[1] CON-NS: control diet with no phytogenic water additive (PWA) supplemented, CON-LS: control diet with a low dose of PWA (4 mL/L of water) supplemented, CON-HS: control diet with a high dose of PWA (8 mL/L of water) supplemented, LP-NS: low-protein diet with no PWA added, LP-LS: low-protein diet with a low dose of PWA (4 mL/L of water) added and LP-HS: low-protein diet with a high dose of PWA (8 mL/L of water) added. The *p*-values for the overall model effect of protein, PWA, week, protein × PWA, protein × week, PWA × week and protein × PWA ×week for BWG were 0.01, 0.61, 0.01,0.07, 0.85, 0.48, and 0.12, for CFI were 0.35, 0.31, 0.01, 0.68, 0.86, 0.19, and 0.05, for CPI were 1.00, 0.94, 0.01, 0.01, 0.09, 0.11, and 0.01, for CWI were 0.26, 0.18, 0.01, 0.20, 0.39, 0.57and 0.06, for G:F were 0.05, 0.87, 0.26, 0.42, 0.01, 0.01 and 0.57, and for G:P were 0.66, 0.52, 0.01, 0.07, 0.42, 0.01 and 0.01, respectively. Values are the means. n = 8/diet. [2] SEM: standard error of the mean. [3] PWA: phytogenic water additive. [4] BWG: body weight gain (weekly); CFI: cumulative feed intake; CPI: cumulative protein intake CWI: cumulative water intake; G:F: gain: feed; G:P: gain: protein. [b] $p \leq 0.05$ CON-NS vs. CON-HS, [c] $p \leq 0.05$ CON-NS vs. LP-NS, and [e] $p \leq 0.05$ LP-NS vs. LP-HS. *[#$] Within a row, values with a common superscript symbol tend to be different ($0.05 < p \leq 0.1$).

3.2. Muscle Fat and Lean Content

Muscle lean% and fat% are shown in Table 3. There was a significant effect of protein, PWA, and protein × PWA ($p < 0.01$) on muscle lean% and fat%. Pigs in the LP group had higher muscle lean% (85.1%) and lower fat% (13.9%) than those in the CON group (81.5% and 17.8% for lean and fat%, respectively). Supplementing diets with PWA dose-dependently increased muscle lean% (81.5%, 82.0%, and 86.6% for NS, LS, and HS, respectively) and decreased muscle fat% (17.9%, 17.1% and 12.7% for NS, LS, and HS, respectively). While there was no difference in muscle lean% and fat% of pigs in CON-LS, CON-HS, and CON-NS, pigs fed with LP-HS had 13% more lean and 55% less fat compared to those fed with LP-NS (Table 3).

3.3. Plasma Nitrogen-Containing Compounds

Plasma nitrogen-containing compounds are shown in Table 5. There was a significant effect of protein ($p < 0.05$) on the majority of plasma AA concentration. Overall, pigs fed LP diets had a lower concentration of plasma leucine, isoleucine, phenylalanine, histidine, arginine, valine, tyrosine, proline, and creatinine, and had higher plasma lysine, methionine, tryptophan, threonine, alanine, and glutamic acids than those fed with CON diets. Pigs fed with LP-NS had lower plasma valine, isoleucine, histidine and 3, methylhistidine, and higher lysine, serine, glycine, sarcosine, and alanine than CON-NS pigs ($p < 0.05$). Overall, pigs in the HS group had a higher concentration of methionine, tryptophan, leucine, isoleucine, valine, phenylalanine, arginine, serine, glutamine, and tyrosine compared to LS and NS groups. Pigs fed CON-HS had a higher plasma concentration of valine, isoleucine, leucine, phenylalanine, tryptophan, lysine, histidine, arginine, serine, glycine, alanine, tyrosine, asparagine, proline, and sarcosine and lower glutamic acid, aspartic acid, and taurine than CON-NS. Moreover, the plasma concentration of leucine, serine, glycine, proline, sarcosine, and alanine was higher, and methionine and lysine tended to be higher in pigs fed with

CON-LS than those fed with CON-NS. Pigs fed with LP-LS had a higher ($p < 0.05$) plasma valine, histidine, and alanine and tended to have a higher plasma leucine, isoleucine, phenylalanine, and ornithine compared to those fed with LP-NS. The plasma concentration of histidine, serine, glutamic acid, taurine, and ammonia were lower and lysine and proline tended to be lower in the LP-HS group when compared to LP-NS ($p < 0.05$).

Table 5. The concentration (nmol/mL) of plasma nitrogen-containing compounds of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive.

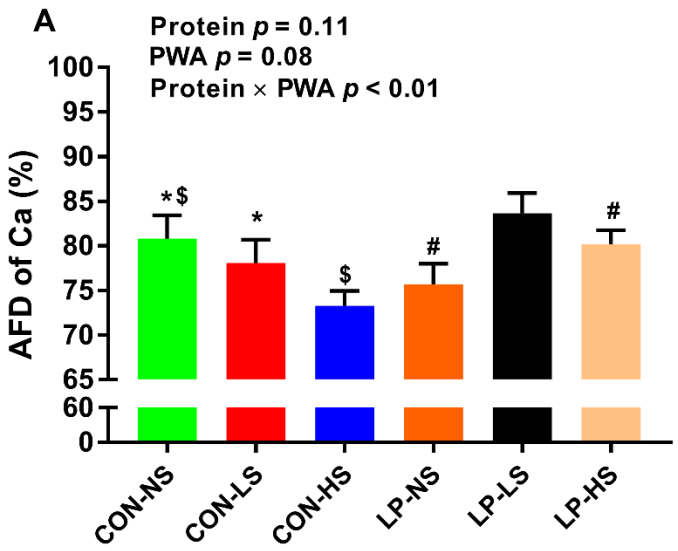
Item	Diets [1]						SEM [2]	*p*-Value		
	CON-NS	CON-LS	CON-HS	LP-NS	LP-LS	LP-HS		Protein	PWA	Protein × PWA
Valine	343.7	372.7	518.0[b]	130.1[c]	246.6[d]	86.5	26.3	0.01	0.01	0.01
Methionine	41.2*	59.8*	68.6	64.5	72.3	73.0	3.2	0.02	0.04	0.41
Threonine	328.3[$]*	388.4	460.0[$]	631.5*	660.2	590.7	28.9	0.01	0.10	0.26
Isoleucine	171.9	190.6	301.3[b]	100.4[c#]	121.7 [#]	75.9	13.4	0.01	0.01	0.01
Leucine	268.9	314.1[a]	428.6[b]	255.9 [#]	294.7 [#]	214.3	12.9	0.01	0.01	0.01
Phenylalanine	126.4*	146.2	194.7[b]	100.8*[#]	118.8 [#]	89.0	6.6	0.01	0.01	0.01
Tryptophan	55.8	70.4	109.4[b]	81.2	93.7	112.8	4.9	0.02	0.01	0.42
Lysine	189.6*	267.6*	353.6[b]	529.0[c]*	618.3	365.7*	29.0	0.01	0.09	0.01
Histidine	136.3	137.3	182.2[b]	74.2[c]	95.3[d]	50.4[e]	8.0	0.01	0.28	0.01
Arginine	39.7	105.2	393.8[b]	63.8	79.4	124.6	11.9	0.01	0.01	0.01
Aspartic acid	79.2	76.7	41.67[b]	58.3	78.6	30.17	4.6	0.16	0.01	0.42
Serine	307.4	382.4[a]	401.3[b]	395.7[c]	420.5	285.6[e]	11.9	0.84	0.02	0.01
Glutamic acid	444.0	485.8	261.6[b]	499.9	594.9	330.3[e]	24.9	0.03	0.01	0.81
Glutamine	632.8	683.9	840.5	770.4	892.4	862.8	29.9	0.06	0.05	0.64
Glycine	1420.1	1772.4[a]	1701.3[b]	2168.0[c]	2027.7	1887.3	54.5	0.01	0.45	0.01
Alanine	778.4	917.4[a]	989.1[b]	1054.4[c]	1262.6[d]	915.5	34.0	0.01	0.01	0.01
Tyrosine	167.3	199.6	305.1[b]	159.2	181.9	129.9	10.5	0.01	0.03	0.01
Asparagine	169.3	208.7	291.9[b]	164.0	181.4	150.1	11.9	0.01	0.10	0.02
Proline	457.2	528.6[a]	676.9[b]	505.6[#]	538.3	396.8[#]	17.6	0.01	0.07	0.01
Ammonia	367.3	452.7	245.3	403.1	424.0	232.1[e]	19.1	0.62	0.01	0.25
Creatinine	115.6	131.1	161.7	112.9	129.8	81.7	6.2	0.01	0.43	0.01
Taurine	266.7	281.0	141.4[b]	273.8	357.3	96.4[e]	19.1	0.27	0.01	0.01
Sarcosine	50.7	66.1[a]	62.7[b]	73.6[c]	80.9	74.0	2.2	0.01	0.02	0.31
3,methylhistidine	10.9	10.2	12.5	6.71[c]	7.6	6.8	0.4	0.01	0.42	0.08
1,methylhistidine	58.4	70.3	67.3	56.7	57.9	60.2	3.0	0.26	0.63	0.78
Ethanolamine	38.1	47.1	25.0	29.4	20.3	17.9	3.0	0.01	0.01	0.26

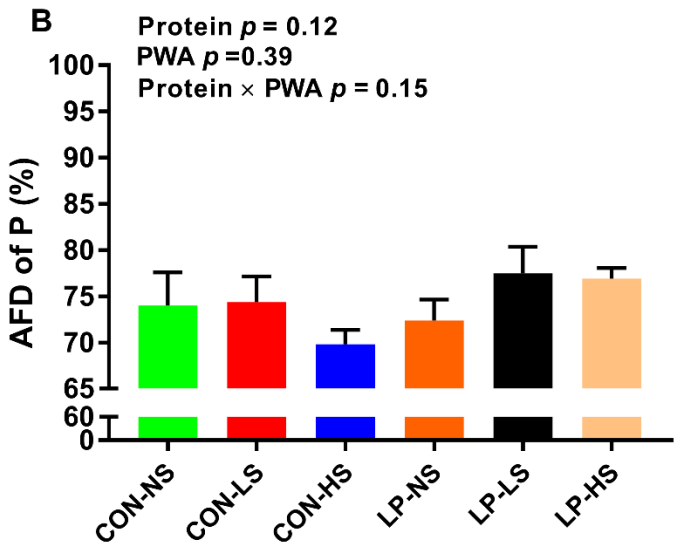
Carnosine	31.9	29.7	30.4	22.7	23.1	19.2	1.7	0.01	0.81	0.84
Hydroxylysine	11.5	14.6	14.1	9.6	9.8	12.5	0.5	0.01	0.03	0.24
Citrulline	74.5	89.6	96.4	70.1	75.8	79.1	2.7	0.02	0.04	0.53
Ornithine	352.8[$]	360.5	285.5[$]	251.5[#]	311.4[#]	125.2	17.2	0.01	0.01	0.16
α-aminobutyric acid	35.6	41.0	13.4	39.6	34.0	17.7	3.1	0.93	0.01	0.63

[1] CON-NS: control diet with no phytogenic water additive (PWA) supplemented, CON-LS: control diet with a low dose of PWA (4 mL/L of water) supplemented, CON-HS: control diet with a high dose of PWA (8 mL/L of water) supplemented, LP-NS: low-protein diet with no PWA added, LP-LS: low-protein diet with a low dose of PWA (4 mL/L of water) added and LP-HS: low-protein diet with a high dose of PWA (8 mL/L of water) added. Values are the means. n = 6/diet. [2] SEM: Standard error of the mean. [a] $p \leq 0.05$ CON-NS vs. CON-LS, [b] $p \leq 0.05$ CON-NS vs. CON-HS, [c] $p \leq 0.05$ CON-NS vs. LP-NS, [d] $p \leq 0.05$ LP-NS vs. LP-LS, and [e] $p \leq 0.05$ LP-NS vs. LP-HS. *[#$] Within a row, values with a common superscript symbol tend to be different ($0.05 < p \leq 0.1$).

3.4. Apparent Fecal Digestibility of Calcium, Phosphorus, and Nitrogen

Pigs fed with CON-LS and CON-HS tended to have a lower AFD of Ca compared to those fed with CON-NS (Figure 1A). Additionally, pigs fed with LP-HS tended to have a higher AFD of Ca compared to pigs fed with LP-NS (Figure 1A). There were no differences in AFD of P (Figure 1B) and N (Figure 1C) across dietary groups.


A
Protein p = 0.11
PWA p = 0.08
Protein × PWA p < 0.01
AFD of Ca (%)
100
95
90
85
80
75
70
65
60
0
*$
*
$
#
#
CON-NS
CON-LS
CON-HS
LP-NS
LP-LS
LP-HS


B
Protein p = 0.12
PWA p =0.39
Protein × PWA p = 0.15
AFD of P (%)
100
95
90
85
80
75
70
65
60
0
CON-NS
CON-LS
CON-HS
LP-NS
LP-LS
LP-HS

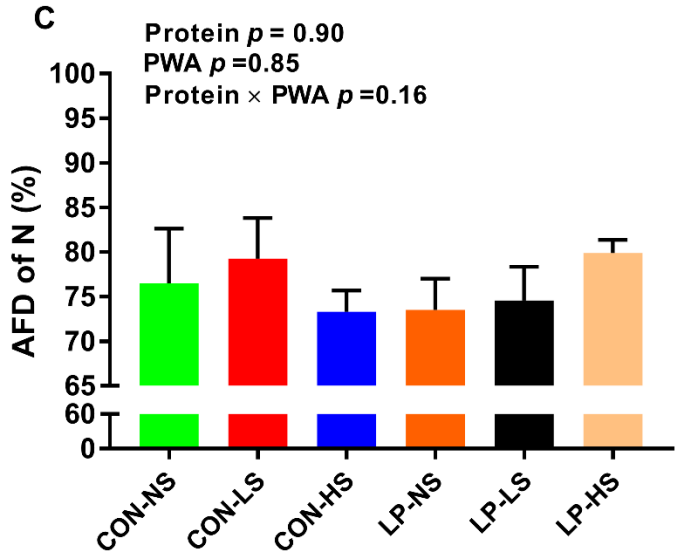

C
Protein p = 0.90
PWA p =0.85
Protein × PWA p =0.16
AFD of N (%)
100
95
90
85
80
75
70
65
60
0
CON-NS
CON-LS
CON-HS
LP-NS
LP-LS
LP-HS

Figure 1. Apparent fecal digestibility (AFD) of (**A**) calcium (Ca), (**B**) phosphorus (P) and (**C**) nitrogen (N) of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive (PWA). CON-NS: control diet with no PWA supplemented, CON-LS: control diet with a low dose of PWA (4 mL/L of water) supplemented, CON-HS: control diet with a high dose of PWA (8 mL/L of water) supplemented, LP-NS: low-protein diet with no PWA added, LP-LS: low-protein diet with a low dose of PWA (4 mL/L of water) added and LP-HS: low-protein diet with a high dose of PWA (8 mL/L of water) added. $^{*\$\#}$Among groups, values with a common superscript symbol tend to be different ($0.05 < p \leq 0.1$). Values are the means ± SEM. n = 8/dietary group.

3.5. Serum Calcium, Phosphorus, and Alkaline Phosphatase and Blood Urea Nitrogen

There was a significant effect of protein, PWA and protein × PWA on serum Ca concentration ($p < 0.05$; Figure 2A). The serum Ca of pigs fed with LP was higher than CON pigs (14.14 vs. 12.50 mg/dl, respectively). The serum Ca concentration for pigs fed with NS, LS and HS were 12.08, 14.57 and 13.30 mg/dl, respectively. Pigs fed with CON-LS, CON-HS and LP-NS had higher serum Ca than animals fed with CON-NS. In addition, LP-HS pigs had a lower serum Ca compared to LP-NS ones (Figure 2A). The effect of PWA and protein × PWA on serum P was significant ($p < 0.05$; Figure 2B). Supplementation of PWA dose-dependently increased the serum P concentration (5.59, 6.03, and 9.72 mg/dl for NS, LS, and HS, respectively). Pigs fed with CON-HS had a higher ($p < 0.05$) serum P compared to those fed with CON-NS, but pigs in CON-LS group had lower serum P compared to those in CON-NS group (Figure 2B). Additionally, pigs fed LP-LS and LP-HS had an increased serum P compared to those fed with LP-NS ($p < 0.05$; Figure 2B). Alkaline phosphatase was used as a marker of bone turnover in the current study. No differences in serum ALP concentration were observed across groups (Figure 2C). The effect of protein on BUN tended to be significant ($p = 0.08$) with a decrease in serum BUN of LP compared

to CON pigs (6.78 and 15.75 mg/dl, respectively) (Figure 2D). Pigs fed with CON-HS had a higher serum BUN compared to those fed with CON-NS. Additionally, pigs fed with LP-NS had a lower serum BUN compared to pigs fed with CON-NS (Figure 2D).

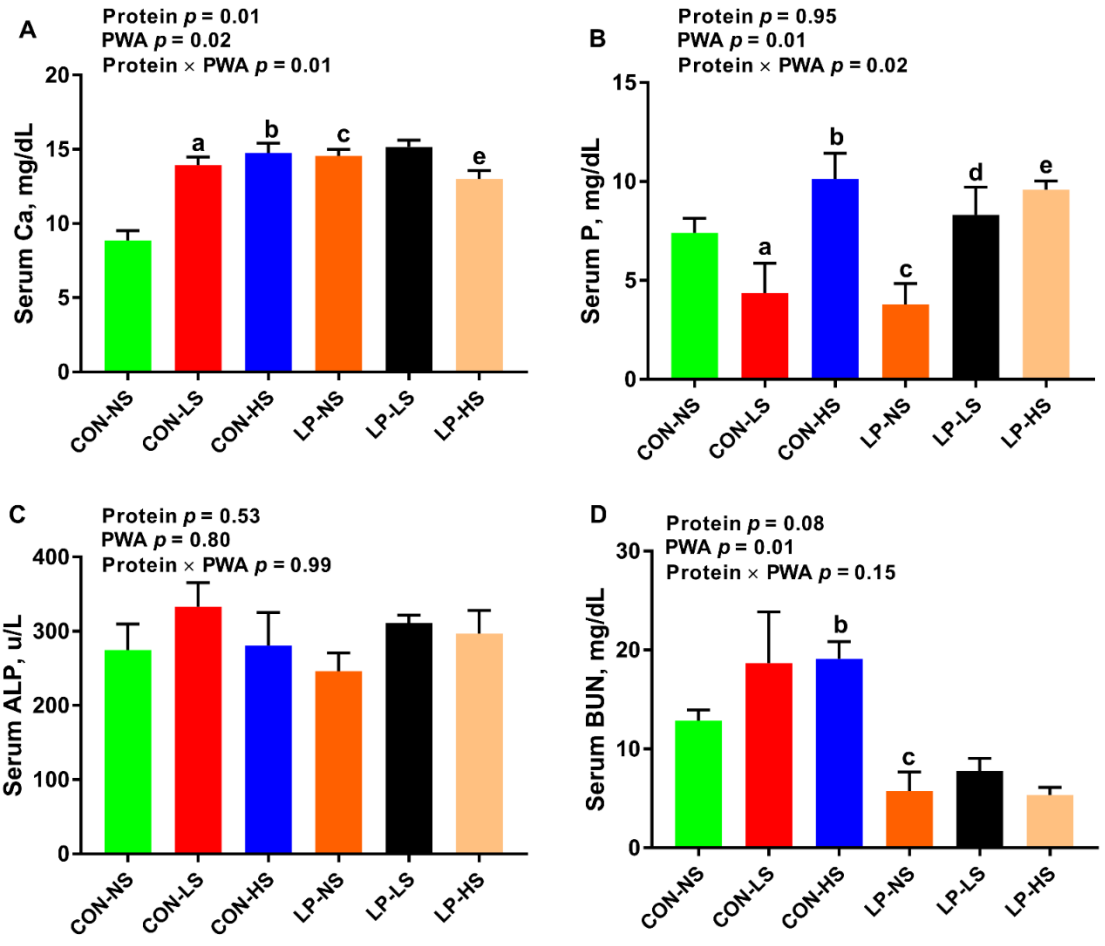


Figure 2. Serum (**A**) calcium (Ca), (**B**) phosphorus (P), (**C**) alkaline phosphatase (ALP) and (**D**) blood urea nitrogen (BUN) of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive (PWA). CON-NS: control diet with no PWA supplemented, CON-LS: control diet with a low dose of PWA (4 mL/L of water) supplemented, CON-HS: control diet with a high dose of PWA (8 mL/L of water) supplemented, LP-NS: low-protein diet with no PWA added, LP-LS: low-protein diet with a low dose of PWA (4 mL/L of water) added and LP-HS: low-protein diet with a high dose of PWA (8 mL/L of water) added. [a] $p \leq 0.05$ CON-NS vs. CON-LS, [b] $p \leq 0.05$ CON-NS vs. CON-HS, [c] $p \leq 0.05$ CON-NS vs. LP-NS, [d] $p \leq 0.05$ LP-NS vs. LP-LS, and [e] $p \leq 0.05$ LP-NS vs. LP-HS. Values are the means ± SEM. n = 7–8 for each dietary group.

3.6. The mRNA Abundance of Amino Acid Transporters in the Jejunum and Skeletal Muscle

The effect of PWA and protein × PWA on mRNA abundance of solute carrier family 7 member 11 (*SLC7A11*) and solute carrier family 6 member 19 (*SLC6A19*) in the jejunum (Figure 3B,C) was significant. The relative mRNA abundance of *SLC7A11* was 0.88, 0.76, and 0.61 for NS, LS, and HS, respectively. Further, the effect of PWA on solute carrier family 7 member 8 (*SLC7A8*) was significant ($p < 0.05$), with the relative mRNA abundance of 0.86, 0.81, and 0.56 for NS, LS, and HS, respectively (Figure 3G). Relative to CON-NS, the abundance of the *SLC7A11* transcript was decreased ($p < 0.05$) in the jejunum of CON-LS, CON-HS, and LP-NS (Figure 3B). Pigs fed with CON-HS had a decreased mRNA abundance of *SLC6A19* compared to those fed with CON-NS (Figure 3C). Pigs fed with LP-LS had an increased transcript of *SLC6A19* compared to the LP-NS group (Figure 3C). No differences were seen among the treatments for the abundance of the transcript of solute carrier family 15 member 1 (*SLC15A1*), solute carrier family 3 member 1 (*SLC3A1*), solute carrier family 7 member 1 (*SLC7A1*), and solute carrier family 7 member 9 (*SLC7A9*) (Figure 3A,D–F) in the jejunum.

The effect of PWA on the mRNA abundance of *SLC7A8*, *SLC3A1*, *SLC7A1*, and *SLC7A11* in skeletal muscle was significant (Figure 4B,D–F). The relative mRNA abundance for *SLC7A8* were 1.14, 0.80 and 0.54, for *SLC3A1* were 0.73, 0.87 and 0.37, for *SLC7A1* were 1.07, 0.90, and 0.45 and for *SLC7A11* were 1.32, 0.75 and 0.54 for NS, LS and HS, respectively. Pigs fed with CON-HS tended to have a lower *SLC7A1* gene expression compared to those fed with CON-NS (Figure 4E). Additionally, pigs fed with LP-HS tended to decrease the abundance of the transcript of *SLC7A1* compared to LP-NS (Figure 4E). No differences in the mRNA abundance of *SLC15A1* and *SLC6A19* among dietary treatments were observed in skeletal muscle (Figure 4A,C).

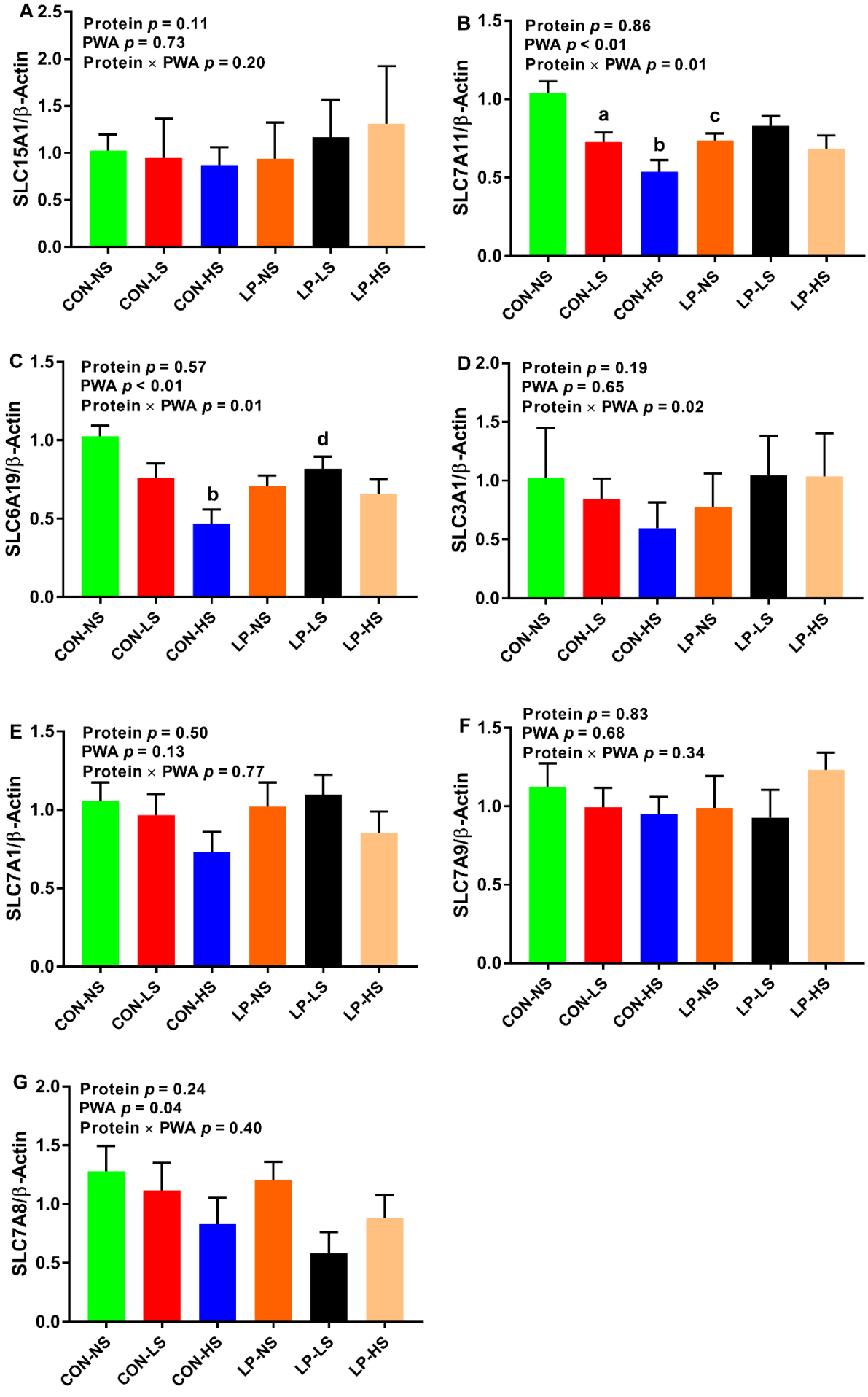

A
Protein p = 0.11
PWA p = 0.73
Protein × PWA p = 0.20
SLC15A1/β-Actin
B
Protein p = 0.86
PWA p < 0.01
Protein × PWA p = 0.01
a
b
c
SLC7A11/β-Actin
C
Protein p = 0.57
PWA p < 0.01
Protein × PWA p = 0.01
b
d
SLC6A19/β-Actin
D
Protein p = 0.19
PWA p = 0.65
Protein × PWA p = 0.02
SLC3A1/β-Actin
E
Protein p = 0.50
PWA p = 0.13
Protein × PWA p = 0.77
SLC7A1/β-Actin
F
Protein p = 0.83
PWA p = 0.68
Protein × PWA p = 0.34
SLC7A9/β-Actin
G
Protein p = 0.24
PWA p = 0.04
Protein × PWA p = 0.40
SLC7A8/β-Actin
CON-NS
CON-LS
CON-HS
LP-NS
LP-LS
LP-HS

Figure 3. Relative mRNA abundance of (**A**) solute carrier family 15 member 1 (*SLC15A1*), (**B**) solute carrier family 7 member 11 (*SLC7A11*), (**C**) solute carrier family 6 member 19 (*SLC6A19*), (**D**) solute carrier family 3 member 1 (*SLC3A1*), (**E**) solute carrier family 7, member 1 (*SLC7A1*), (**F**) solute carrier family 7 member 9 (*SLC7A9*), and (**G**) solute carrier family 7 member 8 (*SLC7A8*) in the jejunum of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive (PWA). CON-NS: control diet with no PWA supplemented, CON-LS: control diet with a low dose of PWA (4 mL/L of water) supplemented, CON-HS: control diet with a high dose of PWA (8 mL/L of water) supplemented, LP-NS: low-protein diet with no PWA added, LP-LS: low-protein diet with a low dose of PWA (4 mL/L of water) added and LP-HS: low-protein diet with a high dose of PWA (8 mL/L of water) added. [a] $p \leq 0.05$ CON-NS vs. CON-LS, [b] $p \leq 0.05$ CON-NS vs. CON-HS, [c] $p \leq 0.05$ CON-NS vs. LP-NS, [d] $p \leq 0.05$ LP-NS vs. LP-LS. Values are the means ± SEM. $n = 8$ for each dietary group.

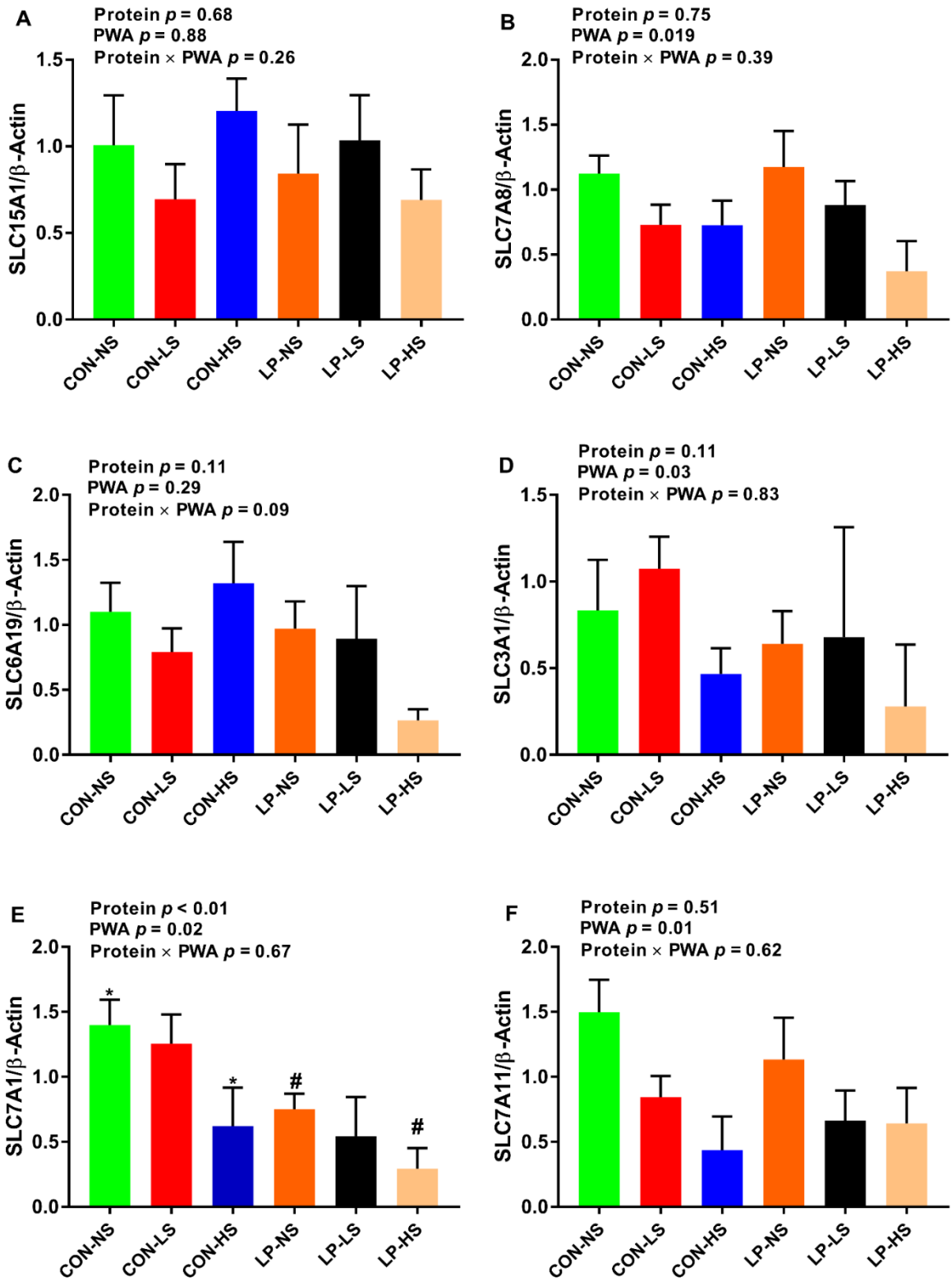


Figure 4. Relative mRNA abundance of (**A**) solute carrier family 15member 1 (*SLC15A1*), (**B**) solute carrier family 7 member 8 (*SLC7A8*), (**C**) solute carrier family 6 member 19 (*SLC6A19*), (**D**) solute carrier family 3 member 1 (*SLC3A1*), (**E**) solute carrier family 7, member 1 (*SLC7A1*), and

(**F**) solute carrier family 7 member 11 (*SLC7A11*) in the skeletal muscle of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive (PWA). CON-NS: control diet with no PWA supplemented, CON-LS: control diet with a low dose of PWA (4 mL/L of water) supplemented, CON-HS: control diet with a high dose of PWA (8 mL/L of water) supplemented, LP-NS: low-protein diet with no PWA added, LP-LS: low-protein diet with a low dose of PWA (4 mL/L of water) added and LP-HS: low-protein diet with a high dose of PWA (8 mL/L of water) added. *[#]Among groups, values with a common superscript symbol tend to be different ($0.05 < p \leq 0.1$). Values are the means ± SEM. $n = 8$ for each dietary group.

3.7. Serum Total Antioxidant Capacity

No differences in serum TAC were observed among dietary treatments, although the effect of protein × PWA on TAC was significant (Figure S2).

4. Discussion

The objective of this study was to assess the effect of a PWA on growth performance of nursery pigs fed with low-protein/high-carbohydrate or standard protein diets and the factors involved in this process. Our study revealed several important findings: (1) overall, supplemental PWA increased the ADG and tended to improve BW in CON-HS, but not in LP pigs, suggestive of a positive effect of PWA on growth performance when dietary protein is adequate; (2) regardless of dietary protein content, supplementing PWA increased muscle lean% and decreased muscle fat%, but this effect was significant when PWA was supplemented to LP diets at high dose (LP-HS), but not to CON diets, indicative of a promising effect of PWA on muscle composition when dietary protein is deficient; (3) supplementing CON pigs with a low or high dose of PWA increased the concentration of the majority of plasma essential AA and in LP pigs, a low dose of PWA (LP-LS) recovered the reduced AA levels such as branched-chain AA, phenylalanine, and histidine suggestive of improved digestion and absorption of proteins and AA by PWA; (4) supplementing LP pigs with PWA at high dose (LP-HS) reduced serum Ca concentration while supplementing CON pigs with both low and high doses of PWA increased the serum Ca concentration and decreased the AFD of Ca; (5) CON and LP pigs supplemented with a high

dose of PWA had higher serum P concentration; (6) supplementing CON pigs, but not LP pigs, with a high dose of PWA decreased the mRNA abundance of *SLC7A11* and *SLC6A19* in the jejunum and supplementing both CON and LP pigs with a high dose of PWA tended to reduce the abundance of the *SLC7A1* transcript in skeletal muscle. In summary, supplemental PWA improved growth performance when the dietary protein was adequate and the muscle composition when dietary protein was deficient, improved plasma AA profile, and produced differential effects on blood Ca and its digestibility depending on the level of dietary protein.

Little is known about whether supplemental phytogenic additives would improve the growth performance and meat composition of pigs fed with LP diets. Supplementation of PWA improved the ADG and body weight of pigs when diets contained recommended amounts of protein in the current study. Similarly, previous studies have shown a beneficial effect of phytogenic additives on growth performance in early weaned [16] and growing pigs [17] when diets with standard protein levels were fed. In the current study, PWA did not improve the performance of pigs fed with low-protein/high-carbohydrate diets. Likewise, others showed that supplementing very low-protein diets with a plant extract YGF251 did not influence the growth performance of growing pigs [20]. In the present study, the beneficial effects of PWA in pigs fed with CON diet could be due to the increased concentration of blood essential AA that may contribute to improving the growth of pigs, within their genetic potential. Others have reported an enhanced nutrient digestibility in response to supplemental phytogenic additives when pigs are fed with adequate-protein diets [18,24–26]. In parallel with our data, an increased serum Ca and other minerals were reported in broiler chicks following herbal extract supplementation [46]. In the current study, the concentration of blood Ca was dramatically increased in experimental diets up to ~15 mg/dL. It has been shown that most animals show systemic hypercalcemia signs when the concentration of Ca is greater than 15 mg/dL [47]. In another study, the concentration of Ca was increased to similar levels seen in our study with no clinical signs of illness when pigs were fed with calcium-replete and P-deficient diets [48]. Similarly, no clinical signs of hypercalcemia were observed in our animals. Hypercalcemia induced renal vasoconstriction, which is characterized by reduced renal

blood flow and glomerular filtration rate was shown to occur in dogs with blood Ca concentration higher than 20 mg/dL [47]. Given the subclinical levels of blood Ca and no clinical signs of hypercalcemia in animals in the current study, no failure in kidney function was assumed in our animals. Further, the blood ALP as a marker of bone turnover did not change across dietary groups in the present study. Additional research will be needed to determine the effect of PWA on bone parameters such as bone ash, mineral contents and density. The reduced AFD of Ca in CON pigs supplemented with PWA is likely due to a negative feedback loop that inhibits the release of parathyroid hormone and vitamin D3 and Ca absorption when blood Ca is rising. Little is known about the effect of phytogenic additives on nutrient digestibility when pigs are offered LP diets. We observed a differential response of PWA on blood Ca and P concentration in the current study. Further research is warranted to elucidate the interaction of the dietary protein with PWA on nutrient digestibility. The data on the effect of PWA on muscle composition and its interaction with dietary protein are scarce. Our study showed that regardless of dietary protein content, supplementing PWA increased the lean% and decreased the fat% in the muscle in a dose-dependent manner but this effect was significant when PWA was supplemented to low-protein/high-carbohydrate diet at a high dose. In a recent study, treating porcine primary muscle cell lines with natural phytogenic compounds from fruit peel increased the expression of genes involved in muscle development, and in live animals, these compounds decreased back fat thickness [49]. The data from this study provide evidence that a novel PWA could be used to improve the growth performance and meat composition of nursery pigs. Whether or not the used PWA in the current study could potentially alter back fat thickness or the whole body composition of the carcass, additional research is required to answer that question. While our data show improvement in blood AA profile and increased Ca and P concentration following PWA supplementation, the mode of action of the PWA used in the present study on growth performance and muscle composition has not been fully explored and further studies are needed to better understand the pathways involved.

The positive effects of phytogenic additives on the growth performance of pigs have been linked with the beneficial effects of these additives on eating behavior [17,21], immune function [22,23],

antioxidant capacity [24], and intestinal development, ecosystem, and nutrient digestibility [18,24–26]. However, less attention has been paid towards the changes in circulating AA while blood AA profile is highly associated with animal health and growth [50]. In the current study, regardless of the level of dietary protein, supplementing PWA improved the plasma AA concentration. More specifically, supplementing CON pigs with a low or high dose of PWA and LP pigs with a low dose of PWA improved the majority of plasma essential AA concentration. Similarly, others reported an increase in serum AA concentration when a phytogenic additive was added to a diet of weaned pigs fed with standard protein diets [51]. In support of data obtained in the present study, an increase in ileal AA digestibility was reported in weaned pigs supplemented with herbal extract, and that improvement was attributed to the positive effect of phytogenic additives on enhancing the functions of digestive enzymes in the gut [14,52]. The improved performance of pigs fed with an adequate-protein diet supplemented with PWA in the current study might be due to the increased concentration of essential AA in blood. Due to the complexity of the composition of herbal extracts containing a wide variety of compounds such as flavonoids, fatty acids, and protein, it remains to be determined whether a group of active compounds or a single chemical moiety of phytogenic additives is stimulating the increased blood AA concentration.

Amino acids are sensed and transported by specific AA transporters [53,54]. It is known that nutrient composition can alter intestinal AA transporter expression in pigs fed protein-deficient diets [32], but little is known about interactive effects of dietary protein and phytogenic additives on dynamics of these transporters in the gut and skeletal muscle. Supplementing CON pigs with a high dose of PWA decreased the abundance of the *SLC7A11* and *SLC6A19* transcripts in the jejunum and supplementing both CON and LP pigs with a high dose of PWA reduced the abundance of the *SLC7A1* transcript in skeletal muscle. In contrast, others showed that incubating jejunal cells with the combination of essential oils and saponins at medium and high doses stimulated solute carrier family 15 member 1 (*SLC15A1*) recruitment to the cytoplasmic membrane in growing broilers [33]. These controversial results on the effect of PWA on the expression of AA transporters might be due to the differences in the composition

and the dose of PWA used. *SLC7A11* is a cystine/glutamate exchanger [55] and is specifically involved in the cysteine and glutamate transport system. The downregulation of jejunal *SLC7A11* in the CON-HS group is likely contributing to the reduced plasma glutamic acid concentration in this group. *SLC6A19* is the major neutral AA transporter in the small intestine and kidney [56]. Given the increased concentration of neutral AA including alanine, asparagine, glycine, isoleucine, leucine, phenylalanine, serine, proline, threonine, tryptophan, tyrosine, and valine in the CON-HS group, the reduced mRNA abundance of *SLC6A19* in the jejunum is suggestive of a feedback regulatory mechanism in this process. A feedback regulatory mechanism has been described between cycling AA pool and expression of intestinal AA transporters [57]. Although the role of *SLC71* is not very well known in skeletal muscle [58], there is evidence that accounts for arginine uptake [59,60]. The reduced expression of *SLC7A1* in skeletal muscle of CON-HS and LP-HS groups and hence decreased uptake of arginine by skeletal muscle may contribute to the greater concentration of arginine in the circulation for these groups. The role of other basic and acidic transporters in the small intestine that contribute to improved AA profile in pigs supplemented with phytogenic additives requires further investigation.

5. Conclusions

Little research has been conducted to explore the interaction of phytogenic additives and dietary protein on growth performance and related underlying factors in young pigs. Overall, supplemental PWA (8 mL/L) increased the concentration of plasma essential AA and reduced the abundance of the transcript for some of the AA transporters in the small intestine and skeletal muscle, improved growth performance when the dietary protein content was adequate and increased muscle lean%, and reduced muscle fat% when the dietary protein was deficient. Depending on the level of dietary protein, supplementation of PWA had differential effects on plasma Ca concentration and its digestibility. Altogether, although the supplemental PWA used in this study failed to improve the growth performance of nursery pigs fed with LP diets, this additive improved the performance of pigs fed with standard

protein diets likely through improved blood AA profile and had beneficial effects on muscle composition when dietary protein was deficient. Further research is warranted to elucidate the mechanisms controlling the interactive effects of dietary protein with phytogenic additives on nutrient digestibility and AA transport system. Since phytogenic additives contain a wide variety of bioactive compounds, further investigation is required to understand what active compound(s) of these additives is mediating their beneficial effects.

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Table S1. Ingredients and chemical composition of the phytogenic water additive (Herbanimals®) used in this study (as-fed basis)

Items	Herbanimals®
Ingredients, %	
Pandanus amaryllifolius Roxb	4.83
Phyllanthus niruri	24.15
Amomum cardamomum	4.83
Zingiber zerumbet	13.04
Apium Graveolens	14.49
Anethum Graveolens	14.49
Ocimum americanum	4.83
Cinnamomum burmannii Blume	4.83
Myristica fragrans Houtt	4.83
Zingiber officinale roscoe	9.66
Analyzed Chemical Composition, mg/100 g	
Calcium	4.99
Magnesium	4.16
Iron	1.01
Vitamin B6	0.46
Vitamin B12	0.69
Vitamin E	0.86
Vitamin B1	0.04
Vitamin B2	0.19
Arginine	15.58
Vitamin B3	0.14
Aspartic acid	10.45
Pantothenic acid	1.39

Supplementary Figures

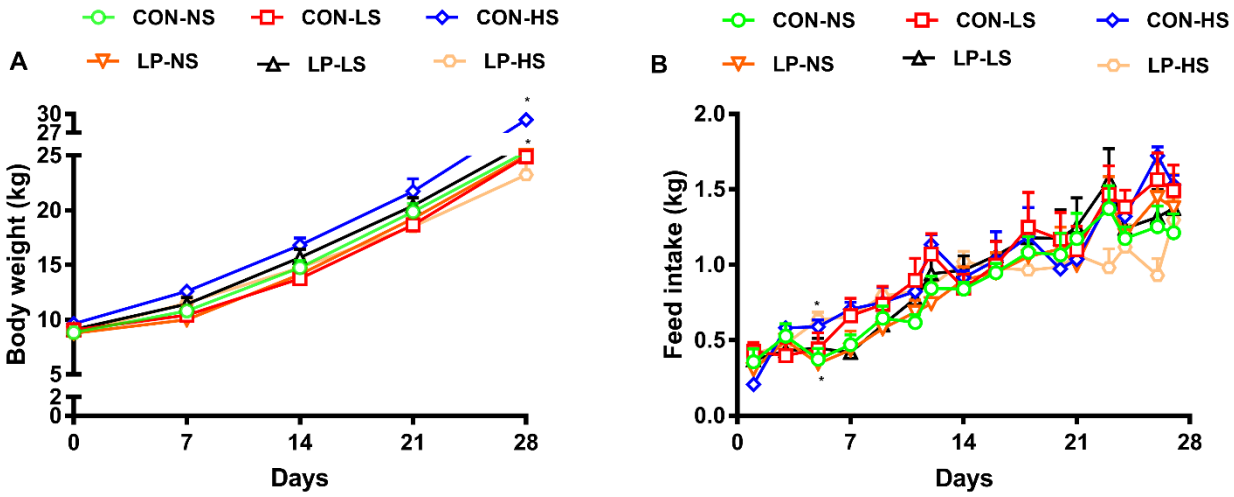

Figure S1. Body weight (A) and feed intake (B) of nursery pigs fed with two levels of dietary protein and three levels of a phytogenic water additive (PWA). The *p* values for the overall model effect of protein, PWA, time, protein × PWA, protein × time, PWA × time and protein × PWA× time for body weight were 0.05, 0,49, 0.01, 0.49, 0.01, 0.05 and 0.01 and for feed intake were 0.07, 0.10, 0.01, 0.37, 0.68, 0.47 and 0.31, respectively. CON-NS: control diet with no PWA supplemented, CON-LS: control diet with a low dose of PWA (4 ml/L of water) supplemented, CON-HS: control diet with a high dose of PWA (8 ml/L of water) supplemented, LP-NS: low protein diet with no PWA added, LP-LS: low protein diet with a low dose of PWA (4 ml/L of water) added and LP-HS: low protein diet with a high dose of PWA (8 ml/L of water) added. *Among groups, values with a common superscript symbol tended to be different ($0.05 < p \leq 0.1$). Values are means ± SEM. *n*=8/dietary group.

VITAE

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Dissertation: EVALUATING THE FACTORS THAT CONTROL THE GROWTH PERFORMANCE OF PIGS FED WITH LOW PROTEIN DIETS SUPPLEMENTED WITH A CORN-EXPRESSED PHYTASE OR PHYTOGENIC FEED ADDITIVE

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