Utilization of Low Crude Protein Diets to Promote the Gastrointestinal Health in Weaned Piglets

by

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ABSTRACT

Lowering dietary protein content has been proposed to reduce post-weaning diarrhea and enhance gut health of weaned pigs. A series of experiments was conducted to evaluate the effects of low crude protein (**LCP**) diets in weaned pigs in relation to different nutritional or environmental factors. First, standardized ileal digestible (**SID**) lysine requirements of 7- to 15-kg weaned pigs were determined using a new genotype of pigs that our research station obtained. The SID lysine requirements for maximum growth of 7- to 15-kg weanling pigs ranged from 1.27 to 1.43%, giving an average value of 1.32%. In the second experiment, the effects of dietary crude protein (**CP**) content were evaluated in relation to resistant starch supplementation. Feeding LCP diets reduced feed efficiency, decreased incidence of diarrhea and digesta ammonia N content, and enhanced ileum histomorphology in weaned pigs. Dietary supplementation of resistant starch increased the production of beneficial microbial metabolites in the small and large intestines of weaned pigs fed the LCP diet. The third experiment explored the effects of LCP diets and crystalline amino acid supplementation patterns in association with different environmental conditions. Pigs fed LCP diets showed decreased incidence of diarrhea and enhanced the immune system by increasing the plasma anti-inflammatory cytokine profile. However, decreased growth performance was observed in pigs fed LCP diets. Poor sanitation resulted in reduced growth performance in the second week of the trial, however, pigs compensated for the lost growth in the subsequent week. Crystalline amino acid supplementation patterns did not influence growth performance, jejunum histomorphology, or immune response in weaned pigs under different sanitary conditions. However, dietary CP, sanitary conditions, and crystalline amino acid supplementation patterns did not influence colonic bacterial composition and diversity. Overall, lowering dietary protein improved

intestinal health of weaned piglets by reducing protein fermentation by the microorganism. Although the growth performance of weaned piglets differed between low- and high-protein diets, feeding LCP diets to weaned pigs can be a nutritional strategy by ameliorating post-weaning diarrhea, improving intestinal morphology, and modulating the microbial profile and metabolites in the gut.

DEDICATION

This thesis is dedicated to my beloved parents, Jaerok Lee and Hwaja Choi; my sister, Juhyeon Lee; and my brother, Jihun Lee.

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FOREWORD

This thesis was written in a manuscript format according to the guidelines of *Journal of Animal Science* and there are four manuscripts corresponding to four chapters. Manuscript I was published in *Animal* journal; Manuscript II is under preparation; Manuscripts III and IV were published in *Journal of Animal Science*.

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LIST OF ABBREVIATIONS

AA	Amino acid(s)
ADFI	Average daily feed intake
ADG	Average daily gain
BCAA	Branched chain amino acids
BCFA	Branched-chain fatty acids
BW	Body weight
CAA	Crystalline amino acids
CD	Crypt depth
CI	Confidence interval
CLDN	Claudin
CP	Crude protein
CSC	Clean sanitary conditions
DM	Dry matter
FD4	Fluorescein isothiocyanate dextran – 4kDa
GC	Goblet cells
G:F	Gain-to-feed ratio
GAPDH	Glyceraldehyde 3-phophate dehydrogenase
HCHO	Formaldehyde
HCP	High crude protein
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IL	Interleukin
KRB	Krebs Ringer buffer

LCP	Low crude protein
LBL	Linear broken-line
MUC	Mucin
NSP	Non-starch polysaccharides
OCLN	Occludin
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis
PUN	Plasma urea nitrogen
PWD	Post-weaning diarrhea
QBL	Quadratic broken-line
RS	Resistant starch
SCFA	Short-chain fatty acids
SDAP	Spray-dried animal plasma
SID	Standardized ileal digestible
TNF	Tumor necrosis factor
TVOC	Total volatile organic compounds
USC	Unclean sanitary conditions
VFA	Volatile fatty acids
VH	Villus height
ZO	Zonula occludens

1. GENERAL INTRODUCTION

Weaned pigs are subjected to nutritional (e.g., solid feeds instead of sow milk) and environmental stressors (e.g., transportation to new rooms and rearrangement of hierarchy), and due to these various stressors, physiological, microbiological, and immunological changes in the gastrointestinal tract of young piglets occur around weaning (Lallès et al., 2007). Post-weaning diarrhea (**PWD**) is a typical multifactorial disease in weaned pigs associated with the proliferation of enterotoxigenic *Escherichia coli* (Laine et al., 2008). Nursery diets fortified with sub-therapeutic levels of antimicrobial growth promoters have been used to minimize adverse effects of weaning and PWD, however, the use of in-feed antibiotics has been banned in swine production due to its considerable concerns for the developing antibiotic-resistant of bacteria (Yang et al., 2009). Thus, many efforts have been made to investigate alternatives to antimicrobial growth promoters.

Weaned pigs were traditionally provided with high protein diets at the starter phase to complement reduced feed intake and impaired growth after weaning. However, diets with high dietary protein content increase substrates availability for protein fermentation, thereby increasing the proliferation of pathogenic bacteria, including *Escherichia coli* (Opapeju et al., 2008). Therefore, feeding a diet with low dietary protein content is utilized as one of the major strategies to control PWD in weaned piglets. However, little information is available on the effects of low crude protein (**LCP**) diets in combination with other strategies that could influencing growth performance and gut health in weaned pigs. Furthermore, accurate amino acids (**AA**) requirements should be determined to provide enough AA for growth in LCP diets for weaned pigs. Especially, determination of the optimal lysine requirement is essential because lysine is the reference amino

acid, and the rest of the indispensable amino acids are expressed as a percentage of lysine in the ideal protein concept (NRC, 2012).

Therefore, the main objective of this study was to evaluate the effects of LCP diets in weaned pigs in relation to different nutritional or environmental factors. To achieve this main objective, the following specific objectives were formulated:

i) To determine the standardized ileal digestible lysine requirement of 7- to 15-kg weanling pigs (TN70 × TN Tempo; Topigs Norsvin) fed a corn-soybean meal-based diet.

ii) To evaluate the effects of dietary protein content and resistant starch supplementation on growth performance, histomorphology, and microbial metabolites of weaned pigs.

iii) To investigate the effects of dietary protein content and crystalline amino acids supplementation patterns on growth performance, intestinal histomorphology, and immune response in weaned pigs raised under different sanitary conditions.

iv) To investigate the effects of dietary protein content and crystalline amino acids supplementation patterns on intestinal bacteria and their metabolites in weaned pigs raised under different sanitary conditions.

2. LITERATURE REVIEW

2.1. INTRODUCTION

Dietary crude protein (**CP**) plays an important role in piglet health. Weaning is one of the most stressful phases for young pigs, which changes the structure and function of the gastrointestinal tract, decreases growth performance, and increases the occurrence of diarrhea (Lallès et al., 2007; Pluske et al., 2018). Post-weaning diarrhea is a multifactorial gastrointestinal disease showing the discharge of watery feces during the first 2 weeks after weaning (Laine et al., 2008). Antimicrobial growth promoters have been utilized to reduce the proliferation of pathogens and ameliorate PWD in weaned pigs, however, in-feed antibiotics are no longer allowed for use in livestock production because of increased concerns about the development of antibiotic-resistant bacteria (Yang et al., 2009). Therefore, alternative dietary interventions have been studied to prevent PWD, and feeding LCP diets is one of the major strategies to control PWD in weaned piglets. Due to reduced feed intake and weight gain typically observed after weaning, weaned pigs were traditionally fed high CP (**HCP**) diets at the starter phase. However, a HCP diet increases the substrate for protein fermentation, thereby increasing the proliferation of pathogenic bacteria and thus impairs gut health in weaned pigs (Opapeju et al., 2008). Therefore, feeding LCP diets with supplementation of crystalline amino acids (**CAA**) to meet the indispensable AA requirements has been investigated for decades as a strategy to enhance gut health in weaned piglets in an antibiotic-free feeding system (Opapeju et al., 2008, 2009a). Due to reduced soybean meal in LCP diets, indispensable CAA have to be included in LCP diets for optimal pig growth. However, some CAA, such as His and Phe, are not commonly available in feed- or food-grade forms in the industry, which in turn leads to increased feed cost in LCP diets. Despite the importance of the evaluation of the optimum

supplementation patterns of CAA in the LCP diets, recently published information is extremely limited. Also, accurate requirements of AA must be determined to formulate LCP diets without an indispensable AA deficiency.

Although extensive research has been carried out to investigate the effects of feeding LCP to weaned pigs on growth performance and gut health during last decade, there has been comparatively limited study of the effects of LCP diets in combination with other strategies that may affect growth performance and gut health in weaned pigs. Quantifying the relationships between feeding LCP and other potential impact factors is an essential step to develop strategies to improve post-weaning performance in swine industry. Therefore, the objective of this review is to provide an overview of current knowledge on LCP diets and AA requirements for weaned pigs.

2.2. DIGESTION OF PROTEIN

Digestion of food is the breakdown process of large food into smaller particles that can be absorbed by the small intestine. Although the process is well written in many books (Yen, 2011), protein digestion will be introduced briefly. Dietary protein is ingested through the mouth and mechanical digestion begins by chewing, which is the physical degradation of large particles into smaller pieces. The food has a more exposed surface by this mechanical process and digestive enzymes can easily access it for chemical digestion. Chemical digestion is the enzymatic cleavage of proteins into oligopeptides and then into smaller AA, dipeptides, or tripeptides (Yen, 2011). The chemical digestion of protein begins in the stomach by gastric proteases and hydrochloric acid, and these enzymes denature protein into large peptides. Pepsinogen, an inactive proenzyme, is secreted and hydrochloric acid activates pepsinogen to pepsin. Proteolytic activity of these gastric

proteases declines when gut pH is increased to pH 3.5 and the activity is absent when pH is above 6.0 (Yen, 2011).

Large peptides are broken down by pancreatic proteases in the lumen of the small intestine. Those pancreatic proteases are secreted in the form of inactive proenzymes, such as trypsinogen, chymotrypsinogen, proelastase, and procarboxypeptidases. Enterokinase is secreted from a duodenal brush border and activates trypsinogen to trypsin in the duodenum. And then this activated trypsin is involved in the activation process of other pancreatic proenzymes, chymotrypsinogen, proelastase, and procarboxypeptidases into chymotrypsin, elastase, and carboxypeptidases, respectively. These enzymes break down large peptides into smaller oligo-, tri-, dipeptides or AA in the small intestine. The final stage of protein digestion in the small intestine is carried out by a brush border peptidase and cytosolic peptidase in the enterocyte (Alpers, 1987). The brush border peptidase hydrolyzes oligopeptides of more than three AA, and both brush border peptidase and cytosolic peptidase break down tri- and dipeptides into the free form of AA.

2.3. WEANING STRESS AND ITS INFLUENCE ON PIGLETS

Weaning causes nutritional, social, and environmental disorders that challenge the health of piglets. Weaning of piglets in commercial swine production systems typically occurs at a young age (about 2 to 5 weeks of age) compared to that in nature, which aggravates various stresses in young, immature pigs (Lallès et al., 2007). During the weaning period, piglets have to deal with socialization with other litters in new environments after abrupt separation from their mother, and their diet is switched from readily digestible milk to dry, complex solid feeds (Lallès et al., 2007). Piglets' distress also comes from handling and transportation management (Campbell et al., 2013). For these reasons, piglets usually show low feed intake and poor growth performance after

weaning, which most likely results in a carryover effect on subsequent performance. Body weights (**BW**) of piglets at weaning impact lifetime growth performance as piglets with heavy weaning weight keep their weight advantage over medium or light weaning groups until 123 days of age (Collins et al., 2017).

There are some changes in key AA in the pig intestine before and after weaning. Sow's colostrum and milk contain large amounts of glutamate and glutamine (Wu and Knabe, 1994), thus, those AA can be easily found in the intestine of the suckling pigs. Glutamate, glutamine, and aspartate are major fuels for the intestinal mucosa (Alpers, 2000). Glutamate can be readily converted into many other AA in swine by transamination (Wu, 2009). Both glutamate and glutamine contribute to yield ATP with very high efficiency (Wu et al., 1995; Wu, 1998), and they are major substrates for the synthesis of ornithine, citrulline, and arginine, which are very important AA for various metabolism in the neonatal period (Wu et al., 1995). At or after weaning, however, the importance of other AA such as threonine or tryptophan is increased to maintain the gut health of piglets. Threonine has great importance in maintaining gut health in the post-weaning period because it is a major AA component of mucins and immunoglobulin (Li et al., 2007a). Many studies showed that dietary threonine supplementation could improve the immune function and intestinal morphology of weaned pigs by increasing immunoglobulin A concentration in jejunal mucosa, maintaining jejunal integrity, and repairing villus damage under pathogen-challenged model (Mao et al., 2014; Ren et al., 2014). Koo et al. (2020) reported that threonine supplementation resulted in the downregulation of IL-6 gene expression and upregulation of tight junction protein gene expression in the jejunum of weaned pigs. Supplementation of dietary tryptophan improves small intestinal morphology without affecting permeability and increases the gastrointestinal robustness of the piglets during the weaning phase (Koopmans et al., 2006).

Moreover, tryptophan is the sole precursor of serotonin, which is a key neurotransmitter in the gut-brain axis that modulates central neurotransmission such as emotional control, food intake, sleep, and pain processing (Israelyan and Margolis, 2018). This serotonin is mostly located in the gastrointestinal tract, especially in the hindgut, and is mainly produced from enterochromaffin cells which is the intestinal epithelial enteroendocrine cells (Mawe and Hoffman, 2013).

2.3.1. CHANGES IN GUT STRUCTURE AND FUNCTION

Changes in gut structure and function are one of the major challenges for piglets at weaning. First, there are changes in morphology after weaning in the piglet intestine. Weaning induces both acute and long-lasting structural and functional changes in the small intestine (Pluske et al., 1997; Boudry et al., 2004). The epithelial lining of the small intestine consists of finger-shaped villi, which serve to absorb nutrients, and crypts which are tubular glands lining at the base of the villi (Tappenden, 2006). Longer villi are desirable for the optimal function of the small intestine, which indicates a greater absorptive capability of nutrients (Yang and Liao, 2019). An increased crypt depth (**CD**; crypt hyperplasia) is considered a demerit because an increased rate of crypt cell proliferation leads to faster tissue turnover for villus renewal, which may be required in response to inflammation caused by the pathogens (Yang and Liao, 2019). For the structural changes in piglets after weaning, decreased villus height (**VH**; villous atrophy) in the jejunum and increased CD both jejunum and ileum were found from 2 days to 15 days after weaning (Boudry et al., 2004), and PWD has been suggested as the main factor for these changes (Pluske et al., 1997).

As a change in function of the small intestine, the activities of brush border enzymes are markedly reduced after weaning (Boudry et al., 2004; Marion et al., 2005). Enterocytes account for 95% and 90% of the epithelial cells in the villus and crypt, respectively (Cheng and Leblond, 1974), and digestive enzymes, such as lactase, sucrase, and maltase, are secreted from the brush

border surface of enterocytes, which are important indicators of maturation and digestive capacity in weaned pigs (Pácha, 2000). Although lactase activities were consistently reduced in the small intestine of pigs after weaning in previous studies (Hampson and Kidder, 1986; Boudry et al., 2004; Marion et al., 2005), there was a discrepancy in the literature on the effects of weaning on the activities of other brush border enzymes. For instance, some studies reported increased maltase and/or sucrase activities after weaning (Fan et al., 2002; Marion et al., 2005), whereas others observed decreased sucrase activity after weaning (Hampson and Kidder, 1986; Bruininx et al., 2002) or no difference between before and after weaning on maltase activity (Boudry et al., 2004). These discrepancies could be due to variations in experimental design, age of pigs at weaning, or experimental diets after weaning. Moreover, intestinal barrier functions, including paracellular permeability and permeability to macromolecules, are influenced by weaning. Weaning decreases transepithelial resistance in pig jejunum, indicating increased paracellular permeability and impaired intestinal barrier (Boudry et al., 2004; Hu et al., 2013). Increased gut permeability after weaning results from the fact that weaning is a stressor that is accompanied by reduced feed intake, and increased stress. Starvation is known to affect mucosal integrity by increasing epithelial paracellular transport (Spitz et al., 1996; Kiliaan et al., 1998; Yang et al., 1999).

Along with the functional change in the small intestine, it has been investigated that the abundance of intestinal AA transporter in piglets decreases after weaning compared to the suckling phase. It is known that AA absorption in the intestine is much more effective in newborn animals than in adults (Himukai et al., 1980; Guandalini and Rubino, 1982). Buddington et al. (2001) reported that AA absorption rates in pigs were highest at their birth and decreased by about 30% during the first 24 hours. Previous studies showed neutral AA transporters such as B^0AT1 and SNAT2 protein abundances in jejunum decreased dramatically after weaning compared to the

early suckling phase of piglets (Yang, 2011; Yang et al., 2012; Li et al., 2015a). Yang (2011) investigated the underlying reasons for this decline in AA transporter activity at weaning. A few possible mechanisms for the developmental decreases of the intestinal apical neutral AA transporter activity have been postulated, such as the limitation of transcriptional level (i.e., limited by mRNA gene abundance of AA transporter) or translational level (i.e., limited by efficiency or capacity of AA transporter protein synthesis). Yang (2011) found that mRNA abundance of B^0AT1 in the proximal jejunum increased from d 1 to d 12 of birth and then at d 70, the mRNA abundance decreased to a similar level to the d 1, which indicated that transcriptional limitation might not be the reason of decline of AA transporter activity during the postnatal development of pigs. It is speculated that the reduction of AA transporter activity after weaning could be a part of the body's regulatory mechanism to adapt to the new dietary conditions. Solid feed consumption after weaning does not require rapid absorption of AA like milk consumption during the suckling phase, which leads to a downregulation of transporter activity.

2.3.2. CHANGES IN MICROBIAL COMMUNITY STRUCTURE

Although the small intestine is typically considered important due to its function as the key site for nutrient digestion and absorption, the effect of weaning on the large intestine is of equal importance due to its function in fluid and electrolyte absorption and physical barrier against the pathogen. Therefore, the structure and function of the large intestine play an essential role in the incidence of PWD in piglets (Hopwood and Hampson, 2003). Especially, changes in the microbial ecology of the gastrointestinal tract are directly related to the nutrition and pathology of weaning pigs (Hao and Lee, 2004). Newborn piglets have a sterile gut, and their gut becomes colonized by microbiota, such as lactic acid producing bacteria, Enterobacteriaceae, and *Streptococcus*, from the sow and the environment (Lallès et al., 2007). Colostrum and milk help to establish a beneficial intestinal

microbiota and contain bioactive molecules, including immunoglobulins and growth factors, which help to stimulate gastrointestinal tissue growth and gut maturation and develop the immune system of suckling pigs (Xu et al., 2002; Maga et al., 2013). Lactic acid producing bacteria, such as *Lactobacillus*, are considered beneficial bacteria and one of the main representing fecal microbiota of suckling pigs (Chen et al., 2017). However, diminished *Lactobacillus* species were found in ileal and colon digesta after weaning compared to pre-weaning (Konstantinov et al., 2006). Weaning increases the number of *Escherichia coli* in the gastrointestinal tract, thereby decreasing the number of *Lactobacilli* (Jensen, 1998; Nabuurs, 1998). Unlike adult animals, weaned piglets are highly susceptible to enteric diseases because their microbiota composition is unstable compared to the before weaning period (Konstantinov et al., 2006).

2.3.3. CHANGES IN THE IMMUNE SYSTEM

Because neonatal piglets can generate only limited T-cell and B-cell responses when they are challenged with pathogens, maternal colostrum or milk supplies various immune factors to young pigs (Lallès et al., 2007). However, weaning can have a negative impact on the immune system in young piglets. First, dietary antigens such as glycinin and beta-conglycinin in soybean meal derived from solid feed can have an adverse immune response by weaned pigs by enhancing the production of immunoglobulins and proliferation of lymphocytes (Sun et al., 2008). In addition, intestinal inflammation is caused by pathogenic infections such as *Escherichia coli* and *Salmonella* spp. occurs PWD and intestinal damage in weaning pigs (Fedorka-Cray et al., 2000; Luppi, 2017). Intestinal barrier inflammation and dysfunction are directly related to increased permeability derived from toxins, viruses, or bacteria during weaning (Lallès et al., 2004). As mentioned above, weaning increases paracellular permeability in the gastrointestinal tract, and antigens or toxins can pass the lamina propria and enter systemic tissues due to increased permeability, thereby resulting

in inflammation (Deitch, 1993; Spreeuwenberg et al., 2001). Spreeuwenberg et al. (2001) reported that translocation of antigens decreased CD4 to $CD8^+$ T-cell ratio at days 2 and 4 postweaning which indicates a weak immune system, and this result might have led to a further increase of paracellular transport in weaned pigs. Moreover, expansion of $CD8^+$ T-cells likely stimulates the secretion of proinflammatory cytokines, and a systemic increase of proinflammatory cytokines may reduce feed intake, leading to starvation in weaned pigs (Johnson, 1997). The increased immune response is associated with the growth performance of piglets, not only by decreasing feed intake, but also by increasing competition between protein deposition and immune function (Le Floc'h et al., 2009). Studies carried out over the past years in nursery pigs showed that immune system stimulation increases the requirements for sulfur-containing AA (Kahindi et al., 2017b), threonine (Ren et al., 2014; Jayaraman et al., 2015), and tryptophan (Le Floc'h et al., 2008; Jayaraman et al., 2017).

2.4. POST-WEANING DIARRHEA AND ITS PREVENTION IN WEANED PIGS

Post-weaning diarrhea is a multifactorial gastrointestinal disease showing the discharge of watery feces during the first 2 weeks after weaning (Laine et al., 2008). The proliferation of one or more strains of enterotoxigenic *Escherichia coli* is one of the major factors causing PWD (Fairbrother et al., 2005). The enterotoxigenic *Escherichia coli* fimbriae bind to the porcine intestinal brush borders and secrete enterotoxins which disrupt fluid homeostasis and hypersecretion of fluid and electrolytes into the intestinal lumen, thereby resulting in the excess absorptive capacity of the large intestine (Nagy and Fekete, 2005; Zhang et al., 2006). Also, solid feeds are poorly digested and absorbed due to a temporary shortage of digestive enzymes at weaning, which leads to a fertile environment for *Escherichia coli* proliferation (Nabuurs, 1998). After weaning, a series of adverse

reactions is initiated, such as diarrhea, dehydration, reduced feed intake and nutrient digestibility, and retarded growth performance in piglets. Therefore, key factors of dietary or environmental management to minimize PWD are reducing the number of *Escherichia coli* and preventing adhesion of the enterotoxigenic *Escherichia coli* to enterocytes (Heo et al., 2013).

Various dietary interventions have been investigated to prevent PWD in weaned pigs. Antimicrobial growth promoters have been used in the swine industry to reduce the proliferation of pathogenic bacteria and ameliorate the PWD in weaned pigs, thereby promoting pig growth. However, due to increased concerns about the development of antibiotic resistance of bacteria, in-feed antibiotics have been banned in livestock production (Yang et al., 2009). Therefore, an alternative to antibiotics has been investigated for weaned pigs. Probiotics are live microbial feed supplements and are used for competitive exclusion of pathogenic bacteria by modulating gut microbiota and favoring gut eubiosis or by their immunomodulatory effects on the intestinal immune response (Lallès et al., 2007; Brousseau et al., 2015; Yang et al., 2016). Among probiotics, lactic acid producing bacteria such as *Lactobacillus* spp. or *Bifidobacterium* spp. are considered promising beneficial bacteria to produce antimicrobial substances or antioxidants (Ljungh and Wadström, 2006). The primary effect of probiotics is the barrier function of the intestinal mucosa. Increased adhesion of lactic acid producing bacteria to the intestinal epithelial cells causes inhibition of that of pathogenic bacteria as a result of an ability to increase the production of intestinal mucins (Liévin-Le Moal and Servin, 2006; Quinto et al., 2014). Also, *Lactobacillus* spp. increase the mRNA expression of mucin genes or mucin-binding proteins, thus, inhibiting the attachment of enterotoxigenic *Escherichia coli* (Mack et al., 2003; Macías-Rodríguez et al., 2009). Apart from the modulation of gut microbiota, another mode of action of probiotics is their ability to modulate host immune system (Roselli et al., 2007; Sauerwein et al., 2007). Lactic acid

producing bacteria have local or systemic effects on immune response by upregulating the expression of genes related to an adaptive immune response in the small intestinal mucosa, increasing blood immunoglobulins, and decreasing lymphocytes and acute phase protein in the blood of weaned pigs after enterotoxigenic *Escherichia coli* challenge (Luise et al., 2019; He et al., 2020).

Another significant strategy to reduce the proliferation of pathogenic bacteria and control PWD is feeding dietary fiber or prebiotics to weaned pigs. Post-weaning diarrhea is influenced by dietary non-starch polysaccharides (**NSP**) components, which increase viscosity of the intestinal contents (Hopwood et al., 2004). Non-starch polysaccharides are a class of dietary fibers that can change the physicochemical environment for microbial fermentation in the gut (Jensen and Jørgensen, 1994). The solubility, viscosity, or fermentability of dietary fiber modulates the intestinal environment, thereby affecting the expression of infectious intestinal diseases. The results of experiments evaluating the effects of dietary fiber on PWD are variable depending on solubility of the fiber sources. For example, fiber from the oat hulls, wheat bran, or wheat middlings are rich in insoluble fiber, which ameliorated protein fermentation and decreased the severity of PWD (Kim et al., 2008a; Berrocoso et al., 2015). On the other hand, high soluble fiber sources, such as sugar beet pulp or guar gum, are associated with increased susceptibility of weaned pigs to PWD due to increased digesta viscosity (McDonald et al., 1999; Molist et al., 2014). The inclusion of dietary soluble NSP in diets increases digesta viscosity and decreases digesta passage rate and nutrient digestibility, thereby increasing endogenous nitrogen flow in the small intestine (Kim et al., 2012). More substrates from dietary and endogenous nitrogen and increased proliferation time for bacteria can increase the proliferation of proteolytic microbes such as Escherichia coli, increasing the incidence of PWD in piglets. However, part of the reasons for the

increased incidence of diarrhea in weaning pigs fed soluble fiber is the limited digestive capacity of the piglets (Molist et al., 2014). Therefore, soluble fiber sources can be gradually included in the nursery diets once the pigs adapt to the solid feed to provide the environment for healthy fermentation. The fermentability of dietary fiber sources also influences the incidence of PWD in pigs. Dietary fiber fermentation generates short-chain fatty acids (**SCFA**) in the gut, which are considered beneficial components for intestinal development and gut health in pigs (Jha and Berrocoso, 2015). Soluble fiber sources are rapidly fermented in the gastrointestinal tract of pigs and are generally more fermentable than insoluble sources (Agyekum and Nyachoti, 2017). The fermentation of soluble fiber typically occurs in the proximal colon, whereas the fermentation of insoluble fiber is sustained until the end of the colon (Choct, 1997). Some studies reported that highly fermentable sources could increase the incidence of PWD in pigs because of the limited capacity of piglets at this age to ferment fiber, which results in accumulated non-fermentable materials in the gut (Pluske et al., 1998; McDonald et al., 2001; Molist et al., 2014). Therefore, feeding insoluble or a combination of soluble and insoluble fiber may be more preferable strategies to promote a superior response on indices of gut health in weaned pigs (Molist et al., 2009; Chen et al., 2020). Prebiotics are defined as "a selectively fermented ingredient that allows specific changes, both in the composition and activity in the gastrointestinal microflora, that confer benefits" (Gibson et al., 2004). All prebiotics are fiber sources including inulin, resistant starch, fructo-oligosaccharides, and galacto-oligosaccharides (Slavin, 2013). Previous studies investigated that the incidence of diarrhea was reduced along with decreased populations of *Escherichia coli* by increasing population of beneficial bacteria, when weaning piglets fed diets containing fructo-oligosaccharide (Liu et al., 2020b; Zhang et al., 2022) or chito-oligosaccharide (Liu et al., 2008a;

Liu et al., 2010). Thus, the effects of prebiotics can be synergistically enhanced with probiotics supplementation to reduce the incidence of diarrhea in weaned pigs (Krause et al., 2010).

2.5. DIETARY PROTEIN TO MANAGE POST-WEANING DIARRHEA

Weaning is one of the most stressful phases for young pigs because of social stress and stress coming from diet and environmental change, which generates changes to the structure and function of the gastrointestinal tract as well as decreases feed intake and increases the occurrence of PWD (Pluske et al., 2018). Dietary CP content plays a vital role in young piglet health. Reduced feed intake and weight gain are typically observed after weaning, and to resolve this poor performance, weaned pigs were traditionally fed with HCP diets at the starter phase. However, a diet containing a high CP level increases the substrates for protein fermentation, which increases the proliferation of pathogenic bacteria such as *Escherichia coli*, which impairs intestinal health in weaned pigs (Opapeju et al., 2008). Therefore, feeding LCP diets with supplementation of CAA to meet the indispensable AA requirements has been investigated as a strategy to enhance gut health in weaned piglets in an antibiotic-free feeding system (Opapeju et al., 2008, 2009a). In fact, to recover from the impact caused by weaning stressors, piglets need a high nutritional diet. Thus, risk such as reduced growth performance is accompanied by the reduction of dietary CP, and the LCP diets should be carefully formulated with the inclusion of a sufficient amount of indispensable AA (Le Floc'h et al., 2018).

2.5.1. DIETARY PROTEIN LEVEL AND POST-WEANING DIARRHEA

Lowering dietary CP content can modulate gut health in piglets by changing microbial populations and activities (Figure 2.1). High dietary CP content potentially generates more non-digested compounds, which can be used for microbial fermentation. Substrate availability is known as a

major driver for bacterial fermentation pathways (Pieper et al., 2016). Various pathogens, including enterotoxigenic *Escherichia coli*, prefer to use fermentable protein for their metabolism and energy sources, therefore, HCP diets promote the growth of pathogenic bacteria and associated production of toxic metabolites such as ammonia and biogenic amines in the large intestine (Ball and Aherne, 1987; Buddle and Bolton, 1992). In this regard, feeding a LCP diet to weaned pigs has been investigated to reduce protein fermentation and its metabolites, thereby decreasing diarrhea incidence (Nyachoti et al., 2006; Opapeju et al., 2009).

The roles of LCP diets in different growth stages of pigs have been extensively researched (Table 2.1). Most studies were conducted using weaned pigs, and growth performance and gut health were the primary parameters in this phase. Major response criteria in growing pigs were nutrient utilization and digestibility, microbial composition, and growth performance, and the finishing phase was focused on N excretion and ammonia emissions in slurry.

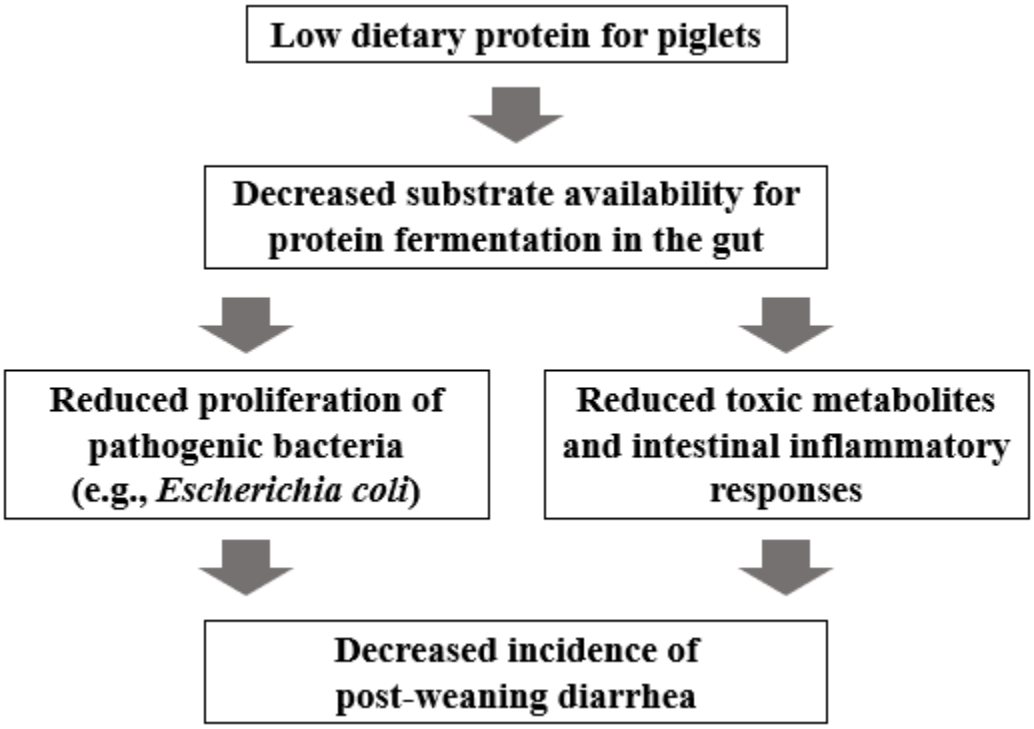

Figure 2.1 Mechanisms of action of low dietary CP content as a function of managing post-weaning diarrhea. Adapted from Opapeju (2010).

Table 2.1 Effects of low crude protein (**CP**) diets on growth performance, gut health, and immune response in pigs

Age, day (BW)	CP content	CP difference	Effects of low CP diets	Reference
14 (4.9 kg)	24, and 20%	4% units	↓microbial metabolites (ammonia N, biogenic amine, and SCFA)	Htoo et al. (2007)
17 (6.4 kg)	21, 19, and 17%	2% units	↓crypt hypertrophy ↓ammonia N concentration in large intestine ↓small intestine weight ↓growth performance	Opapeju et al. (2008)
17 (5.3 kg)	22.5, and 17.6%	4.9% units	↓growth performance before *E. coli* challenge ↑relative composition of butyrate producing bacteria in the colon after *E. coli* challenge	Opapeju et al. (2009a)
18 (6.2 kg)	23, 21, 19, and 17%	-	↓ADG and ADFI in 19 and 17% CP ↓feed efficiency in 17% CP linear ↓ammonia N ↓SCFA concentrations	Nyachoti et al. (2006)
18 (6.8 kg)	23.1, 21.2, 18.9, and 17.2%	-	↓ADG and feed efficiency in 17.2% CP, but no difference on growth performance until 18.9% ↓fecal score 18.9% CP did not affect intestinal morphology	Yue and Qiao (2008)
19 (7.0 kg)	22.3, and 17.3%	5% units	no difference on ADFI no negative effect on the development of jejunal brush border enzymes	Opapeju et al. (2009b)
19 (7.0 kg)	22.2, and 17.3%	4.9% units	↓*E. coli* proliferation and attachment in the intestinal mucosa ↓expression of sodium-coupled glucose transporter 1	Opapeju et al. (2015)
19 (7.5 kg)	24, and 20%	4% units	no difference on growth performance or fecal consistency scores	Htoo et al. (2007)
20 (5.5 kg)	22, 19, and 16%	3% units	↓fecal score ↓expression of genes associated with inflammation ↑VH in the jejunum ↓CD in the ileum ↓feed efficiency in 16% CP	Limbach et al. (2021)

Table 2.1 Effects of low crude protein (**CP**) diets on growth performance, gut health, and immune response in pigs (continued)

Age, day (BW)	CP content	CP difference	Effects of low CP diets	Reference
21 (5.6 kg)	21, 19.5, 18, and 16.5%	1.5% units	Linear ↓ ADG and feed efficiency Linear ↓fecal dry matter	Batson et al. (2021)
21 (5.9 kg)	25.6, and 17.5%	8.1% units	↓fecal ammonia N ↓incidence of diarrhea ↑fecal dry matter content no difference on growth performance	Heo et al. (2009)
21 (6.1 kg)	24.3, and 17.3%	7% units	↓fecal score ↓fecal ammonia N no difference on total tract digestibility of dry matter, energy, and CP	Heo et al. (2008)
21 (8.2 kg)	21, and 17%	4% units	no difference on growth performance, enzyme activity ↓fecal score ↑VH:CD ratio	Zhou et al. (2020)
24 (7.4 kg)	20 and 16%	4% units	↓ADG and feed efficiency ↓fecal score ↓butyric acid concentration in feces	Lynch et al. (2009)
24 (6.9 kg)	19 and 16%	3% units	↓ADG and feed efficiency	Wellington et al. (2023)
25 (9.1 kg)	24.4, and 18.7%	5.7% units	no significant difference on ADG ↓fecal score ↓ileal bacterial community richness and diversity inhibited the growth of protein-fermenting bacteria	Pollock et al. (2019)
26 (6.5 kg)	21, and 18%	3% units	no improvement in fecal score ↑relative abundance of Enterobacteriaceae ↑bacterial diversity	Rattigan et al. (2020)
28 (6.7 kg)	19.1, 16.6, and 14%	2.5% units	↓incidence of diarrhea ↓ADG, but no difference in feed efficiency	Lynegaard et al. (2021)
28 (8.0 kg)	21, and 17%	4% units	↓ADG, ADFI, and feed efficiency ↓VH (villous atrophy) ↓immune response	Ren et al. (2015)

Table 2.1 Effects of low crude protein (**CP**) diets on growth performance, gut health, and immune response in pigs (continued)

Age, day (BW)	CP content	CP difference	Effects of low CP diets	Reference
28 (8.6 kg)	21.1, and 18.6%	2.5% units	↓fecal protein, ammonia, pH and butyrate concentration	Bindas et al. (2019)
28 (9.6 kg)	20, 17, and 14%	3% units	↓nitrogen emissions ↓digestive capacity ↓VH and VH:CD no reduction more than 3% CP due to maladaptive changes in intestinal morphology and enzyme activity	Yu et al. (2019)
28 (11.9 kg)	22.4, 20.4, 18.4, and 16.9%	-	no difference in ADG and feed efficiency no difference in fecal consistency no difference in energy utilization and body composition by reducing CP 5.5% units	Le Bellego and Noblet (2002)
30 (8.3 kg)	22, and 19%	3% units	no difference in piglet performance ↓ammonia N, cecal *E. coli* counts ↓serum interleukin-1 and tumor necrosis factor-α	Wang et al. (2011)
weaned pigs (6.8 kg)	21.7, and 19.7%	2% units	↓ADG and feed efficiency ↓emissions of ammonia and hydrogen sulfide ↓SCFA concentrations ↓nutrient digestibility	Lee et al. (2017)
weaned pigs (9.6 kg)	20, and 17%	3% units	no difference in intestinal mucosal barrier function and morphology in the jejunum	Li et al. (2019)
nursery pigs (13.6 kg)	19.5, and 16%	3.5% units	↓ADG and ADFI no difference on N digestibility	Cho et al. (2008)
45 (13.5 kg)	20, 17.2, 15.3, and 13.9%	-	moderate reduction (2.8% units) of dietary CP level may benefit large intestinal bacterial community and its metabolites production, which was negatively affected by extremely low CP diet (15.3 and 13.9%)	Peng et al. (2017)
63 (19.9 kg)	16 and 13%	3% units	↑protein expression of pro-inflammatory cytokine	Wang et al. (2021)
growing pigs (19.6 kg)	18, and 15%	3% units	no significant difference on growth performance ↑protein, amino acid, and energy utilization may be due to the improvement of intestinal microbiota	Liu et al. (2021)

Table 2.1 Effects of low crude protein (**CP**) diets on growth performance, gut health, and immune response in pigs (continued)

Age, day (BW)	CP content	CP difference	Effects of low CP diets	Reference
growing barrows (21.7 kg)	20.5, and 15.3%	5.3% units	↓abundances of microflora in the hindgut of growing pigs, which weakened the fermentation capacity ↓nutrient digestion efficiency in the hindgut ↓total tract nutrient digestibility	Zhou et al. (2022)
growing (37 kg) and finishing pigs (62 kg)	18, 15, and 12% (growing), 16, 13, and 10% (finishing)	3% units	↓growth performance when dietary CP reduced by 6% units ↓excretion of N with maintaining ADG and feed efficiency when dietary CP reduced by 3% units Maintaining mRNA levels of digestive enzymes and ileal AA digestibility when dietary CP reduced by 3% units	He et al. (2016)
growing (37 kg) and finishing pigs (62 kg)	18, 15, and 12% (growing), 16, 13, and 10% (finishing)	3% units	↓ADG and feed efficiency in growing pigs no difference in feed efficiency when dietary CP reduced by 3% units in finishing pigs Improved meat quality of growing and finishing pigs through regulation of intramuscular fat and fatty acids composition in longissimus dorsi muscle	Li et al. (2018)
growing pigs (45 kg)	20.8, 17, 15, and 12.3%	-	quadratic ↑ADG until 15% and a deterioration thereafter quadratic ↓urinary N and N utilization	Carpenter et al. (2004)
growing (50 kg) and finishing pigs (88 kg)	17, and 15% (growing), 15, and 14% (finishing)	1-2% units	↓urinary and total N excretion	Hernández et al. (2011)
finishing pigs (62 kg)	16, 13, and 10%	3% units	↓intestinal SCFA and biogenic amines concentrations ↑expression of tight junction proteins in 13% CP ↓VH and ↑CD in 10% CP ↓expression of biomarkers of intestinal stem cells in 10% CP	Fan et al. (2017)
finishing pigs	21, 19, 16, and 13%	-	↓ammonia emissions and odor emission rate in 16 and 13% CP	Leek et al. (2007)

2.5.2. LOW PROTEIN DIETS AND CRYSTALLINE AMINO ACIDS

High protein levels in nursery diets have been attributed to both gut health and environmental impacts in swine industry. Ammonia emission in pig manure originates mainly from the urea (Lenis and Jongbloed, 1999). Because of urease activity of fecal microbiome, urea is easily converted to ammonia, which causes a concern such as aerial pollution and acidification of the soil. Environmental benefits of reducing CP level in diets are expected by lowering N waste. However, piglet performance could deteriorate when CP is reduced in the diets due to a limited availability of AA. Thus, the provision of CAA in the diets to meet indispensable AA requirements for optimal growth has been utilized in LCP swine diets. Non-bound AA (synthetic and CAA) in LCP diets can replace intact protein sources such as soybean meal in high CP diets (Heo et al., 2009; Selle et al., 2020). Dietary CP can be lowered without retardation of growth performance in the nursery by utilizing the proper AA ratios with CAA supplementation (Goodband et al., 2014). Htoo et al. (2007), Yue and Qiao (2008), and Liu et al. (2021) reported the growth performance of pigs fed LCP diets formulated by replacing soybean meal with CAA to meet the AA requirements was not affected when CP was lowering 3 to 4% units from those fed high protein diets. Moreover, there are potential advantages to nutrient digestion and gut health with the increased use of CAA rather than intact protein sources (Yu et al., 2019). While the AA in the intact protein from need to be digested and are not completely released by the digestive enzymes, those in free form such as CAA can be fully available and rapidly absorbed in the intestine (Yen et al., 2004). Low CP diets contain more CAA and less intact protein, therefore, they do not need high enzymes activity to degrade intact protein, which might be attributed to increased digestibility of CP of weaned pigs fed LCP diets (Yu et al., 2019). Also, AA digestibility can affect intestinal morphology because villi is the main unit of AA absorption in the small intestine where the AA transporters are located

(Gao et al., 2000; Salgado et al., 2002). Therefore, higher VH indicates an increased total intestinal surface area for AA absorption (Eugenio et al., 2022). Eugenio et al. (2022) investigated that highly digestible diet using free form of AA resulted in higher VH and VH to CD ratio in the jejunum compared to the diet using intact protein sources. Although it is difficult to find studies regarding the effects of LCP diets on gas emissions in nursery pigs, potential reduction of greenhouse gas emission from swine manure by using LCP diets supplemented with CAA has been investigated. Feeding reduced CP diets formulated by replacing soybean meal with CAA did not impact growth performances of grow-finisher pigs and is effective to reduce NH_3 emissions compared to HCP diets (Hernández et al., 2011; Li et al. 2015b). However, limited studies have been carried out on the effects of different supplementation patterns of CAA in the LCP diets fed to nursery pigs.

2.5.3. LOW PROTEIN DIETS AND GROWTH PERFORMANCE IN WEANED PIGS

Several studies demonstrated that feeding LCP diets did not impair piglet growth, however, inconsistency in the results still has been found for the effects of LCP diets on growth performance in weaned pigs. Nyachoti et al. (2006), Htoo et al. (2007), Yue and Qiao (2008), Wang et al. (2011), Pollock et al. (2019), and Zhou et al. (2020) reported the growth performance of pigs fed LCP diets by lowering CP 3 to 4% units did not differ from those fed high protein diets, whereas others investigated impaired growth performance in weaned pigs when CP levels were lowered by 2 to 5% units (Cho et al., 2008; Opapeju et al., 2008, 2009a; Lee et al., 2017; Lynegaard et al., 2021). This discrepancy could be explained by differences in the weaning age of pigs and the presence of an adaptation period before starting the experiment. Leibbrandt et al. (1975) showed that pigs weaned at 4 weeks could adapt to the diet and environment faster than pigs weaned at 2 weeks, and Leliveld et al. (2013) reported that post-weaning growth performance increased as weaning age increased from 3 to 5 weeks. Also, the presence of an adaptation period prior to the start of the

experiment may affect the growth performance of weaned pigs by providing time to get used to solid feeds and new facilities. For example, although Nyachoti et al. (2006) and Opapeju et al. (2008) used pigs with similar weaning dates (18-day-old vs. 17-day-old) and BW (6.2 vs. 6.4 kg), pigs used in the Nyachoti et al. (2006) study were provided a 7-day adaptation period before the experiment began, which may lead to better growth compared to a 4-day adaptation period for the pigs used in the Opapeju et al. (2008). Indeed, the growth performance of weaned pigs fed LCP (until 4% units lower CP) in the study by Nyachoti et al. (2006) did not differ from that of pigs fed HCP, whereas the feed efficiency of the pigs fed LCP diets was lower than that of pigs fed HCP diet in the study by Opapeju et al. (2008). In addition, one of the most important factors affecting the growth performance of weaned pigs is the ideal ratio of indispensable AA to lysine in LCP diets. A balance of AA is essential for protein retention, thus, a deficiency of any one of the indispensable AA in diets or dietary imbalance of indispensable AA results in impaired growth performance of pigs. Because only small amounts of protein sources such as soybean meal are included in LCP diets, LCP diets would most likely not fulfill all indispensable AA requirements without supplementation of CAA. For example, the studies providing only crystalline lysine, methionine, threonine, and tryptophan to meet those AA requirements in LCP diets showed poor growth performance in weaned pigs compared to those in HCP diets (Cho et al., 2008; Lee et al., 2017), which might be due to insufficient AA supply in those LCP diets. Although some studies, which did not find differences in growth performance between pigs fed the HCP and LCP diets, did not provide all indispensable AA as CAA in LCP diets to meet all indispensable AA requirements (Nyachoti et al., 2006; Htoo et al., 2007; Zhou et al., 2020), these studies provided crystalline lysine, methionine, threonine, tryptophan, isoleucine, and valine to meet AA requirements in LCP diets. Indeed, Ren et al. (2015) showed supplementation of the branched-

chain amino acid (**BCAA**) in LCP diets could maintain growth performance compared to the HCP diet in weaned pigs. However, Opapeju et al. (2008) showed contrary results in the poor performance of pigs fed the LCP diets compared to those fed the HCP diet despite supplementation of isoleucine and valine in LCP diets, which indicated other indispensable AA, including leucine, histidine, or phenylalanine, could have been limited in LCP diets. Recent research by Wellington et al. (2023) investigated lower ADG and feed efficiency in the pigs fed LCP diets than those fed a HCP diet despite providing 10% higher level of all indispensable CAA except Phe. Thus, they concluded that poor growth performance in pigs fed LCP diets might be due to either limiting Phe or the high indispensable AA:total N ratio in the experimental diets, which compromises the efficient use of AA for protein synthesis and growth. Formulation of weaned pig diets to be low in CP should be done carefully because dispensable AA could be insufficient in LCP diets. Lowering CP content potentially reduces not only indispensable but also dispensable AA as well, therefore, standardized ileal digestible (**SID**) Lys:CP ratio should be considered for LCP diets formulation. Nemechek et al. (2014) demonstrated that the growth performance among pigs fed different SID Lys:CP ratio diets from 6.2 to 6.9% did not differ. Moreover, according to Htoo (2017), a maximum dietary SID Lys:CP ratio of 6.9% should be maintained to avoid adverse effects on pig performance due to the deficiency of non-essential AA or nitrogen for non-essential AA synthesis at a higher SID Lys:CP ratio.

2.5.4. LOW PROTEIN DIETS AND GUT HEALTH IN WEANED PIGS

The positive effects of LCP diets with supplementation of appropriate AA are mostly related to gut health in weaned pigs, and intestinal bacterial fermentation has a key role in gut health. Undigested and endogenous proteins are utilized for microbial metabolism in the gut via deamination and decarboxylation (Pieper et al., 2016). Bacterial protein catabolism is related to

gut pH because deaminases are active at neutral or slightly alkaline pH, unlike decarboxylase, which is active under acidic conditions (Blachier et al., 2007). The large intestine has acidic pH in normal physiological conditions, therefore, carbohydrate fermentation is preferred over protein (Pieper et al., 2016). In the distal colon, however, carbohydrates are depleted, and the fermentation pattern is shirted from saccharolytic to proteolytic fermentation from the proximal to distal colon due to increased pH (above 6) as a consequence of the released alkaline ammonia (Pieper et al., 2016; Vieira-Silva et al., 2016). Carbohydrate fermentation is considered beneficial for the host because of SCFA production, such as acetate, propionate, and butyrate. Acetate takes the highest concentrations among the SCFA and is rapidly absorbed and transported to the liver to be used for lipogenesis after entering the systemic circulation (Wong et al., 2006). Propionate is a substrate for hepatic gluconeogenesis, and butyrate plays an important role in the regulation of cell proliferation and differentiation as a major energy source for colonocytes (Wong et al., 2006). Unlike carbohydrate fermentation, microbial protein fermentation is considered to be detrimental to the host. High dietary CP increases the substrates for the proliferation of pathogenic bacteria that usually colonize the gut after weaning, such as *Escherichia coli*, therefore inducing PWD (Ball and Aherne, 1987). The endotoxins produced by enterotoxigenic *Escherichia coli* can change the physiological characteristics of the gastrointestinal tract, linking to poor water and fluid absorption and ion secretion, resulting in an increased incidence of diarrhea (Sun and Kim, 2017). Decreased intestinal *Escherichia coli* counts have been found in weaned pigs fed LCP diets than in HCP diets (Wang et al., 2011), and pigs fed a LCP diet showed a depression of *Escherichia coli* proliferation and attachment in the intestinal mucosa compared to a HCP diet under *Escherichia coli* challenge model (Opapeju et al., 2015).

Deamination of AA contributes to the generation of SCFA and branched-chain fatty acids (**BCFA**) productions and the formation of ammonia (Blachier et al., 2007). Ammonia is a typical metabolite produced via deamination of AA, and it can be used by bacteria for their metabolism and own protein synthesis (Windey et al., 2012). Deamination of BCAA yields BCFA, including isovalerate (from leucine), 2-methylbutyrate (from isoleucine), and isobutyrate (from valine). In contrast, decarboxylation of AA results in the production of biogenic amines such as cadaverine (from lysine), histamine (from histidine), tyramine (from tyrosine), and tryptamine (from tryptophan) (Pieper et al., 2016). Polyamines, including putrescine, spermine, and spermidine, are either generated by bacteria or synthesized from the degradation of AA, such as arginine, ornithine, and methionine (Minois et al., 2011). Bacterial degradation of aromatic AA, such as tyrosine, phenylalanine, and tryptophan, produces phenolic and indolic compounds (e.g., phenol, *p*-cresol, and skatole) in the colonic lumen (Windey et al., 2012). In addition, hydrogen sulfide is produced as a result of bacterial degradation of dietary and mucinous sulfur-containing AA such as methionine, cystine, cysteine, and taurine (Lewis and Cochrane, 2007). Hydrogen sulfide is a typical toxic gas that has been attributed to deleterious effects such as genomic DNA damage with high concentrations (Davila et al., 2013). Reduced ammonia and hydrogen sulfide emissions were found in weaned pigs fed the LCP diet (Lee et al., 2017).

These proteolytic fermentation metabolites have been associated with gut health impairment. For example, high ammonia N concentration can damage the mucosal cell and gut structure and impair gut function (Lin and Visek, 1991; Jensen, 1998). Also, ammonia increases epithelial permeability (Villore Tudela et al., 2015) and can interfere with the metabolism of SCFA in the colon, leading to inducing energy deficiency in the cell (Blachier et al., 2007). Many previous studies showed that LCP diets reduce ammonia N in the small or large intestines of

weaned pigs (Nyachoti et al., 2006; Htoo et al., 2007; Opapeju et al., 2008; Wang et al., 2011), and Opapeju et al. (2008) investigated reduced crypt hypertrophy in pigs fed LCP diets which could be related with reduced ammonia concentration. Others also showed similar results that increased VH, decreased CD, and increased VH to CD ratio in the small intestine of weaned pigs fed LCP diets (Opapeju et al., 2009a; Zhou et al., 2020; Limbach et al., 2021). On the contrary, several studies reported villus atrophy (Ren et al., 2015; Yu et al., 2019) or no significant difference in intestinal morphology (Li et al., 2019) in the pigs fed LCP diets compared to those fed HCP diets. These results could be due to protein deficiency which made pigs unable to maintain the gut architecture of intestinal epithelium (Yu et al., 2019). Also, intestinal stem cell growth was inhibited in adult pigs when dietary protein content decreased, which could be related to the suppression of villus growth (Fan et al., 2017). Hughes et al. (2008) reported that phenol and ammonia derived from colonic bacteria increased paracellular permeability and impaired barrier function in the Caco-2 cell *in vitro* model, and Andriamihaja et al. (2015) revealed that *p*-cresol impairs mitochondrial oxidative metabolism in human colonic epithelial cell line and increases DNA damage. Although little information about the role of biogenic amines is available, few biogenic amines have been reported to function as a promoter of diarrhea. Histamine induces chloride secretion into the gut lumen in the proximal colon of pigs, leading to diarrhea (Ahrens et al., 2003). Kröger et al. (2013) demonstrated histamine concentration was lower with low dietary fermentable protein diets compared to high fermentable protein diets. Also, high cadaverine and putrescine concentrations can reduce the metabolism of histamine in pig epithelium and increase the detrimental effects of histamine, which is associated with an increased incidence of diarrhea in weaning pigs (Teti et al. 2002). Feeding LCP diets decreased ammonia and cadaverine concentrations in colonic digesta as well as the reduced abundance of *Escherichia coli* in the colon

of nursery pigs (Wang et al., 2019). Collectively, these metabolites derived from protein fermentation negatively affect gut health in weaned pigs, and by reducing the above-mentioned toxic metabolites, LCP diets could reduce the incidence of PWD in weaned pigs (Yue and Qiao, 2008; Zhou et al., 2020; Limbach et al., 2021).

Although the effects of LCP diets on gut health have been studied recently, there is still limited research on gut microbial population in weaned pigs. Both Gram-positive (e.g., *Streptococcus, Lactobacillus, Bifidobacterium,* and *Clostridium*) and anaerobe Gram-negative bacteria (e.g., *Fusobacterium, Bacteroides, Selenomonas, Butyrivibrio* and *Prevotella*) compose the gastrointestinal microbiota in pigs (Rist et al., 2013). Because of the existence of mutual interactions between host animal and gastrointestinal microbiota, there is growing attention on bacterial ecology of the gastrointestinal tract in nursery pigs. The interaction between host animal and gut bacteria can be not only microbial metabolism which can be influenced by host originated dietary substrates as mentioned above, but also supportive effects of gastrointestinal microbiota on host animal through immune modulation and pathogen protection (Fanning et al., 2012). A few studies have been conducted to investigate the effects of LCP diets on microbial composition in weaned pigs. Lynch et al. (2009) reported that dietary CP concentrations did not affect bacterial population (enterobacteria and lactobacilli) in the feces of weaned pigs. Similarly, no differences were found between dietary treatments of either 19 or 15% CP levels on counts of coliforms, enterococci, enterobacteria and lactobacilli in the feces of piglets in a study conducted by Hermes et al. (2009). However, Opapeju et al. (2009a) reported higher counts of carbohydrate-fermenting, butyrate-producing *Roseburia* in colonic digesta of pigs fed LCP than HCP diet on day 7 post challenge with *Escherichia coli* K88. In the study by Zhou et al. (2016), the effects of a long-term LCP diet on the microbial composition and metabolomic profile in the hindgut of the pig were

assessed. In this study, feeding LCP diet significantly decreased the relative abundance of *Lactobacillus*, and increased the abundance of *Prevotella* and *Coprococcus* in the cecal digesta of pigs. *Prevotella* is known as the predominant fiber-degrading bacterial species in the intestinal tracts of pigs (Varel, 1987) and *Coprococcus* is one of the major butyrate-producing bacteria via carbohydrate fermentation (Holdeman and Moore, 1974), therefore, feeding LCP diets for a long-term could beneficially modulate the microbial composition in pigs. Altogether, results in studies with piglets are rather inconsistent, thus, more research is required to enable us to determine the effects of feeding LCP diets on microbial composition in weaned pigs.

2.5.5. LOW PROTEIN DIETS AND IMMUNE SYSTEMS IN WEANED PIGS

The toxins secreted by *Escherichia coli* affect not only the physiological characteristics of the gastrointestinal tract but also the immune system of weaned pigs. The bacterial structural components initiate a cascade of immune stimulation, resulting in intestinal and systemic inflammation (Bannerman and Goldblum, 2003; Gil-Cardoso et al., 2019). Lipopolysaccharide is the component on the surface of the outer membrane of Gram-negative bacteria, including *Escherichia coli* (Alexander and Rietschel, 2001), and the lipopolysaccharide is a highly pro-inflammatory molecule that elicits up-regulation of cytokines and induces endothelial cell death (Bannerman and Goldblum, 2003). Receptors that respond to lipopolysaccharide are present in the innate immune cells such as macrophages, and the activation of the receptors elicits the biosynthesis of inflammatory mediators (Raetz and Whitfield, 2002). Feeding a high dietary CP diet can increase the proliferation of *Escherichia coli*, therefore, the HCP diet has been correlated with intestinal and systemic inflammation in weaned pigs. Wang et al., (2011) reported that high serum concentrations of pro-inflammatory cytokines, including interleukin (**IL**)-1 and tumor necrosis factor (**TNF**)-α were observed in weaned pigs fed HCP diets compared to those fed LCP

diets. Similarly, feeding LCP diets decreased protein expressions of Toll-like receptor-4, nuclear factor-κB, IL-1β, and TNF-α in colonic tissue of nursery pigs (Wang et al., 2019). A toll-like receptor is the receptors that respond to lipopolysaccharide, thus, the expression of the Toll-like receptor decreased in pigs fed LCP diets. Nuclear factor-κB is considered a mediator of the pro-inflammatory signaling pathway, and IL-1β and TNF-α are pro-inflammatory cytokines, which are also reduced in pigs fed LCP diets.

Some proteolytic fermentation metabolites are related to increased inflammatory responses in weaned pigs. A study by Villodre Tudela et al. (2015) showed that ammonia derived from the HCP diet downregulated monocarboxylate transporter 1 gene expression and stimulated pro-inflammatory cytokine expression in the colon of piglets. Although polyamines have beneficial effects on the gut, such as serving as an energy source for enterocytes or protecting mitochondrial membrane integrity in the small intestine (Bekebrede et al., 2020), they could be an indicator of immune response based on their prominent role in the regulation of cell proliferation and development in immune cells such as T-cell, B-cell, and macrophage (Hesterberg et al., 2018). For instance, macrophages are produced in response to infection or inflammatory stimulation, and spermine uptake is increased in activated macrophages under inflammatory stimulation (Zhang et al., 2000).

On the contrary, others reported feeding LCP diets to weaned pigs resulted in an impaired immune response. Ren et al. (2015) revealed that reducing dietary CP levels significantly increased the number of intra-epithelial lymphocytes in the small intestine and decreased immunoglobulin concentrations in the jejunum and ileum tissues, which could be due to AA deficiency in pigs fed LCP diets. Intra-epithelial lymphocytes are the immune cells lining the gastrointestinal epithelium and majorly consist of T cells which have a functional role in the development of tolerance and

adaptive response to luminal antigens (Lundqvist et al., 1995). The nutritional requirement, especially AA and glucose, for intestinal epithelium increased in post-weaning pigs due to the synthesis of immune-related protein (Dugan et al., 1994; Lallès et al., 2007), and thus, AA deficiency in pigs fed LCP diets can impair immune response.

2.5.6. DIETARY PROTEIN SOURCES AND POST-WEANING DIARRHEA

The dietary protein source has also been proposed to influence gut health in weaned piglets (Pluske et al., 2002). Animal protein sources such as spray-dried animal plasma (**SDAP**), whey protein, and fish meal have high digestibility of protein and AA than plant-origin protein sources (Yun et al., 2005; Yoo et al., 2009), thus, the growth performance of weaned pigs fed diets containing animal protein was higher than those fed plant-origin such as soybean meal (Chae et al., 1999; Yun et al., 2005). Pigs fed animal protein sources also showed better gut morphology than those fed plant sources in the post-weaning period (Yun et al., 2005). Legume protein sources such as soybean meal, lupins, and peas have been studied to have a negative impact on growth performance and gut health in weaned pigs. Soybean contains various anti-nutritional factors, such as protease inhibitors and allergenic compounds, which hinder digestion and absorption of nutrients (Cervantes-Pahm and Stein, 2010) and activate the immune system (Sun et al., 2008). The allergenic compounds such as glycinin and β-conglycinin induce an inflammatory response by increasing immunoglobulin and pro-inflammatory cytokine production in intestinal mucosa in weaned pigs (Sun et al., 2008). Inflammation initiates a cascade of intestinal effects, including alteration of the intestinal mucosa and impairment of gut barrier function (Peuhkuri et al., 2010). Inflammation also can cause a reduction in feed intake in pigs, which negatively alters the intestinal morphology in pigs (Pluske et al., 1997). Although only limited information on the effects of dietary protein sources on intestinal microbial composition is available, a few studies showed the

potential positive effects of animal protein on microbial structure in weaned pigs. Compared to feeding pea protein isolate diets, feeding SDAP diets reduces the incidence and severity of diarrhea in weaned pigs challenged with *Escherichia coli* (Owusu-Asiedu et al., 2003). Also, Che et al. (2020) demonstrated improved microbial diversity and increased the abundance of Firmicutes but decreased the Proteobacteria found in colonic digesta of weaned pigs fed SDAP diet than those fed soy protein concentrate diet, which were associated with a high abundance of *Lactobacillus* and low abundance of *Escherichia–Shigella* in weaned pigs. Lowering CP content in weaned pig diets is typically occurred by reducing the soy protein contents, thus, LCP diets can ameliorate immune stress and improve gut health and PWD in weaned pigs (Opapeju et al., 2008; Heo et al., 2009; Peng et al., 2016).

2.5.7. LOW PROTEIN DIETS IN COMBINATION WITH ANOTHER FACTOR

In several recent studies with newly weaned piglets, effects of LCP diets were tested with additional dietary factors such as carbohydrate sources or feed additives. As was reviewed by de Lange et al. (2010), the use of fermentable carbohydrates can be the most promising approach to beneficially change the composition and activity of the intestinal microbiota in pigs. Therefore, effects of dietary supplementation of fermentable carbohydrates in different CP diets have been investigated to possibly reduce protein fermentation in the gut. According to Hermes et al. (2009), the addition of dietary fiber in the LCP diet reduced the fecal score and increased the antibiotics interventions, whereas the opposite was observed in pigs fed the HCP diet. However, this interaction between dietary levels of CP and fiber was not observed on the growth performance or the microbial counts in the colonic digesta of weaned pigs. In contrast, Lynch et al. (2009) observed no significant interaction between dietary CP and inulin supplementation on fecal score, fecal microbial populations and their metabolites concentrations (volatile fatty acids

concentrations) in weaned pigs. Overall, results of currently available studies on the interaction between dietary CP content and supplementation of fermentable carbohydrate source on growth performance and gut health in weaned pigs are inconsistent, and more research is needed in this area.

In addition to carbohydrates, some studies have reported to investigate the effects of LCP with feed additives in weaned pigs. Tang et al. (2019) obtained interaction effects between dietary CP content and probiotics supplementation, whereby a greater ADG and higher villus in ileum of weaned pigs when they fed LCP diets with the addition of probiotics (*Bacillus subtilis*). According to a study of Wang et al. (2021), pigs fed LCP diets supplemented with casein hydrolysate showed improved intestinal barrier function and decreased pro-inflammatory cytokine expression in the small intestine of growing pigs.

Not only dietary factors but also environmental factors may affect growth performance and gut health in weaned pigs. In a study by van der Meer et al. (2016), if the performance and immune status of pigs kept under different sanitary conditions is influenced by protein intake and AA supplementation were tested from nursery to finishing phase. Increasing the dietary CP concentration increased serum haptoglobin concentrations in pigs kept under the low sanitary conditions but not in those under the high sanitary conditions. However, no significant interaction effects between sanitary conditions and dietary CP content were detected on growth performance of pigs. Although the environmental factor may affect growth performance and gut health in weaned pigs, no studies were performed yet to investigate the interactive effects of dietary CP and sanitary conditions in young piglets.

2.6. AMINO ACID REQUIREMENT FOR PIGLETS

Protein is a relatively expensive nutrient and for this reason, investigating a balance between AA supply and AA demand of animals is highly necessary. Moreover, excess dietary protein contributes to nitrogen excretion and increases environmental concern in the world. Therefore, determining the accurate AA requirements is essential not only economically to reduce feed costs and achieve optimal growth of pigs but also environmentally to maintain sustainable swine production.

2.6.1. METHODS FOR DETERMINATION OF AMINO ACID REQUIREMENTS

Empirical and factorial methods are the most common approaches to determining the AA requirements of pigs (NRC, 2012). The AA requirements in the empirical approach are defined as the minimal amount of AA needed to maximize (e.g., weight gain or feed efficiency) or minimize (e.g., plasma urea nitrogen; **PUN**) responses during a certain experimental period (Hauschild et al., 2010). Critical components for empirical determination of AA requirements include: 1) a deficient amount of test AA in the basal diet, 2) sufficient amounts of other nutrients except the test AA in the basal diet, 3) more than four graded levels of test amino acids with two levels for each below and above the estimated requirement, 4) appropriate experimental period, and 5) an appropriate statistical model selection (NRC, 2012). Breakpoint methodology is commonly used for the determination of AA requirements (Robbins et al., 2006). The empirical method allows us to obtain precise nutrient requirements based on the actual population responses of animals. However, there are some limitations of the empirical approach because it is a time-dependent method. Animo acids requirements for pigs keep changing as pigs grow, and the requirements cannot be estimated empirically for the entire lifetime of pigs, therefore, the factorial approach was established for the determination of AA requirements of pigs. The AA requirements are obtained as the sum of the

requirement for body maintenance and protein retention in the factorial approach (NRC, 2012). Components such as basal endogenous origin AA from intestinal protein and skin and hair losses are included for body maintenance (van Milgen et al., 2008; NRC, 2012), and body protein deposition is influenced by several factors such as BW, gender, or supplementation of ractopamine (NRC, 2012). The inefficiency of AA utilization is also considered for body protein deposition, such as minimum and inevitable AA catabolism and between-animal variation (Pomar et al., 2003; NRC, 2012). However, the growth model does not generate AA requirements in nursery pigs due to insufficient information on the biological relationship between AA utilization and growth in young piglets, thus, NRC (2012) provides the AA requirement based on empirical data. A comprehensive review of empirical studies for nursery pigs is critical for model development in the factorial approach.

2.6.2. RESPONSE CRITERIA FOR AMINO ACID REQUIREMENTS

It is important to note that appropriate response criteria should be considered to determine AA requirements in nursery pigs. Growth performance, including average daily gain (**ADG**) and gain-to-feed ratio (**G:F**), is mostly considered as criteria for nutrient requirement studies. Determination of AA requirements based on ADG is favored for practical purposes, however, the estimates may not be accurate if the experiment is not carried out for a long period of time (van Milgen and Dourmad, 2015). Metabolic traits such as PUN, plasma AA concentrations, N balance, and indicator AA oxidation have also been used as response criteria for the estimation of AA requirements (Lenehan et al., 2004; Moehn et al., 2008; Nieto et al., 2015). The PUN and individual AA concentrations in plasma are common criteria used to estimate AA requirements because they typically show consistent results with the expected responses when titrating limiting AA (Lenehan et al., 2004). Also, the indicator AA oxidation technique has been chosen for the

response criteria of AA requirement with its benefit that measurements are obtained after an adaptation period of 2 days, which would require at least a few weeks up to several months for growth rate or N balance assays (Moehn et al., 2008).

2.6.3. FACTORS AFFECTING AMINO ACID REQUIREMENTS

Several factors have to be considered to maximize whole body protein deposition, such as dietary composition, differences in pig growth potential associated with the genotype, differences in health status derived by environmental sanitation, or statistical model, which are leading to changes in AA requirements of nursery pigs. A dietary component such as dietary fiber concentration in nursery diets can influence the AA requirements because the high inclusion of fiber increases the AA requirement for maintenance by enhancing endogenous losses (Blank et al., 2012). Moreover, different growth rates and AA requirements were observed in pigs with different genetic potential for protein deposition (Bikker et al., 1994; Schneider et al., 2010). The experimental duration should be considered carefully together to estimate AA requirement in different genotypes because Taylor et al. (2012) demonstrated that genotype did not affect the growth rate of weaner pigs in the first 2 weeks after weaning, but the effect was distinguishable from the third week. Sanitary conditions are one of the critical factors to be considered for AA requirements determination. Increased AA requirements were obtained in pigs kept under poor (unclean) sanitation due to the shift of use of AA from growth towards immune response under poor sanitation (Jayaraman et al., 2015; Jayaraman et al., 2017). Different models are used to interpret the response of the animal as dietary AA increases. The most frequently used models for determination of AA requirement are linear-plateau (one slope broken-line) and curvilinear-plateau (quadratic broken-line) models (Robbins et al., 2006). The difference between the two models is that the linear-plateau model has a constant marginal response below the AA requirement, whereas the marginal response below the

AA requirement value is a linear function for the curvilinear-plateau model (van Milgen and Dourmad, 2015). Therefore, the estimates obtained from the curvilinear-plateau model are always greater than those from the linear-plateau model.

2.7. CONCLUSION

Weaning is a complex and very stressful period that causes various detrimental effects on growth performance, gut health, and the immune system of young pigs. During the weaning period, piglets encounter several difficulties, including decreased VH, increased permeability, impaired intestinal barrier function in the intestine, and a weak immune system, which are linked to growth retardation. Post-weaning diarrhea is a condition of weaned pigs showing the frequent discharge of watery feces for 2 weeks after weaning. Recent studies have shown various dietary interventions such as supplementation of probiotics or prebiotics are effective in controlling PWD.

Adjustment of dietary protein levels or sources is one of the major strategies to reduce PWD in weaned pigs. Lowering dietary CP content modulates gut health by changing microbial populations and activities in weaned pigs. A HCP diet has greater substrate availability for protein fermentation which is preferred by pathogenic bacteria such as enterotoxigenic *Escherichia coli*. Therefore, feeding a HCP diet promotes the proliferation of pathogenic bacteria and produces toxic metabolites in weaned pigs, which is related to immune stimulation. For this reason, feeding a LCP diet to weaned pigs has been utilized to reduce protein fermentation and its metabolites, which in turn decreases the incidence of PWD. Various studies showed that feeding LCP diets by lowering dietary CP contents by approximately 4% units in diets has positive effects on gut health and immune function in weaned pigs without growth retardation.

Moreover, plant-origin protein sources contain a few anti-nutritional factors, including protease inhibitors and allergenic compounds, which impede nutrient digestion and absorption, activate the immune system, and modulate microbiota composition in pigs. Lowering dietary CP content is typically occurred by reducing the soy protein contents, thus, LCP diets can ameliorate immune stress and improve gut health and PWD in weaned pigs. To support optimal use of LCP diets, accurate AA requirements should be determined for weaned pigs.

3. HYPOTHESES AND OBJECTIVES

The studies in this dissertation tested the following hypotheses:

1. Feeding LCP diets with another dietary factor (i.e., supplementation of resistant starch) would show synergistic effects on gut health and function in weaned pigs.
2. Feeding LCP diets would improve gut health and function in weaned pigs under different environmental factors (i.e., sanitary conditions)
3. Feeding LCP diets would positively modulate microbial structure in weaned pigs.
4. Formulating LCP diets with different CAA supplementation patterns would affect growth performance and gut health in weaned pigs.

The overall objective was to evaluate the effects of LCP diets in combination with other factors that may affect gut health and function of weaned pigs.

The specific objectives were:

1. To determine the standardized ileal digestible lysine requirement of 7- to 15-kg weanling pigs (TN70 × TN Tempo; Topigs Norsvin) fed a corn-soybean meal-based diet.

   Note: Lysine requirement had to be determined before the test effects of low protein diets because our research station obtained a new genotype of pigs. Determination of the optimal lysine requirement is essential because lysine is the reference amino acid, and the

rest of the indispensable amino acids are expressed as a percentage in the ideal protein concept.

2. To evaluate the effects of dietary CP content and resistant starch supplementation on growth performance, histomorphology, and microbial metabolites of weaned pigs.
3. To investigate the effects of dietary CP content and CAA supplementation patterns on growth performance, intestinal histomorphology, and immune response in weaned pigs raised under different sanitary conditions.
4. To investigate the effects of dietary CP content and CAA supplementation patterns on the bacteria and their metabolites in the intestine of weaned pigs raised under different sanitary conditions.

4. MANUSCRIPT I

Evaluating the standardized ileal digestible lysine requirement of 7- to 15-kg weanling pigs fed corn-soybean meal-based diets

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4.1. ABSTRACT

Continued genetic improvement necessitates the verification of nutrient requirements for newly developed pig genotypes. Therefore, the objective of this research was to determine the standardized ileal digestible (**SID**) Lys requirement of 7- to 15-kg weanling pigs (TN70 × TN Tempo; Topigs Norsvin) fed a corn-soybean meal-based diet. A total of 144 piglets with an initial BW of 6.51 ± 0.56 kg (mean ± SD) were assigned to 1 of 6 diets using a randomized complete block design based on body weight to give 8 replicate pens with 3 pigs per pen. The six diets contained 1.00, 1.16, 1.32, 1.48, 1.64, and 1.80% SID Lys, achieved by adding crystalline L-Lys·HCl at the expense of cornstarch. Other indispensable amino acids were provided to meet the requirements. Piglets had free access to diets and water for 21 days. Individual BW of pigs and feed disappearance were recorded weekly and blood samples were collected on day 1, 14, and 21. Average daily gain (**ADG**) and average daily feed intake were not affected by dietary SID Lys content during the first 7 days. However, the addition of dietary SID Lys quadratically increased ($P < 0.05$) gain:feed (**G:F**) during the first 7 days of experiment. A quadratic increase ($P < 0.05$) was found in both ADG and G:F when SID Lys content increased in the diets from day 14 to 21. During the overall experimental period, increasing dietary Lys content quadratically increased ($P < 0.05$) ADG and G:F, whereas plasma urea nitrogen quadratically decreased ($P < 0.05$) as SID Lys content increased. The SID Lys requirements were estimated for linear and quadratic broken-line models. In conclusion, the SID Lys requirement for optimal growth performance of 7- to 15-kg weanling pigs fed corn-soybean meal-based diets based on linear and quadratic broken-line models were 1.27% (95% confidence interval (**CI**): [1.01, 1.53]) and 1.30% (95% CI: [0.94, 1.66]) for ADG and 1.27% (95% CI: [1.14, 1.40]) and 1.43% (95% CI: [1.11, 1.75]) for G:F, respectively, thus giving an overall average value of 1.32%.

.**Key words:** amino acid, growth performance, lysine, plasma urea nitrogen, swine

4.2. INTRODUCTION

Dietary Lys is mainly used for protein accretion and the efficiency of available Lys for protein deposition is 75% in young piglets (Möhn et al., 2000). Also, Lys is typically the first-limiting amino acid (**AA**) in corn-soybean meal-based swine diets. It is, therefore, considered as a reference AA for the relative amounts required for the other AA (NRC, 2012). Previous studies reported that standardized ileal digestible (**SID**) AA contents should be used in feed formulation because they are additive in a mixture of feed ingredients (Stein et al., 2007). Thus, NRC (2012) suggested that the dietary Lys requirement should be expressed as SID Lys for each phase of growth. However, due to insufficient published data and information about factors to model AA requirement, the SID Lys requirement for weanling pigs was determined by extrapolation using an average value from previous estimates based on SID Lys requirements per kg of body weight (**BW**) gain.

The NRC (2012) reported the SID Lys requirement for 7- to 11-kg pigs to be 1.35%. Similarly, Kahindi et al. (2017a) reported an average value of 1.32% SID Lys for optimal ADG in 7- to 16-kg pigs fed wheat-corn-soybean meal-based diets. However, Park and Kim (2015) reported slightly higher estimates that ranged from 1.39 to 1.49%, with a recommendation of 1.43% SID Lys based on feed efficiency for pigs from 6- to 10-kg body weight. Nemechek et al. (2012) indicated that SID Lys requirement for feed efficiency was 1.37% when using one-slope broken line analysis and 1.54% when using quadratic broken-line analysis, respectively. This discrepancy could be attributable to several factors, including genetic capacity for protein deposition (Bikker et al., 1994; Schneider et al., 2010), sanitary conditions of experimental environment (Jayaraman et al., 2017), and the statistical methods used for data analysis (Nemechek et al., 2012; Park and Kim, 2015).

To further enhance the accuracy with which swine diets are formulated with respect to AA supply, it is critical that requirement values for each stage of growth are determined. However, such values for weanling pigs have seldom been reported. Thus, the objective of this study was to determine the SID Lys requirements for 7- to 15-kg weanling pigs fed corn-soybean meal-based diets. The piglets used in the study were offspring of a recently developed cross bred genotype (TN70 × TN Tempo; Topigs Norsvin).

4.3. MATERIALS AND METHODS

The methods and procedures used in the present study were reviewed and approved by the University of Manitoba Animal Care Committee (AC11406), and pigs were handled according to the guidelines from the Canadian Council on Animal Care (2009).

4.3.1. ANIMALS, HOUSING, AND EXPERIMENTAL DESIGN

A total of 154 piglets [TN Tempo × TN70 (boars and gilts); Topigs Norsvin, Winnipeg, MB, Canada] were weaned at approximately 21 days of age with a BW of 6.15 ± 0.58 kg. Piglets were given a 5-day adaptation period before the experiment was started. One hundred and forty-four piglets (initial BW = 6.51 ± 0.56 kg) were used for the current experiment after adaptation period and randomly allocated to 1 of 6 dietary treatments using a randomized complete block design based on BW to give 8 replicate pens with 3 pigs per pen. Sex was balanced within each replicate (4 replicates for each male and female). Piglets were housed in a room with 1.2 × 1.8 m expanded plastic-coated metal floor pens. Pigs were allowed to have free access to feed and water for 3 weeks. Room temperature was maintained at 29 ± 1°C for the first week and reduced by 1°C per week.

Table 4.1 Composition of experimental diets fed to 7-15 kg weanling pigs (as-fed basis)

	SID Lys[1], %
Item[2]	1.00
Ingredient, %	
Corn	54.58
Soybean meal	28.23
Dried whey	10.00
Vegetable oil	2.50
Cornstarch	1.03
Limestone	1.22
Monocalcium phosphate	1.02
Salt	0.40
Vitamin-mineral premix[3]	0.15
$_{L}$-Lys HCl	0.09
$_{DL}$-Met	0.30
$_{L}$-Thr	0.23
$_{L}$-Trp	0.09
$_{L}$-Ile	0.02
$_{L}$-Val	0.16
Total	100

[1]Different concentrations of L-Lysine.HCl were supplemented to the diets at the expense of cornstarch at 0, 0.21, 0.41, 0.62, 0.82, and 1.03% of the diet to create 6 levels of SID Lys as 1.00, 1.16, 1.32, 1.48, 1.64, and 1.80% SID Lys in the experimental diets. The SID coefficients of each ingredient provided by NRC (2012) were used to predict the SID amino acid contents from analyzed total amino acids in the diets.

[2]SID, standardized ileal digestible; Lys, lysine; Met, methionine; Thr, threonine; Trp, tryptophan; Ile, isoleucine; Val, valine.

[3]Provided the following nutrients (per kg of diet): Vitamins: A, 2,200 IU; D3, 220 IU; E, 16 IU; K, 0.5 mg; B_1, 1 mg; B_2, 3.5 mg; B_6, 7 mg; B_{12}, 17.5 µg; calcium pantothenate, 10 mg; folic acid, 0.3 mg; niacin, 30 mg; biotin, 50 µg. Minerals: Cu, 6 mg (as copper sulfate); I, 0.14 mg (as calcium iodate); Fe, 100 mg (as ferrous sulfate); Mn, 4 mg (as manganese oxide); Se, 0.3 mg (as sodium selenite); Zn, 100 mg (as zinc oxide).

4.3.2. EXPERIMENTAL DIETS

Six experimental diets based on corn, soybean meal, and dried whey were prepared in a mash form (Table 4.1). Corn, soybean meal, and dried whey were analyzed for dry matter (**DM**), crude protein (**CP**), and AA composition using NIRS before formulating the experimental diets, and then diets were formulated based on SID AA which were calculated using analyzed AA concentrations in each ingredient and SID coefficients provided by NRC (2012). Experimental diets contained 6 increasing concentrations of SID Lys from 1.00 to 1.80% by adding crystalline $_{L}$-Lys·HCl at the expense of cornstarch. Diet 1 (1.00%) and diet 6 (1.80%) were mixed in single batches and diet 2 (1.16%), 3 (1.32%), 4 (1.48%), and 5 (1.64%) were prepared by blending diets 1 and 6 in different proportions, 8:2, 6:4, 4:6, and 2:8, respectively, to meet the targeted SID Lys amount in each diet. The levels of all other indispensable AA were provided as the values suggested by AMINOPig® 1.0 (2011; Evonik Industries, Hanau-Wolfgang, Germany) to avoid deficiency in AA other than Lys. Minerals and vitamins were provided to meet the values recommended by NRC (2012). Calculated net energy concentrations in diets ranged from 10.83 to 10.85 MJ/kg.

4.3.3. SAMPLING AND MEASUREMENTS

The BW of individual pigs and feed disappearance were recorded weekly after overnight fasting to calculate average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain to feed ratio (**G:F**). On day 0, 14, and 21, blood samples (10 ml) were collected via jugular vein puncture into heparinized vacutainer tubes (Becton Dickson, Rutherford, NJ, USA) from the pig with the closest BW to mean BW of each pen after 8 hours fasting to determine plasma urea nitrogen (**PUN**) concentrations. The blood samples were centrifuged at 3600 rpm for 10 minutes at 4°C to harvest plasma and the recovered plasma was immediately stored at −80°C until required for analysis. The PUN measurements were done at the Veterinary Diagnostic Services, Manitoba Agriculture and

Rural Development (Winnipeg, MB, Canada) using the Ortho Clinical Vitros 250 Chemistry System (Johnson & Johnson®, New Brunswick, NJ, USA).

4.3.4. SAMPLE PREPARATION AND CHEMICAL ANALYSIS

Diet and ingredient samples were ground and analyzed for DM content according to the AOAC (2006; method 934.01). Crude protein and AA analyses were performed at the laboratory of Evonik Operations GmbH (Hanau-Wolfgang, Germany). The nitrogen content of the diets was determined with a gas combustion method using a Leco FP-2000 Nitrogen Analyzer (Leco Corp., St. Joseph, MI, USA). Concentrations of all indispensable and dispensable AA except for tryptophan and tyrosine were analyzed by ion-exchange chromatography after post-column derivatization with ninhydrin. Amino acids were oxidized with performic acid, which was neutralized with Na metabisulfite (Llames and Fontaine, 1994; Commission Directive, 1998). Cysteine and methionine were oxidized prior to hydrolysis. Oxidation was performed with a performic acid/phenol mixture at 0°C and excess oxidation reagent was decomposed with sodium disulphite. Samples were subjected to a 24-hour hydrolysis in 6 N HCl at 110°C, and AA were quantified with the internal standard by measuring the absorbance of reaction products with ninhydrin at 570 nm. Tryptophan was determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm), after alkaline hydrolysis with barium hydroxide octahydrate for 20 hours at 110°C (Commission Directive, 2000). Tyrosine was not determined.

4.3.5. STATISTICAL ANALYSIS

Data were analyzed by ANOVA using MIXED procedure of SAS (version 9.4; SAS Inst. Inc., Cary, NC, USA) including the SID Lys concentration as the main fixed factor and the block as the random factor. Sex effect was not significant, thus it was excluded from the model. The UNIVARIATE procedure of SAS was used to determine if there were any outliers, and those

outliers were removed from the final statistical analysis. Data were adjusted for calculation of ADG, ADFI and G:F using the individual feed intake estimation procedure (Lindemann and Kim, 2007). Orthogonal polynomial contrasts were used to test the linear and quadratic effects of dietary SID Lys concentration. The SID Lys requirements were estimated for linear broken-line and quadratic broken-line models by NLMIXED procedure of SAS based on the studies by Robbins et al. (2006) and Gonçalves et al. (2016). Results are presented as least squares means for each response variable and the experimental unit was the pen. The significance of the model was set at $P < 0.05$.

Table 4.2 Calculated and analyzed nutrient composition of experimental diets fed to 7-15 kg weanling pigs (as-fed basis)

	SID Lys, %					
Item	1.00	1.16	1.32	1.48	1.64	1.80
Calculated nutrient composition						
Net energy, MJ/kg	10.83	10.83	10.84	10.84	10.85	10.85
Crude protein, %	20.60	20.79	20.99	21.19	21.38	21.58
Total amino acids, %						
Lys	1.13	1.29	1.45	1.61	1.77	1.93
Met	0.60	0.60	0.60	0.60	0.60	0.60
Met + Cys	0.94	0.94	0.94	0.94	0.94	0.94
Thr	1.02	1.02	1.02	1.02	1.02	1.02
Trp	0.34	0.34	0.34	0.34	0.34	0.34
SID amino acids[1], %						
SID Lys	1.00	1.16	1.32	1.48	1.64	1.80
SID Met	0.57	0.57	0.57	0.57	0.57	0.57
SID Met + Cys	0.85	0.85	0.85	0.85	0.85	0.85
SID Thr	0.89	0.89	0.89	0.89	0.89	0.89
SID Trp	0.31	0.31	0.31	0.31	0.31	0.31
SID Ile	0.78	0.78	0.78	0.78	0.78	0.78
SID Val	0.97	0.97	0.97	0.97	0.97	0.97
SID Leu	1.58	1.58	1.58	1.58	1.58	1.58
Analyzed nutrient composition						
Dry matter, %	88.83	88.79	88.93	88.82	88.72	88.70
Crude protein, %	20.52	20.67	21.13	21.69	21.81	22.24
Total amino acids, %						
Arg	1.26	1.22	1.23	1.28	1.28	1.25
His	0.52	0.49	0.50	0.52	0.52	0.50
Ile	0.91	0.88	0.89	0.91	0.91	0.89
Leu	1.81	1.72	1.74	1.78	1.79	1.75
Lys	1.17	1.34	1.52	1.65	1.81	1.99
Met	0.57	0.59	0.60	0.60	0.59	0.59
Met + Cys	0.90	0.92	0.93	0.94	0.93	0.93
Phe	0.99	0.94	0.96	0.99	1.00	0.97
Thr	0.99	0.97	1.00	1.00	1.00	1.00
Trp	0.33	0.34	0.34	0.34	0.34	0.34
Val	1.11	1.10	1.09	1.13	1.13	1.12

[1]SID, standardized ileal digestible; Arg, arginine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Cys, cysteine; Phe, phenylalanine; Thr, threonine; Trp, tryptophan; Val, valine.

4.4. RESULTS AND DISCUSSION

Pigs readily consumed their diets and stayed healthy throughout the experiment. The analyzed nutrient compositions of the experimental diets are given in Table 4.2. The differences between calculated and analyzed total Lys values ranged from 2.26 to 4.83% and analyzed CP and other total essential AA contents were also in agreement with the calculated values. Thus, calculated values were used for data analysis to determine the SID Lys requirement of weanling pigs. Sex effect was excluded from the analysis because there was no significant difference on growth performance between male and female piglets. In general, sex effects on growth performance were not found in weanling pigs because weanling pigs are immature to reveal the differences in growth (Kornegay et al., 1994).

Table 4.3 Effect of dietary SID Lys on growth performance of weanling pigs[1]

	SID Lys[2], %							*P*-values	
Item[3]	1.00	1.16	1.32	1.48	1.64	1.80	SEM	Linear	Quadratic
BW, kg									
day 0	6.50	6.51	6.50	6.53	6.53	6.51	0.199	0.197	0.496
day 7	8.03	8.12	8.25	8.42	8.27	8.18	0.296	0.159	0.050
day 14	10.23	10.77	11.23	11.46	10.94	11.11	0.380	0.010	0.004
day 21	13.32	14.61	15.57	15.88	15.22	15.46	0.516	< 0.001	0.001
ADG, g/day									
day 0 to 7	223	231	250	275	251	239	18.8	0.232	0.054
day 7 to 14	314	379	427	435	382	414	24.4	0.015	0.007
day 14 to 21	441	549	606	631	624	621	29.8	< 0.001	0.003
day 0 to 21	325	386	433	443	414	417	18.5	< 0.001	0.001
ADFI, g/day									
day 0 to 7	283	285	299	302	282	289	18.6	0.830	0.249
day 7 to 14	483	528	596	561	522	553	24.6	0.144	0.010
day 14 to 21	715	774	840	814	796	815	35.4	0.060	0.085
day 0 to 21	475	505	558	540	500	527	25.9	0.235	0.072
G:F, g/kg									
day 0 to 7	788	813	832	911	881	833	29.7	0.038	0.047
day 7 to 14	647	705	718	774	730	754	29.6	0.011	0.128
day 14 to 21	614	714	733	774	787	762	16.5	< 0.001	< 0.001
day 0 to 21	682	770	788	824	810	812	16.1	< 0.001	0.001
PUN, mmol/L									
day 0	3.03	3.10	3.15	3.04	2.86	3.51	0.386	0.588	0.718
day 14	3.94	3.45	1.80	1.46	2.13	1.03	0.398	< 0.001	0.012
day 21	3.58	3.24	1.36	2.01	1.64	1.66	0.273	< 0.001	0.001

[1]Each least squares mean represents eight observations. The lysine provided was feed-grade L-Lys·HCl (contained 78% L-Lys).

[2]SID, standardized ileal digestible; Lys, lysine.

[3]ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed ratio; PUN, plasma urea nitrogen.

The effects of dietary SID Lys on BW, ADG, ADFI, G:F, and PUN are shown in Table 4.3. There were no significant differences among treatments in ADG, and ADFI, except for increased G:F ($P < 0.05$), during the first 7 days of the experiment. Previous studies showed that growth performance was not affected by the SID Lys content during the first week of an experiment (Park and Kim, 2015; Kahindi et al., 2017a). Dong and Pluske (2007) explained that piglets right after weaning experience stress symptoms such as reduced feed intake, which can be related to poor performance. However, Kahindi et al. (2014) showed increased growth during the first 7 days, which is in agreement with the current study. The possible reason for these different growth patterns in first week is a 5-day adaptation period provided right after weaning in the current study. Because the stress caused by weaning and movement to another environment is related to low feed intake and poor growth in weanling pigs, giving time for piglets to adapt to the new solid feed and environment may have a positive effect on growth performance in the first week post-weaning.

The ADG from day 7 to 14 and from day 14 to 21, and G:F from day 14 to 21 increased ($P < 0.05$) quadratically as dietary SID Lys concentration increased, while G:F from day 7 to 14 increased ($P = 0.011$) in a linear fashion. The addition of dietary SID Lys contents quadratically increased ($P = 0.01$) ADFI from day 7 to 14, whereas ADFI did not differ among treatments during the other periods, which agrees with the study of Kahindi et al. (2017a). According to the literature data, it is known that piglets (Kirchgessner et al., 1999) and growing-finishing pigs (Owen et al., 1994) preferentially select AA-adequate diets over AA-deficient diets, which could explain the lower ADFI of pigs fed AA-deficient diets in the present study. Moreover, Ettle and Roth (2009) demonstrated that piglets showed a clear preference for the higher Lys concentration diet. Due to the quadratic response of the ADFI, the BW of piglets showed a quadratic response as well. Regarding this point, Bruininx et al. (2001) reported that piglets consumed diets according to their

BW pattern, and thus, their feed intake increases as well as BW. Another factor that may affect the ADFI is the form of dietary AA. A previous study found that pigs fed diets supplemented crystalline AA (free AA) showed lower ADFI relative to those fed diets with intact protein (Shen et al., 2020). Shen et al. (2020) explained that the diet with intact protein (casein) may influence appetite enhancement of pigs, because gastric emptying can be accelerated by dietary peptides derived from the intact protein. Indeed, compared to the previous study using same genetic pigs fed intact protein-based diets with similar initial BW (Mejicanos et al., 2020), the ADFI from the current study was similar from d 7 to 14, but lower from d 14 to 21. However, Gloaguen et al., (2014) reported that the ADFI was not different between the piglets fed the diets with protein-bound AA and the diets supplemented with free AA, which indicates that the use of free AA does not cause negative effect on feed intake in terms of the efficiency of AA utilization when pigs are offered feed ad libitum. During the overall experimental period, an increase in dietary SID Lys quadratically increased ($P < 0.001$) both ADG and G:F, which agrees with Kahindi *et al.* (2017a) who reported that growth performance was improved with gradual addition of dietary Lys during a 3-week trial with weanling pigs. Similarly, Nemechek et al. (2012) conducted four 2- to 4-week experiments with pigs originating from different sources and housed in different facilities and reported an increase in ADG and/or G:F with increasing dietary SID Lys concentration.

A limitation of the current study is that the contents of CP and other AA in the diets remained constant, while SID Lys concentration increased. Millet et al. (2018) demonstrated that growth in 4- to 9-week-old piglets is limited by protein instead of individual AA, when the SID Lys:CP ratio exceeds 6.4%. Moreover, according to Nemechek et al. (2014), the growth performance among pigs fed different SID Lys:CP ratio diets from 6.2 to 6.9% did not differ. However, other previous studies showed that if the SID Lys:CP ratio in diets is greater than 7.2%,

the growth performance of pigs decreased (Nyachoti et al., 2006; Opapeju et al., 2008). The Lys content constituting body protein is approximately 7% (van Milgen and Dourmad, 2015), therefore, the Lys:CP ratio will not exceed 7% if all AA meet the requirements for optimal protein synthesis and the efficiency of SID Lys is not different with that of other AA in growth. Thus, the SID Lys:CP ratio should be maintained at a maximum of 6.9% to avoid negative effects on pig performance due to the deficiency of non-essential AA or nitrogen for non-essential AA synthesis at a higher SID Lys:CP ratio. The SID Lys:CP ratios were 4.85 (1.00% SID Lys diet), 5.58 (1.16%), 6.29 (1.32%), 6.98 (1.48%), 7.67 (1.64%), and 8.34% (1.80%), respectively. Therefore, it is possible that Lys was not the first limiting AA in diets above 1.64% SID Lys and other non-essential AA might become limiting, leading to the limitation of growth in piglets fed 1.64 and 1.80% SID Lys diets. Ideal protein ratio among essential AA and SID Lys:CP ratio in experimental diets should be considered carefully for the future studies to achieve the best results in pig performance.

Plasma or serum urea nitrogen has been known as an indicator of protein utilization (Pedersen and Boisen, 2001). A decrease in PUN concentration is expected when the concentration of a limiting AA in the diet increases, and the PUN concentration reaches a plateau or increases when the optimum requirement of the AA is achieved. As dietary Lys concentration increased, PUN concentration quadratically declined ($P < 0.05$) on day 14 and 21. On day 21, the PUN concentration in pigs fed 1.32% SID Lys decreased compared to that fed 1.00% SID Lys diet, and increased thereafter. This observation was consistent with results of previous studies on SID Lys requirement (Ho et al., 2019; Zhou et al., 2019).

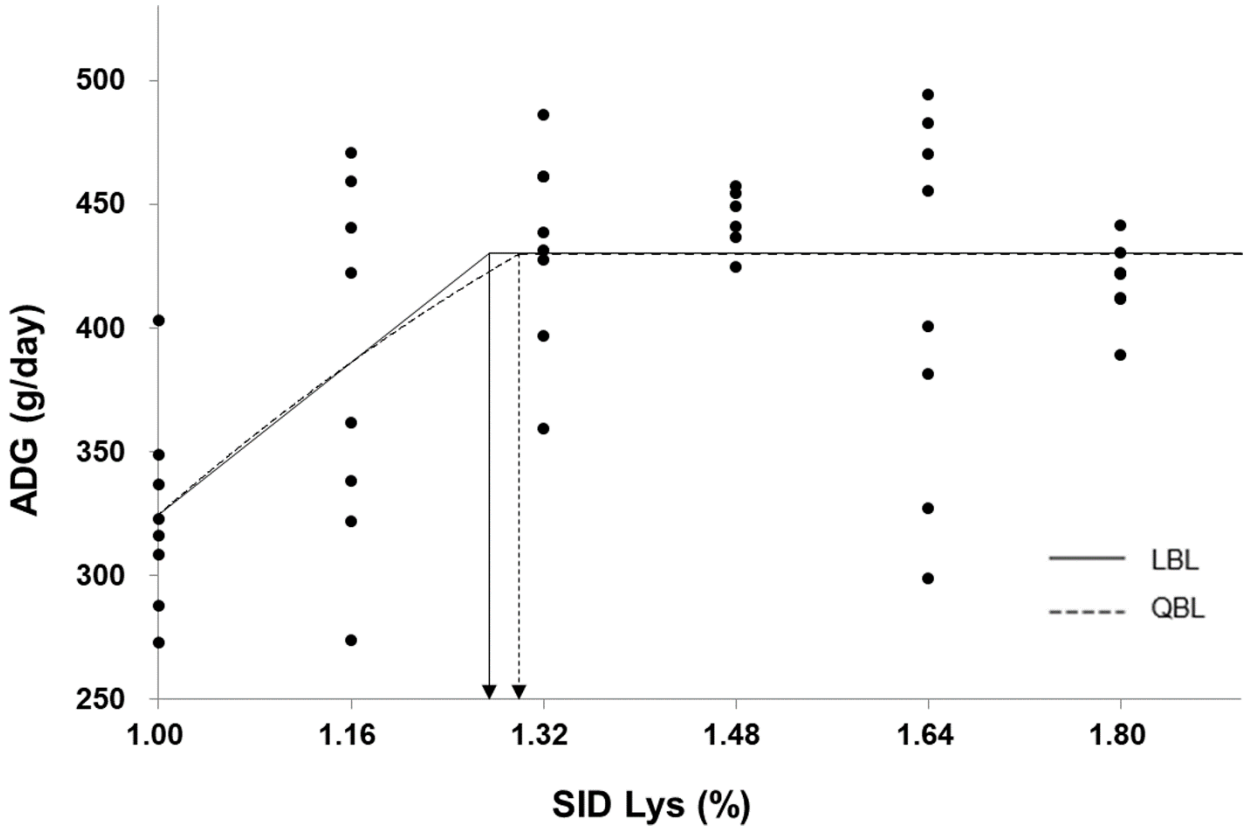

Figure 4.1 Standardized ileal digestible (**SID**) Lys requirement of 7 to 15 kg weanling pigs for average daily gain (**ADG**) with observed mean values for each treatment. The SID Lys requirement with linear broken-line (**LBL**) model was 1.27% [$Y = 429.99 - 384.18 \times (1.2745 - X)$ if $X < 1.2745$; and $Y = 429.99$ if $X \geq 1.2745$]. The SID Lys requirement using the quadratic broken-line (**QBL**) analysis was 1.30% [$Y = 429.99 - 280.5 \times (1.3003 - X) - 235.26 \times (1.3003 - X)^2$ if $X < 1.3003$; and $Y = 429.99$ if $X \geq 1.3003$].

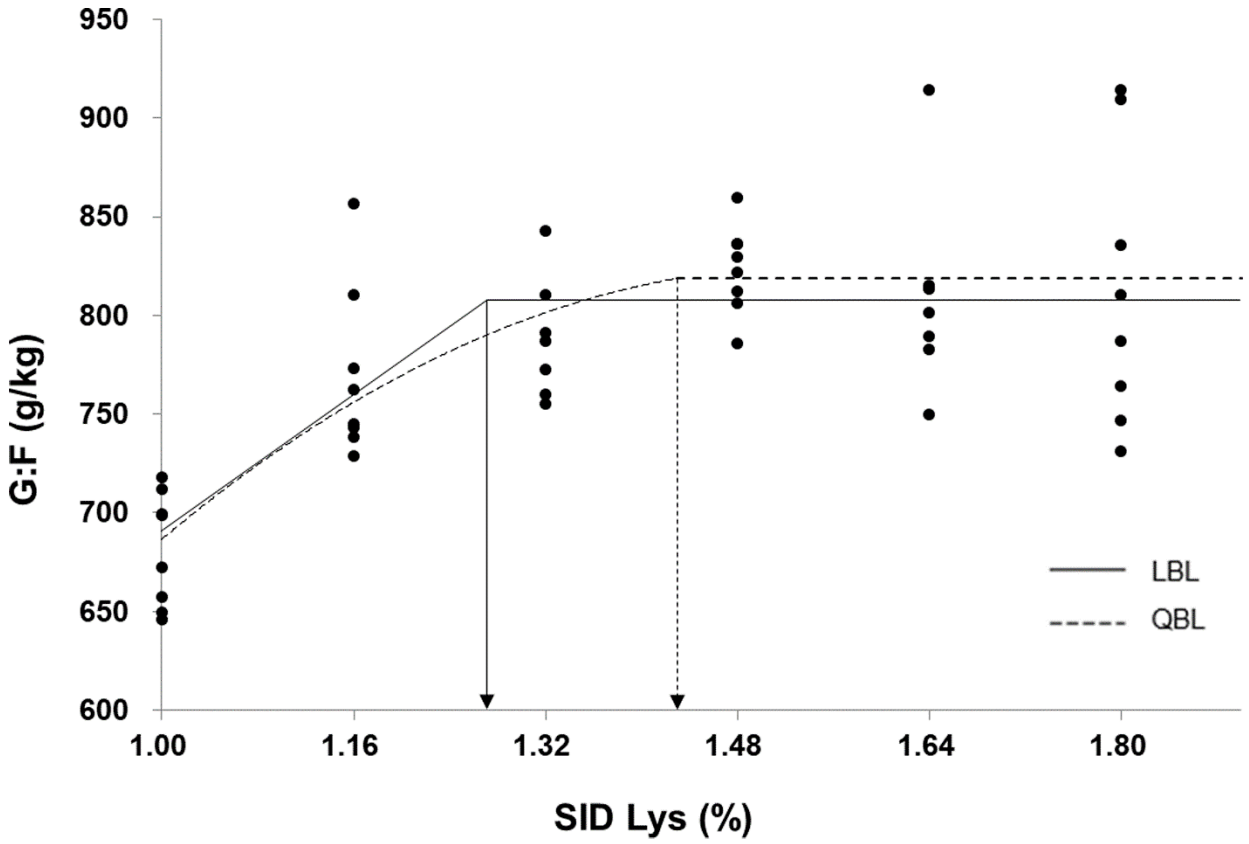

Figure 4.2 Standardized ileal digestible (**SID**) Lys requirement of 7 to 15 kg weanling pigs for gain to feed ratio (**G:F**) with observed mean values for each treatment. The SID Lys requirement with linear broken-line (**LBL**) model was 1.27% [Y = 808.02 – 431.18 × (1.2709 − X) if X < 1.2709; and Y = 808.02 if X ≥ 1.2709]. The SID Lys requirement using the quadratic broken-line (**QBL**) analysis was 1.43% [Y = 818.69 − 105.96 × (1.4295 − X) – 469.01 × (1.4295 − X)2 if X < 1.4295; and Y = 818.69 if X ≥ 1.4295].

The estimated SID Lys requirement for pigs varies depending on the response criteria, statistical method, experimental diet, and environmental conditions. The SID Lys requirement was derived using linear and quadratic broken-line models based on the mean values for both ADG and G:F for the overall experimental period. The linear broken-line analysis showed that the optimum SID Lys requirement estimates were 1.27% for both ADG (Figure 4.1) and G:F (Figure 4.2). The optimum SID Lys requirements were estimated at 1.30% for ADG and 1.43% for G:F, respectively using the quadratic broken-line analysis. The results derived from the current study showed slightly lower SID Lys requirements compared to the values in previous studies by Kahindi et al. (2017a) who reported the SID Lys requirements for ADG using linear and quadratic broken-line models as 1.29 and 1.34%, respectively, for 7-16 kg of piglets and by Nemechek et al. (2012) in which the SID Lys requirements for optimal ADG for 7-14 kg pigs determined using one-slope and quadratic broken-lines were 1.30 and 1.37%, respectively, and SID Lys requirements for optimal G:F were 1.39 and 1.54%, respectively. One possible reason for this discrepancy could be the procedure used for statistical analysis. Unlike the NLIN procedure, which is preferred for data from experiments without blocks, the block effect should be included when using NLMIXED procedure for the data from the experiment which is blocked (Robbins et al., 2006). Also, Gonçalves et al. (2016) demonstrated that heterogeneity of error variances should be considered for the use of linear and nonlinear mixed models because heterogeneity of residual variances is a relatively common event in animal experiments. Robbins et al. (2006) showed that the requirement derived from NLMIXED procedure which included a block effect in the model was lower than the value derived from NLIN procedure with no block effects included, thus, it is possible that the SID Lys requirements by Kahindi et al. (2017a) using NLIN procedure without block effects were higher than the requirements in the current study.

The response criteria used for the estimation can also affect the requirement value. Higher estimates for Lys requirement were commonly observed for feed efficiency compared to BW gain in both poultry and swine (Baker et al., 2002; Nemechek et al., 2012; Zhou et al., 2019), which was also the case in the current study. Baker et al. (2002) explained that once dietary Lys concentration exceeds the requirement point, growth rate remains constant whereas feed consumption decreases, resulting in a higher Lys requirement estimate for feed efficiency than for BW gain which is also shown in the current study.

Because statistical methodology is one of the factors that can influence estimates for nutrient requirement (Baker, 1986), two different approaches (linear and quadratic broken-lines) were used to derive SID Lys requirement in an attempt to avoid any bias due to the statistical method used. One-slope broken-line and quadratic broken-line are the most widely used statistical models in nutrient requirement studies. The one-slope broken-line consists of a straight line with a break point which is usually a reasonable approximation of the ascending portion and is typically considered as the objective requirement value (Pesti et al., 2009). However, this analysis has a weakness in that it is hard to prove whether it is a sharp break between the lines or a smooth transition. Thus, the quadratic broken-line model is used because in reality the ascending portion of the response is expected to be curved in nature. Because the breakpoint in the one-slope broken-line model represents an average of animals among entire populations, the requirement from the one-slope (linear) broken-line analysis generally shows a lower estimate than the requirement derived from the quadratic broken-line model.

The SID Lys requirements also could vary depending on such factors as dietary protein level, genetics, or BW range of the pigs used in each experiment. Protein level and SID Lys:CP ratio in the experimental diets can influence the SID Lys requirements by limiting growth due to

the limitation of non-essential AA or nitrogen as discussed above. Nemechek et al. (2014) reported that pigs fed different SID Lys:CP ratio diets from 6.2% to a maximum of 6.9% showed no differences in growth performance, unlike other experiments showed significant reductions in growth when SID Lys:CP ratio is greater than 7.2% (Nyachoti et al., 2006; Opapeju et al., 2008). Therefore, the SID Lys:CP ratio should not exceed 6.9% to avoid growth limitation in pigs caused by limiting AA other than lysine. Because growth in piglets fed the 1.64 and 1.80% SID Lys diets could be limited in the current study due to inadequate nitrogen for non-essential AA synthesis, the SID Lys requirement could be higher when enough amount of nitrogen for synthesis of non-essential AA was added in the diets. Unlike the present study, Kahindi et al. (2017a) provided sufficient nitrogen in the experimental diets, thus, this could be another explanation why the SID Lys requirements from the current study were slightly lower than the values from the work of Kahindi et al. (2017a).

Many Lys requirement estimates using crossbred pigs [e.g., (Yorkshire × Landrace) × Duroc] were already reported, however, continued genetic progress necessitates the verification of requirement values for recently developed genotypes. Piglets used for the current study were offspring of TN Tempo boars mated to TN70 females (Topigs Norsvin). The TN 70 is a F1 sow that is a crossbred between Landrace and Large White (Yorkshire) and it was developed about 10 years ago which is a relatively recent breed. The TN Tempo boar is developed as a synthetic breed of Yorkshire and Piétrain, and the offspring of these breeds is expected to have robustness, lower mortality, and high growth performance. Previously, different growth rates were obtained as well as Lys requirement estimates depending on the genetic potential for protein deposition (Bikker et al., 1994; Schneider et al., 2010). Although previous study reported that high lean growth pigs are predicted to have 30% greater Lys requirement than pigs with low lean growth rates (Cameron et

al., 2003), the Lys requirement estimates in the current study were similar with the estimates from a previous study in which a 3-way crossbred pigs were used (Kahindi et al., 2017a). It might be because growth performance of nursery pigs might not be different between the piglets from Duroc or Piétrain boars which are the main difference between the current and previous studies. Edwards et al. (2006) reported that there was no difference between Duroc- and Piétrain-sired pigs in BW before 10 weeks of age.

Another factor affecting the requirement value is the different BW range of animals throughout the experiment. As piglets age and increase in BW, the nutrient requirement decreases. For example, compared with the SID Lys requirements from the present study, the lower optimal SID Lys requirements (1.28 and 1.31% for ADG and G:F, respectively, using fitted curvilinear-plateau plot) found by Zhou et al. (2019) could be due to the higher BW range (8-20 kg) compared to the present study (7-15 kg), and the higher SID Lys requirements (1.39 and 1.49% for G:F using linear and quadratic broken-line, respectively) found by Park and Kim (2015) could be due to the lower BW range (6-10 kg) of the piglets. The SID Lys requirement suggested by NRC (2012) is 1.35% for 7- to 11-kg weaning pigs, which is a higher requirement value with a lower BW range compared with the 1.32%, the averaged SID Lys requirement value from this study.

Lastly, environmental factor influences the AA requirements, because the competition of AA utilization between protein accretion and immune response happens when pigs raised under unclean sanitary condition (Jayaraman et al., 2017). Kahindi et al. (2014) showed weanling pigs raised under poor sanitation results in decreased feed intake and depressed growth, therefore, the SID Lys requirement derived from the pigs raised under unclean condition could be higher than the value derived from the present study.

Reporting on the growth performance in weanling pigs below the estimated SID Lys

requirements is as important as determining what the SID Lys requirements are (van Milgen et al., 2012). The response curve of growth performance needs to be fully understood to make cost effective decisions in feed formulation. When Lys is 10% deficient compared to the SID Lys requirements for ADG, pigs might show 11% reductions in ADG (381 g gain/day) using the equation from the linear broken-line model and 9% reduction (390 g gain/day) using the quadratic broken-line model for calculation. An 10% reduction in Lys concentration (10% below the SID Lys requirements) results in 7% (753 g gain/kg feed intake) and 3% reductions (794 g gain/kg feed intake) in G:F using linear and quadratic broken-lines, respectively, which derives the 5% and 7% reduction in ADFI. This deficiency of Lys may have an impact on growth performance, thus, SID Lys should be carefully considered for diet formulation.

4.5. CONCLUSIONS

The SID Lys requirement for maximum growth of 7- to 15-kg weanling pigs ranged from 1.27 to 1.30% for ADG and 1.27 to 1.43% for G:F using linear and quadratic broken-lines, respectively, thus giving an average value of 1.32%. The average value of 1.32% corresponds to 395 g for ADG and 514 g for ADFI, respectively, and when expressed on a g/day and mg/g gained basis, the SID Lys requirement was 6.78 g SID Lys/day and 17.18 g SID Lys/kg gain, respectively.

TRANSITION STATEMENT

Experiment 1 determined standardized ileal digestible lysine requirement of 7- to 15-kg weanling pigs, and these lysine requirement values were used in Experiments 2 and 3. Experiments 2 and 3 were designed to investigate the effects of low dietary crude protein diets in combination with other strategies that could influence growth performance and gut health in weaned pigs. First, Experiment 2 investigated the effects of dietary crude protein content without or with resistant starch supplementation on growth performance and gut function in weaned pigs.

5. MANUSCRIPT II

Effects of dietary crude protein content and resistant starch supplementation on growth performance, histomorphology, and microbial metabolites in weaned pigs

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Charles Martin Nyachoti: conceptualization, supervision, project administration, writing–review & editing.

5.1. ABSTRACT

A 4-week study was conducted to evaluate the effects of dietary crude protein (**CP**) content and resistant starch (**RS**) supplementation on growth performance, intestinal histomorphology, and microbial metabolites of weaned pigs. A total of 96 pigs (7.06 ± 0.45 kg BW) were assigned to 1 of 4 diets in a randomized complete block design involving a 2 (CP levels) × 2 (without or with RS) factorial arrangement to give 8 replicate pens and 3 pigs per pen. Body weight and feed disappearance were recorded weekly, and fecal consistency score was determined every morning. Blood was sampled on days 1, 14, and 28 from one pig per pen, and the same pig was euthanized on day 28 to collect ileal tissue and ileal and colon digesta. Data were analyzed using the MIXED procedure of SAS. The average daily gain and gain:feed ratio were lower ($P < 0.05$) in pigs fed low crude protein (**LCP**) diets compared to those fed high CP (**HCP**) diets during week 3 and overall period. The analyzed Lys, Met+Cys, and Thr in feed were lower than calculated values, particularly in LCP diets, which may have affected performance. Pigs fed the LCP diets had longer ($P < 0.05$) ileal villi and higher villus height (**VH**) to crypt depth ratios than those fed the HCP diets, and RS supplementation increased ($P < 0.05$) ileal VH. Interactions ($P < 0.05$) between dietary CP content and RS inclusion were observed for short-chain fatty acids concentration in the ileum and colon in phase 2. There was no difference in propionic acid (ileum) or butyric acid (colon) concentrations among pigs fed HCP diets, however, butyric acid concentration increased in pigs fed the LCP diet when supplemented with RS. Reducing dietary CP lowered ($P < 0.05$) fecal score, plasma urea nitrogen, and digesta ammonia content. Overall, feeding LCP diets reduced growth performance but improved gut morphology in weaned pigs. Feeding LCP diet with RS supplementation modulated concentrations of intestinal microbial metabolites in weaned pigs.

Key words: gut health, low protein diet, piglets, resistant potato starch

5.2. INTRODUCTION

Feeding a low crude protein (**LCP**) diet with supplementation of crystalline amino acids (**AA**) can be used as one of the strategies to ameliorate post-weaning diarrhea by reducing the amount of available protein for fermentation by intestinal microorganism, which may produce harmful metabolites (Heo et al., 2008; Opapeju et al., 2008; Heo et al., 2010b). Apart from lowering the crude protein (**CP**) content in swine diets, another dietary tool to decrease protein fermentation is the inclusion of fermentable carbohydrates such as fiber and resistant starch (**RS**) (Govers et al., 1999; Bikker et al., 2006; Kim et al., 2008a). Non-digestible carbohydrate fractions may be utilized for microbial metabolism in the large intestine as a prebiotic source, yielding beneficial products for the host gut health (Gibson et al., 2004; Birt et al., 2013). The end products from carbohydrate fermentation, such as short-chain fatty acids (**SCFA**), can be an energy source for the enterocytes in pigs (Wu, 2018). Several studies have shown that the inclusion of dietary fiber reduces metabolites of protein fermentation as well as the incidence of post-weaning diarrhea (Kreuzer et al., 1998; Bikker et al., 2006; Kim et al., 2008a), and a significant interaction was found between dietary inclusion of fermentable protein and fiber on bacterial counts in weaned piglets (Pieper et al., 2012).

Resistant starch is the starch that consists of high amylose and low amylopectin contents, and its digestion in the small intestine by endogenous enzymes of pigs is limited, thus, it is most likely to be fermented in the large intestine (Wu, 2018; Tiwari et al., 2019). According to the work of Haenen et al. (2013), the high RS diet increased butyrate production in the proximal colon of pigs and decreased a relative abundance of *Escherichia coli* in the colon compared to the high digestible starch diet. Inclusion of RS in the diet increases carbohydrate substrates for microbial fermentation, thereby increasing SCFA concentrations in the intestine. Pathogenic bacteria such

as *Escherichia coli* prefer protein as an energy source, and thus, decreased abundance of *Escherichia coli* in pig colon by including high RS in the diet showed that fermentation pattern is changed from protein to carbohydrate with RS inclusion, thereby reducing protein fermenters. Also, it is indicated in both *in vitro* and *in vivo* studies that the fermentation of RS produces more butyrate compared to the fermentation of other non-digestible carbohydrate sources such as non-starch polysaccharides (Topping et al., 2003; Souza da Silva et al., 2013), thus, dietary RS has a potential to be a better prebiotic source to improve gut health than other non-digestible carbohydrates. However, there are few studies on the effects of resistant starch supplementation on the gut health in weaned pigs, and its interactive effects with CP level on intestinal fermentation patterns have not been investigated yet. Moreover, variations were observed in terms of growth performance of pigs fed diets containing RS in previous studies. For instance, Li et al. (2007b) and Regmi et al. (2011) showed a decreased daily gain, feed intake, and feed efficiency in weaned and growing pigs fed diets containing RS, respectively, however, Nofrarías et al. (2007) and Doti et al. (2014) reported that growth performance of growing pigs was not affected by including RS in the diets. Thus, this study aimed to investigate the effects of dietary CP content and RS supplementation on growth performance, intestinal histomorphology, and microbial metabolites in weaned pigs.

Table 5.1 (a) Composition of experimental diets (original formulation; as-fed basis)[1]

	Phase 1				Phase 2			
	HCP		LCP		HCP		LCP	
Item RS:	–	+	–	+	–	+	–	+
Ingredients, %								
Corn	51.47	50.15	63.66	62.34	59.61	58.29	71.01	69.77
Soybean meal	29.80	30.08	17.95	18.24	27.35	27.63	16.28	16.49
Soy protein concentrate	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Raw potato starch	–	1.00	–	1.00	–	1.00	–	1.00
Whey powder	10.00	10.00	10.00	10.00	5.00	5.00	5.00	5.00
Vegetable oil	2.20	2.25	0.82	0.87	1.62	1.66	0.33	0.37
Limestone	1.25	1.24	1.27	1.27	1.25	1.25	1.28	1.28
Monocalcium phosphate	1.19	1.20	1.35	1.35	1.10	1.11	1.25	1.25
Salt	0.40	0.40	0.40	0.40	0.35	0.35	0.35	0.35
Vitamin-mineral premix[2]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
L-Lysine HCl	0.27	0.26	0.63	0.62	0.29	0.28	0.63	0.62
DL-Methionine	0.18	0.18	0.28	0.28	0.17	0.17	0.26	0.26
L-Threonine	0.06	0.06	0.22	0.21	0.07	0.07	0.22	0.22
L-Tryptophan	0.04	0.04	0.10	0.10	0.04	0.04	0.10	0.10
L-Valine	-	-	0.17	0.17	-	-	0.15	0.15
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Calculated nutrient composition, %								
Net energy, kcal/kg	2,560	2,560	2,560	2,560	2,560	2,560	2,560	2,560
Crude protein	23.0	23.0	19.3	19.3	22.0	22.0	18.5	18.5
SID[3] Lys	1.35	1.35	1.35	1.35	1.28	1.28	1.28	1.28
SID Met	0.49	0.49	0.54	0.54	0.46	0.46	0.51	0.51
SID Met + Cys	0.81	0.81	0.81	0.81	0.77	0.77	0.77	0.77
SID Thr	0.85	0.85	0.85	0.85	0.80	0.80	0.80	0.80
SID Trp	0.30	0.30	0.30	0.30	0.28	0.28	0.28	0.28
SID Ile	0.91	0.91	0.72	0.72	0.85	0.85	0.67	0.67
SID Val	0.95	0.95	0.92	0.92	0.90	0.90	0.87	0.87

[1]HCP = high crude protein diet without RS inclusion; LCP = low crude protein diet without RS inclusion; HCP-RS = high crude protein diet with RS inclusion; LCP-RS = low crude protein diet with RS inclusion.

[2]Provided the following nutrients (per kg of air-dry diet): Vitamins: A, 2000 IU, D_3 200 IU, E, 40 mg, K, 2 mg, B_1, 1.5 mg, B_2, 7 mg, B_6, 2.5 mg, B_{12}, 25 µg, calcium pantothenate, 14 mg, folic acid, 1 mg, niacin, 21 mg, biotin, 70 µg. Minerals: Cu, 10 mg (as copper sulphate), iodine, 0.4 mg (as potassium iodine), iron, 120 mg (as ferrous sulphate), Mn, 10 mg (as manganous oxide), Se, 0.3 mg (as sodium selenite), Zn, 110 mg (as zinc oxide).
[3]SID = standardized ileal digestible.

Table 5.1 (b) Composition of experimental diets (actual formulation; as-fed basis)[1]

	Phase 1				Phase 2			
	HCP		LCP		HCP		LCP	
Item RS:	–	+	–	+	–	+	–	+
Ingredients, %								
Corn	51.47	50.15	63.66	62.34	59.61	58.29	71.01	69.77
Soybean meal	29.80	30.08	17.95	18.24	27.35	27.63	16.28	16.49
Soy protein concentrate	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Raw potato starch	–	1.00	–	1.00	–	1.00	–	1.00
Whey permeate	10.00	10.00	10.00	10.00	5.00	5.00	5.00	5.00
Vegetable oil	2.20	2.25	0.82	0.87	1.62	1.66	0.33	0.37
Limestone	1.25	1.24	1.27	1.27	1.25	1.25	1.28	1.28
Monocalcium phosphate	1.19	1.20	1.35	1.35	1.10	1.11	1.25	1.25
Salt	0.40	0.40	0.40	0.40	0.35	0.35	0.35	0.35
Vitamin-mineral premix[2]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
L-Lysine HCl	0.27	0.26	0.63	0.62	0.29	0.28	0.63	0.62
DL-Methionine	0.18	0.18	0.28	0.28	0.17	0.17	0.26	0.26
L-Threonine	0.06	0.06	0.22	0.21	0.07	0.07	0.22	0.22
L-Tryptophan	0.04	0.04	0.10	0.10	0.04	0.04	0.10	0.10
L-Valine	-	-	0.17	0.17	-	-	0.15	0.15
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Calculated nutrient composition, %								
Net energy, kcal/kg	2,608	2,608	2,608	2,608	2,608	2,608	2,608	2,608
Crude protein	22.2	22.2	18.5	18.5	21.6	21.6	18.2	18.2
SID[3] Lys	1.31	1.31	1.31	1.31	1.26	1.26	1.26	1.26
SID Met	0.48	0.48	0.53	0.53	0.46	0.46	0.50	0.50
SID Met + Cys	0.78	0.78	0.78	0.78	0.75	0.75	0.75	0.75
SID Thr	0.79	0.79	0.79	0.79	0.78	0.78	0.78	0.78
SID Trp	0.28	0.28	0.28	0.28	0.27	0.27	0.27	0.27
SID Ile	0.86	0.86	0.67	0.67	0.83	0.83	0.65	0.65
SID Val	0.90	0.90	0.88	0.88	0.88	0.88	0.85	0.85

[1]HCP = high crude protein diet without RS inclusion; LCP = low crude protein diet without RS inclusion; HCP-RS = high crude protein diet with RS inclusion; LCP-RS = low crude protein diet with RS inclusion.

[2]Provided the following nutrients (per kg of air-dry diet): Vitamins: A, 2000 IU, D_3 200 IU, E, 40 mg, K, 2 mg, B_1, 1.5 mg, B_2, 7 mg, B_6, 2.5 mg, B_{12}, 25 µg, calcium pantothenate, 14 mg, folic acid, 1 mg, niacin, 21 mg, biotin, 70 µg. Minerals: Cu, 10 mg (as copper sulphate), iodine, 0.4 mg (as potassium iodine), iron, 120 mg (as ferrous sulphate), Mn, 10 mg (as manganous oxide), Se, 0.3 mg (as sodium selenite), Zn, 110 mg (as zinc oxide).
[3]SID = standardized ileal digestible.

Table 5.2 Analyzed nutrient composition of experimental diets (as-fed basis)[1]

		Phase 1				Phase 2			
		HCP		LCP		HCP		LCP	
Item, %	RS:	–	+	–	+	–	+	–	+
Dry matter		88.9	89.1	88.0	88.5	89.3	87.5	88.6	88.4
Starch		33.8	32.7	38.6	38.7	38.7	38.1	45.4	44.4
Resistant starch		0.83	1.79	0.85	1.70	0.89	1.68	0.94	1.56
Crude protein		20.8	21.1	17.4	17.7	20.9	20.3	16.9	17.0
Total amino acid									
Arg		1.34	1.35	1.00	1.00	1.32	1.32	0.94	0.96
His		0.53	0.54	0.42	0.42	0.53	0.53	0.41	0.42
Ile		0.88	0.89	0.68	0.68	0.87	0.87	0.65	0.67
Leu		1.78	1.80	1.54	1.53	1.84	1.82	1.54	1.58
Lys		1.32	1.34	1.23	1.27	1.32	1.29	1.20	1.27
Met		0.48	0.48	0.50	0.50	0.46	0.48	0.47	0.48
Met + Cys		0.77	0.78	0.74	0.75	0.76	0.78	0.72	0.73
Phe		1.02	1.03	0.81	0.81	1.03	1.02	0.79	0.81
Thr		0.84	0.86	0.78	0.79	0.85	0.86	0.77	0.79
Trp		0.28	0.29	0.26	0.27	0.28	0.28	0.26	0.26
Val		0.96	0.97	0.89	0.89	0.96	0.96	0.88	0.88

[1]HCP = high crude protein diet without RS inclusion; LCP = low crude protein diet without RS inclusion; HCP-RS = high crude protein diet with RS inclusion; LCP-RS = low crude protein diet with RS inclusion.

5.3. MATERIALS AND METHODS

5.3.1. EXPERIMENTAL DIETS

Four dietary treatments arranged in a 2 × 2 factorial were used in the study. The factors consisted of 2 concentrations of CP (i.e., high CP (**HCP**) and LCP) with or without RS supplementation (HCP-RS and LCP-RS) (Table 5.1; a). The CP concentrations were 23 and 19% for HCP and LCP diets in phase 1 (1 to 14 days) and 22 and 18% for HCP and LCP diets in phase 2 (15 to 28 days), and 1% of RS was supplemented to the RS diets for both phase 1 and 2. Experimental diets consisted of a corn-soybean meal-based basal diet with the inclusion of protein sources (soy protein concentrate and whey powder) and crystalline AA to meet the indispensable AA to lysine ratio recommended by AMINOPig® 1.0 (Evonik Industries, Hanau-Wolfgang, Hessen, Germany) for 5 to 10 kg of body weight (**BW**) for phase 1 and for 10 to 20 kg of BW for phase 2, respectively. The standardized ileal digestible lysine requirement for phase 2 was obtained from the requirement value by Lee et al. (2021) using 7- to 15- kg weanling pigs. However, there was a mistake when diets were mixed, in which case whey permeate was used instead of whey powder (Table 5.1; b). Thus, the provided dietary AA concentrations could not meet the requirements of AA in both the HCP and LCP diets for phases 1 and 2. All experimental diets were formulated to meet or exceed the NRC (2012) requirements for minerals and vitamins for 5 to 7 kg of BW for phase 1 and 7 to 11 kg of BW for phase 2, respectively. Pigs were fed the assigned diets for 28 days and had free access to feed and water throughout the experiment. The analyzed nutrient composition of experimental diets is shown in Table 5.2.

5.3.2. ANIMALS AND EXPERIMENTAL DESIGN

The experimental protocol was approved by the University of Manitoba Animal Care Committee (AC11406). Pigs were cared for based on the guidelines of the Canadian Council on Animal Care (CCAC; 2009).

A total of 96 piglets (TN70 × TN Tempo; Topigs Norsvin, Winnipeg, MB, Canada) with an initial BW of 7.06 ± 0.45 kg were randomly assigned to 1 of 4 dietary treatments in a randomized complete block design to give 8 replications per treatment and 3 pigs per pen (1.8 m × 1.2 m). Sex was balanced within treatment (4 replicates for each sex). Room temperature was maintained at 29 ± 1ºC during the first week and reduced by 1ºC for each of the following weeks.

5.3.3. SAMPLING AND MEASUREMENTS

Body weight and feed disappearance were recorded weekly to determine average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain to feed ratio (**G:F**). Feces was scored daily at 0830 throughout the experiment to determine fecal consistency scores as described by Nyachoti et al. (2006). Fecal consistency scoring (0 = normal, 1 = soft feces, 2 = mild diarrhea, and 3 = severe diarrhea) was conducted by two trained individuals without information on treatment allocation.

The pig that had the closest BW to the mean BW of each pen was selected for blood sampling on days 1 and 14. Another pig that had the second closest BW to the mean BW of each pen on day 1 was selected for blood collection on day 28. Blood was sampled via jugular vein puncture into 10 mL heparinized vacutainers tubes (BD Vacutainer, Franklin Lakes, NJ, USA) after 10 hours of fasting to analyze plasma urea nitrogen (**PUN**). Blood samples were centrifuged at 3,600 × g at 4˚C for 10 mins to separate plasma and kept at –80˚C freezer until analysis.

The same pigs which were blood collected on days 14 and 28 in each pen were euthanized for ileal tissue and ileal and proximal colon digesta samples. A 3 cm-ileal sample was collected 30

cm away from the ileocecal junction for histomorphology analysis. Samples were washed with phosphate-buffered saline and immediately stored in 10% buffered formalin. After the fixation process in formalin, samples were sent to the Histology Laboratory (University of Manitoba, Winnipeg, MB, Canada) for further processing, as described by Koo et al. (2020). Briefly, the specimens were moved to paraffin wax for sectionalizing into 5 µm after fixation in 10% buffered formalin. Each section was dewaxed and immersed in turn into xylene, 95% ethanol, and 100% ethanol for 5 minutes, and this immersion was repeated twice. The sections were rinsed with water and stained with 0.5% periodic acid solution for 5 minutes, followed by Schiff reagent staining for 10 minutes. After that, the sections were counter-stained with hematoxylin and then dehydrated using alcohol. Villus heights (**VH**) and crypt depths (**CD**) were measured using an Axio Scope A1 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and Image J software (National Institutes of Health, MD, USA) in all distinguishable villi and crypts. Goblet cells (**GC**) were manually counted for each villus.

Ileal and colon digesta for volatile fatty acids (**VFA**) and ammonia-N analyses were collected in sterile bags, immediately snap-frozen, and stored at –80˚C. The SCFA and branched-chain fatty acids (**BCFA**) concentrations were determined using the method described by Erwin et al. (1961) using gas chromatography (Varian Chromatography System, model Star 3400; Varian Medical Systems, Palo Alto, CA). The ammonia-N concentration was analyzed by using the method described by Novozamsky et al. (1974).

5.3.4. CHEMICAL ANALYSES

Diet samples were finely ground and analyzed for dry matter, starch, RS, CP, and AA composition. The dry matter content was measured according to AOAC (method 934.01; 2006), and nitrogen content was determined by the combustion method (method 990.03; AOAC, 2006) using the

LECO N analyzer (model CNS-2000; LECO Corp., St. Joseph, MI) to calculate CP (nitrogen × 6.25). The AA contents in the diets were determined by ion-exchange chromatography with post-column derivatization with ninhydrin. Amino acids were oxidized with performic acid, which was neutralized with sodium metabisulfite (Llames and Fontaine, 1994; Commission Directive, 1998). Amino acids were liberated from the protein by hydrolysis with 6 N HCL for 24 h at 110°C. Amino acids were quantified with the standard internal method by measuring the absorption of reaction products with ninhydrin at 570 nm. Tryptophan was determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm) after alkaline hydrolysis with barium hydroxide octahydrate for 20 h at 110°C (Commission Directive, 2000). Tyrosine was not determined. Starch and RS analyses were performed using the Megazyme total starch assay kit and the K-STAR resistant starch assay kit (Megazyme International Ltd., Wicklow, Ireland).

5.3.5. STATISTICAL ANALYSES

All data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC), except fecal score data which were using the GLIMMIX procedure of SAS. The model included the main effects of CP content and RS inclusion and their interaction, and replicate was included as random effects. There was no sex effect, therefore, it was excluded from the model. Pen was considered as the experimental unit for growth performance and fecal score, and the individual pig was used as the experimental unit for blood, histomorphology, and microbial metabolites. The LSMEANS statement with the Tukey-adjusted PDIFF option was used to calculate and separate the mean values among treatments. Statistical significance was set at $P < 0.05$.

5.4. RESULTS AND DISCUSSION

The current study was conducted to investigate the effects of dietary CP content and RS supplementation on growth performance and gut health in weaned pigs. The term 'healthy gastrointestinal tract' as defined by Kogut and Arsenault (2016) refers to 'absence/prevention/avoidance of disease so that the animal is able to perform its physiological functions in order to withstand exogenous and endogenous stressor'. To maintain a healthy gut, there are several factors that should be considered: 1) effective digestion and absorption of food, 2) absence of gastrointestinal illness, 3) normal and stable intestinal microbiome, 4) effective immune status, and 5) state of well-being (Bischoff, 2011). Post-weaning period highly influences the gut health of young pigs, considering that weaning changes gut function of piglets in many ways. There is a temporary shortage of digestive enzymes at weaning, thus, more non-digested substrates are available for microbial fermentation, leading to a fertile environment for *Escherichia coli* proliferation (Nabuurs, 1998). A decrease of the *Lactobacillus* spp. group has been found in the gut of piglets during the weaning transition, but at the same time, the amounts of *Clostridium* spp. or *Escherichia coli* were increased (Su et al., 2008; Wei et al., 2017; Adhikari et al., 2019). *Clostridium* spp. including *Clostridium difficile* and *Escherichia coli* are known to cause diarrhea as well as infections in weaned pigs (Madec et al., 2000; Songer, 2004). Gut structure and intestinal barrier functions are also influenced by weaning. An acute deterioration of the gut structure and integrity was observed right after weaning by decreasing villus height and brush border enzyme activities and increasing paracellular permeability in pigs (Vente-Spreeuwenberg and Beynen, 2003; Montagne et al., 2007). Inflammation of the intestine is strongly related to increased gut permeability because of increased translocation of toxins, viruses, or bacteria. When bacteria cross the first line of defense of the gut and reach the lamina propria, their metabolites or mediators

cause an inflammatory response (Pluske et al., 2018). These various impacts of weaning should be considered and mitigated with nutritional intervention to achieve a healthy gut in weaned pigs.

Table 5.3 Effects of crude protein (CP) concentration and resistant starch (RS) supplementation on growth performance and fecal score in weaned pigs[1]

		Diets[2]							
		HCP		LCP			*P*-values		
Item	RS:	–	+	–	+	SEM	CP	RS	CP × RS
BW, kg									
Initial		7.05	7.06	7.05	7.06	0.164	0.996	0.972	0.988
day 7		7.26	7.33	7.32	7.23	0.171	0.902	0.929	0.635
day 14		8.68	8.71	8.97	8.43	0.303	0.997	0.407	0.351
day 21		12.83	11.92	12.12	10.87	0.437	0.054	0.020	0.701
day 28		17.52	17.01	17.51	16.09	0.616	0.456	0.128	0.464
ADG, g/day									
Week 1		30	41	40	23	14.6	0.790	0.823	0.296
Week 2		203	198	235	176	22.8	0.823	0.168	0.240
Week 3		534	510	445	384	22.7	< 0.001	0.072	0.410
Week 4		730	728	771	746	30.9	0.352	0.660	0.719
Overall		373	355	373	322	19.1	0.393	0.081	0.399
ADFI, g/day									
Week 1		123	123	135	114	12.0	0.890	0.389	0.389
Week 2		267	270	315	262	23.1	0.401	0.295	0.241
Week 3		658	596	630	557	35.7	0.351	0.071	0.883
Week 4		967	943	1054	1008	42.9	0.088	0.426	0.794
Overall		594	582	646	580	31.3	0.432	0.220	0.396
G:F									
Week 1		0.19	0.23	0.28	0.16	0.096	0.945	0.661	0.368
Week 2		0.75	0.73	0.74	0.71	0.038	0.816	0.479	0.903
Week 3		0.81	0.86	0.71	0.69	0.020	< 0.001	0.364	0.080
Week 4		0.76	0.77	0.73	0.74	0.015	0.112	0.457	0.902

Overall	0.63	0.61	0.58	0.56	0.016	0.003	0.186	0.910
Fecal score[3]								
Week 1	0.45	0.24	0.16	0.04	0.075	0.004	0.041	0.523
Week 2	1.06	0.82	0.31	0.52	0.139	0.001	0.893	0.108
Week 3	0.71	0.69	0.17	0.17	0.080	< 0.001	0.900	0.900
Week 4	0.59	0.35	0.10	0.13	0.093	0.001	0.248	0.128

[1]Values are least square means, n = 8 per treatment.
[2]HCP, high crude protein diet without resistant starch supplementation; LCP, low crude protein diet without resistant starch supplementation; HCP-RS, high crude protein diet with resistant starch supplementation; LCP-RS, low crude protein diet with resistant starch supplementation.
[3]Fecal score was recorded daily during the experimental period for each pen by the two different people as follows: 0 = normal; 1 = soft feces; 2 = mild diarrhea; and 3 = severe diarrhea.

Table 5.4 Effects of crude protein concentration and resistant starch supplementation on plasma urea nitrogen in weaned pigs

		Diets[1]							
		HCP		LCP			*P*-values		
Item	RS:	–	+	–	+	SEM	CP	RS	CP × RS
day 1		2.01	1.80	1.61	1.77	0.136	0.135	0.851	0.167
day 14		4.50	3.46	2.09	2.46	0.587	0.009	0.573	0.226
day 28		2.72	2.64	0.55	0.53	0.214	< 0.001	0.795	0.875

[1]HCP, high crude protein diet without resistant starch supplementation; LCP, low crude protein diet without resistant starch supplementation; HCP-RS, high crude protein diet with resistant starch supplementation; LCP-RS, low crude protein diet with resistant starch supplementation.

5.4.1. GROWTH PERFORMANCE, FECAL SCORE, AND PLASMA UREA NITROGEN

Pigs fed LCP diets had reduced ($P < 0.05$) ADG and G:F in week 3 (Table 5.3). During the overall period, G:F was lower ($P < 0.05$) in pigs fed LCP diets compared to those fed HCP diets. Resistant starch supplementation did not influence the growth performance of weaned pigs except for reducing ($P < 0.05$) BW on day 21. It was hypothesized that feeding a LCP diet with RS supplementation would act synergistically to support a healthy gut and thereby improve growth performance in weaned pigs. However, there was no interaction between CP content and RS supplementation on growth performance in this study. The major limitation of this study was the use of whey permeate in experimental diets instead of whey powder. Whey permeate, which is deproteinized whey, reduced AA and CP contents in all experimental diets, and especially a more severe AA deficiency was found in the LCP diets. The LCP diets could not meet the requirements of major AA suggested by AMINOPig® 1.0, including Lys, Met, and Thr. Thus, the growth performance of pigs fed the LCP diets was poor in week 3 and the overall period compared to those fed the HCP diets. There was no significant effect of RS supplementation on growth performance except for the BW on day 21, and this finding is in agreement with the previous studies by Bhandari et al. (2009) and Heo et al. (2014). The reason for this result could be due to the low inclusion rate of RS (1%) in the diets. Although a previous study showed that supplementing 1% of RS in the diets of weaned pigs enhances gut health (Heo et al., 2014), RS supplementation at low levels does not have growth-promoting effects (Bhandari et al., 2009).

Although there is a limitation to interpret the growth performance data in this study due to the error in diet formulation, this study can be considered meaningful in a sense that it provides the effects of CP content on gut health in weaned pigs such as incidence of diarrhea or gut microbial metabolites. High CP content in diets increased ($P < 0.05$) the fecal score of weaned

pigs throughout the experiment. Resistant starch supplementation reduced ($P < 0.05$) fecal score in week 1, but no significant differences were observed among treatments in other weeks. High CP diets are related to an increased incidence of post-weaning diarrhea in weaned pigs, because feeding HCP diets increases fermentation of undigested protein and proliferation of pathogenic bacteria, thereby potentially increasing toxic products such as ammonia and biogenic amines in pig intestine (Pieper et al., 2012; Gilbert et al., 2018). However, dietary RS supplementation decreased fecal scores in weaned pigs only during week 1 in the current study. This result is in agreement with a previous report by Bhandari et al. (2009), who demonstrated a reduced fecal score in pigs fed RS-included diets during week 1 but not in weeks 2 and 3. Resistant starch supplementation is known to reduce diarrhea because RS can produce SCFA in the colon through carbohydrate fermentation and these SCFA facilitate salt and water absorption via stimulation of sodium-dependent fluid absorption (Yang et al., 2017).

As anticipated, pigs fed LCP diets had lower ($P < 0.05$) PUN concentration compared to the pigs fed HCP diets on days 14 and 28 (Table 5.4), which agrees well with findings of previous studies (Heo et al., 2009, 2010a; Powell et al., 2011). Plasma urea nitrogen is an indicator of dietary N utilization (Powell et al., 2011), thus, more N contents are expected in the blood stream in pigs fed HCP diets. However, no significant effects of RS supplementation and interactions between CP content and RS supplementation occurred for fecal score or PUN, which is consistent with results of others who did not observe interactive effects between dietary protein and fermentable carbohydrate intake on PUN in growing (Zervas and Zijlstra, 2002) and weaned pigs (Jeaurond et al., 2008).

Table 5.5 Effects of dietary crude protein content with or without resistant starch supplementation on histomorphology in the ileum of weaned pigs

	Diets[1]							
	HCP		LCP			*P*-values		
Item[2] RS:	–	+	–	+	SEM	CP	RS	CP × RS
Phase 1								
VH, µm	244	296	320	365	21.3	0.002	0.029	0.866
CD, µm	214	228	204	208	12.4	0.240	0.461	0.689
VH:CD	1.1	1.4	1.7	1.8	0.16	0.002	0.214	0.643
GC/100 µm villus	5.9	6.8	7.1	7.9	0.54	0.037	0.146	0.909
GC/villus	13.9	18.8	22.1	28.8	1.60	<0.001	0.001	0.579
GC/100 µm crypt	5.3	5.1	5.8	6.0	0.31	0.038	0.839	0.538
GC/crypt	11.1	11.3	11.3	12.4	0.71	0.384	0.384	0.485
Phase 2								
VH, µm	301	331	388	389	12.2	<0.001	0.199	0.239
CD, µm	203	197	186	186	6.4	0.054	0.629	0.552
VH:CD	1.8	2.0	2.3	2.3	0.14	0.013	0.385	0.385
GC/100 µm villus	4.5	6.1	5.3	5.9	0.45	0.583	0.018	0.275
GC/villus	13.1	18.6	20.0	22.5	1.68	0.004	0.026	0.385
GC/100 µm crypt	5.9	6.4	6.9	6.5	0.26	0.042	0.815	0.109
GC/crypt	12.1	12.4	12.4	11.6	0.49	0.616	0.616	0.319

[1]HCP, high crude protein; LCP, low crude protein; HCP-RS, high crude protein with resistant starch; LCP-RS, low crude protein with resistant starch.
[2]VH, villus height; CD, crypt depth; GC, goblet cells.

5.4.2. ILEAL HISTOMORPHOLOGY

Pigs fed LCP diets had higher ($P < 0.05$) VH and VH:CD ratios compared to those fed HCP diets at the end of both phases 1 and 2 (Table 5.5). Pigs fed the LCP diets had a greater ($P < 0.05$) number of GC per 100 µm of villus and crypt in phase 1 and GC per villus in phase 2. Increased ($P < 0.05$) VH was found in pigs fed diets with RS supplementation at the end of phase 1 and greater ($P < 0.05$) number of GC per villus were observed in pigs fed RS-supplemented diets at the end of both phases 1 and 2. No interaction between dietary CP content and RS supplementation on histomorphology was found, which means that dietary CP content and RS supplementation affected histomorphology in an additive way. Because longer villi are desirable for the optimal function of the small intestine such as the greater absorptive capability for nutrients (Yang and Liao, 2019), feeding LCP diets had beneficial effects on gut morphology in weaned pigs by increasing ileal VH and VH:CD ratios, which were in agreement with findings of previous studies (Nyachoti et al., 2006; Opapeju et al., 2009; Yin et al., 2021). Increased ileal VH could be due to reduced ammonia concentrations in ileum digesta derived from feeding LCP diets in this study. Ammonia is one of the harmful epithelial irritants, which can disturb gut integrity and maintenance of intestinal barrier function (Lin and Visek, 1991; Kim et al., 2012). A previous study with broilers demonstrated that birds had lower VH and CD in the small intestine when they were exposed to high ammonia concentrations (Wei et al., 2012). Moreover, increased ileal VH (phase 1) was observed in pigs fed RS-supplemented diets, which indicates enhanced absorptive capacity and gut health by feeding RS diets. Similar results were demonstrated in a broiler study by Zhang et al. (2020b), who observed linear and quadratic increases in ileal VH and ileal GC density, respectively, as dietary RS increased.

It has been shown that feeding HCP diets increases the number of GC in the ileum and jejunum of weaned piglets (Gu and Li, 2004; Hermes et al., 2009; Lan et al., 2015). Lan et al. (2015) reported that the HCP diet induces GC hyperplasia and greater intestinal mucus content in the ileum of rats, which might be due to higher threonine concentration in the diet. However, pigs fed LCP diets had increased GC density compared with those fed HCP diets in the current study, which is in agreement with previous research in rats (Meng et al., 2019). Such a result may indicate that feeding LCP diets could improve gut barrier function in weaned pigs as hydrogen sulfide derived from undigested AA in the HCP diets could negatively affect GC density in the current study. Previous studies showed that infusion of ammonia damages the mucus layer in the colon of rats (Lin and Visek, 1991). The mucus layer consists of mucin glycoproteins secreted by GC, which helps avoid the attachment of toxins and pathogens to the epithelial cells (Chalvon-Demersay et al., 2021). Another metabolite from protein catabolism, hydrogen sulfide, can be transformed from the undigested sulfur-containing AA and may have a harmful effect by breaking down the mucus layer, leading to increased permeability of the mucus barrier in the small intestine (Ijssennagger et al., 2015). Aslam et al. (1992) reported that incubation of colonic mucosa with hydrogen sulfide markedly increased epithelial cell apoptosis and destruction of GC in the rat. When pigs were fed RS-supplemented diets, increased GC density in the ileum of weaned pigs was observed in the current study. A similar result was found in a previous study showed that feeding a raw potato starch diet tended to increase GC in the jejunum of growing pigs (Nofrarías et al., 2007). This result can be regarded as an increased expression of mucin genes according to the work of Zhou et al. (2017), who demonstrated an increased expression of mucin genes in the colon of pigs fed a raw potato starch diet compared to those fed a corn starch diet.

Table 5.6 Effects of dietary crude protein content with or without resistant starch supplementation on pH and ammonia N in ileal and colon digesta in weaned pigs

	Diets[1]							
	HCP		LCP			*P*-values		
Item RS:	–	+	–	+	SEM	CP	RS	CP × RS
pH								
Phase 1								
Ileum	6.30	6.54	6.49	6.48	0.141	0.666	0.428	0.386
Colon	6.22[a]	5.93[ab]	5.87[b]	5.94[ab]	0.092	0.073	0.203	0.047
Phase 2								
Ileum	6.51	6.10	6.19	6.18	0.190	0.515	0.272	0.301
Colon	5.52[b]	5.72[ab]	5.86[a]	5.57[ab]	0.086	0.260	0.555	0.004
Ammonia N, mg/L								
Phase 1								
Ileum	27.66[a]	25.68[ab]	24.16[b]	25.32[ab]	0.655	0.004	0.483	0.012
Colon	50.36	38.59	38.78	38.09	4.748	0.205	0.188	0.219
Phase 2								
Ileum	24.33	23.64	22.75	22.95	0.356	0.004	0.491	0.225
Colon	30.99	36.76	25.36	25.35	3.296	0.020	0.384	0.370

[1]HCP, high crude protein; LCP, low crude protein; HCP-RS, high crude protein with resistant starch; LCP-RS, low crude protein with resistant starch.

[a,b]Within a row, means with different superscripts differ ($P < 0.05$).

Table 5.7 Effects of dietary crude protein content with or without resistant starch supplementation on volatile fatty acids (**VFA**) concentrations (mmol/L) in ileal and colon digesta

	Diets[1]							
	HCP		LCP			*P*-values		
Item RS:	–	+	–	+	SEM	CP	RS	CP × RS
Ileum								
Phase 1								
Acetic acid	0.84	0.87	0.85	0.77	0.106	0.691	0.790	0.581
Propionic acid	0.07	0.07	0.08	0.10	0.021	0.399	0.557	0.518
Butyric acid	0.03	0.04	0.04	0.04	0.006	0.210	0.510	0.500
Isobutyric acid	0.01	0.01	0.01	0.01	0.002	0.888	0.998	0.349
Isovaleric acid	0.01	0.01	0.01	0.01	0.001	0.379	0.803	0.606
Valeric acid	0.02	0.02	0.03	0.02	0.003	0.449	0.675	0.309
Total SCFA[2]	0.94	0.98	0.98	0.92	0.113	0.941	0.924	0.687
Total BCFA	0.04	0.04	0.05	0.05	0.005	0.387	0.578	0.517
Phase 2								
Acetic acid	1.27	1.28	1.01	1.35	0.144	0.524	0.246	0.277
Propionic acid	0.08[ab]	0.08[ab]	0.07[b]	0.09[a]	0.004	0.986	0.057	0.012
Butyric acid	0.02	0.03	0.02	0.02	0.006	0.959	0.436	0.333
Isobutyric acid	0.03	0.04	0.02	0.04	0.003	0.141	< 0.001	0.830
Isovaleric acid	0.01	0.01	0.01	0.01	0.001	0.835	0.631	0.053
Valeric acid	0.01	0.01	0.01	0.01	0.001	0.840	0.775	0.262
Total SCFA	1.36	1.39	1.11	1.45	0.148	0.523	0.221	0.306
Total BCFA	0.05	0.06	0.04	0.05	0.003	0.042	0.003	0.982
Colon								
Phase 1								
Acetic acid	11.90	10.33	11.18	11.50	0.688	0.722	0.325	0.142
Propionic acid	5.54	5.39	5.92	5.36	0.364	0.584	0.283	0.527

Butyric acid	2.99	2.29	2.92	3.09	0.254	0.167	0.307	0.101
Isobutyric acid	0.16	0.15	0.15	0.12	0.016	0.135	0.270	0.549
Isovaleric acid	0.20	0.19	0.17	0.15	0.025	0.148	0.395	0.637
Valeric acid	0.59	0.61	0.77	0.79	0.088	0.055	0.786	0.977
Total SCFA	20.42	17.86	19.66	20.04	1.034	0.470	0.268	0.140
Total BCFA	0.95	0.95	1.04	1.06	0.083	0.255	0.886	0.943
Phase 2								
Acetic acid	10.59	10.95	11.04	10.69	0.478	0.801	0.988	0.322
Propionic acid	6.37[a]	5.78[ab]	5.25[b]	6.07[ab]	0.316	0.152	0.685	0.020
Butyric acid	3.69[ab]	3.43[ab]	2.82[b]	3.93[a]	0.325	0.570	0.189	0.041
Isobutyric acid	0.10	0.10	0.10	0.10	0.012	0.903	0.751	0.814
Isovaleric acid	0.12	0.10	0.14	0.11	0.019	0.379	0.218	0.876
Valeric acid	1.46	1.18	0.92	1.29	0.181	0.250	0.808	0.087
Total SCFA	19.45	20.65	19.95	20.02	0.696	0.907	0.238	0.293
Total BCFA	1.48	1.36	1.17	1.54	0.188	0.725	0.513	0.216

[1]HCP, high crude protein; LCP, low crude protein; HCP-RS, high crude protein with resistant starch; LCP-RS, low crude protein with resistant starch.
[2]SCFA = short-chain fatty acids; BCFA = branched-chain fatty acids.
[a,b]Within a row, means with different superscripts differ ($P < 0.05$).

5.4.3. DIGESTA pH, AMMONIA NITROGEN, AND MICROBIAL METABOLITES

Interaction effects were found in colon pH in both phases 1 and 2 (Table 5.6). At the end of phase 1, pigs fed the LCP diet had lower ($P < 0.05$) colon digesta pH compared with those fed the HCP diet among pigs without RS supplementation, but no difference was found between RS-supplemented pigs. In phase 2, however, pigs fed the LCP diet had higher ($P < 0.05$) colon digesta pH than those fed the HCP diet among pigs without RS supplementation, which also showed no significant difference between RS-supplemented pigs. A significant interaction effect ($P < 0.05$) was observed between dietary CP content and RS supplementation on ammonia N content in phase 1, as reduced ammonia N was found in pigs fed a LCP diet than a HCP diet without RS supplementation, but no difference in pigs fed RS-supplemented diets. Pigs fed LCP diets had lower ($P < 0.05$) ammonia N concentrations in both ileal and colon digesta in phase 2 compared to those fed HCP diets, whereas ammonia N concentration was not affected by RS supplementation.

There was no significant difference in VFA concentration in both ileum and colon digesta in phase 1 (Table 5.7). Pigs fed LCP diets had lower ($P < 0.05$) total ileal BCFA concentrations compared to those fed HCP diets for phase 2. Pigs fed RS-supplemented diets had higher ($P < 0.05$) ileal isobutyric acid concentrations in phase 2, therefore total ileal BCFA concentrations, compared with those fed diets without RS supplementation. Also, in phase 2, interaction effects were found indicating that ileal propionic acid and colonic butyric acid concentrations were not different between pigs fed HCP and HCP-RS diets, however, RS supplementation increased ($P < 0.05$) ileal propionate and colonic butyric acid concentrations in pigs fed the LCP diet. Also, higher ($P < 0.05$) propionate concentration was observed in pigs fed a HCP diet than those fed a LCP diet among pigs fed diets without RS supplementation, however, there was no difference between pigs fed the HCP-RS and LCP-RS diets.

The undigested and endogenous nutrients that reach the distal small intestine are used for microbial fermentation in the hindgut. Gut microbiota utilizes undigested carbohydrates and proteins for their metabolism in the large intestine of monogastric animals (Macfarlane and Macfarlane, 2012). Unlike protein fermentation which yields various end products, including ammonia, phenols, indoles, and hydrogen sulfide, many of which have harmful properties, carbohydrate fermentation is considered a beneficial process because of its end product, SCFA, which can be used for host metabolism. A review paper by Tan et al. (2021) described the functions of SCFA indicating that propionate is mainly used for gluconeogenesis in the host organs, including the liver, kidney, muscle, and intestine, and butyrate is a major energy source of colonocytes. Beneficial effects of different fermentable carbohydrate sources such as RS and non-starch polysaccharides have been intensively studied. Bird et al. (2007) showed that pigs fed high-amylose corn RS diets had higher butyrate concentrations in the colon compared to those fed a corn starch diet, and Loh et al. (2006) demonstrated a higher proportion of butyrate contribution in the colon of growing pigs fed a diet with inulin supplementation than without inulin, which is one of the non-starch polysaccharides. Especially, RS is found to have more beneficial effects on butyrate production in pigs than other fermentable carbohydrate sources such as inulin and guar gum (Souza da Silva et al., 2013), therefore, RS was selected for the carbohydrate source in the current study.

The acid-buffering capacity is high in protein feeds (Jasaitis et al., 1987), thus, gastric and intestinal pH remains high in pigs fed a HCP diet (Nyachoti et al., 2006). Moreover, RS supplementation in swine diets can lower digesta pH, especially in the large intestine, by increasing the SCFA production (Heo et al., 2014; Metzler-Zebeli et al., 2019). For this reason, feeding the LCP diet was expected to lower digesta pH compared to the HCP diet whereby supplementation

with RS could decrease digesta pH in piglets fed the HCP diet. In the present study, pigs fed the LCP diet had lower colonic pH compared with those fed the HCP diet among pigs without RS supplementation in phase 1, but no difference was found when RS was supplemented in the diets. However, a discrepancy was found in the results of pH in the colon between phases 1 and 2 in this study. For phase 2, higher colonic pH was observed in the pigs fed the LCP diet than HCP diet among pigs without RS supplementation, but there was no difference between HCP and LCP diets among pigs with RS supplementation. The higher digesta ammonia N concentration observed for piglets fed the HCP diets could be explained by more undigested protein from HCP diets that was available for fermentation. This observation is in close agreement with results of others (Nyachoti et al., 2006; Heo et al., 2008; Opapeju et al., 2009). The observed interaction effect that ammonia N concentration in ileum was reduced in pigs fed LCP diets compared with those fed HCP diets among pigs without RS supplementation, whereas ammonia N concentration did not differ among pigs with RS supplementation indicates that RS supplementation can reduce protein fermentation in piglets fed HCP diets. Although a previous study with pigs did not show any differences in ammonia N concentration in ileal and cecal digesta among pigs fed diets with and without raw potato starch (Heo et al., 2014), Birkett et al. (1996) demonstrated that RS consumption lowers fecal concentrations of ammonia and phenols in humans.

Significant differences in microbial metabolites were only found in phase 2 of the study, which may indicate that pigs need more than 2 weeks to show any effects of CP content on microbiota composition and their metabolites. Indeed, Bikker et al. (2006) and Opapeju et al. (2009) reported that VFA concentrations in weaned pigs were not different between HCP and LCP diets when the digesta samples were collected on day 7 of the experiment. There were interaction effects showing that: 1) RS supplementation increased ileal propionate and colonic butyric acid

concentrations in the LCP diets, whereas the HCP-RS diet did not differ from the HCP diet, and 2) higher propionate concentration in the colon of pigs fed HCP diet compared to those fed LCP diet among pigs without RS supplementation but no difference after RS supplementation, which may indicate beneficial effects of RS supplementation on SCFA production in the intestine. A previous work by Mentschel and Claus (2003) showed raw potato starch supplementation produces more butyrate in the colon of pigs, and a meta-analysis conducted by Metzler-Zebeli et al. (2019) reported that enhanced proportion of propionate in mid-colon was found when RS2 (e.g., raw potatoes and high-amylose cereals) level increased in pig diets. The reason why RS supplementation was effective only for the LCP diet but not the HCP diet could be the lack of protein substrates for microbial fermentation in the LCP. Feeding a HCP diet has the potential to produce not only toxic metabolites but also more SCFA compared to the LCP diet because protein fermentation also produces SCFA in the large intestine (Macfarlane and Macfarlane, 2012). Unlike HCP diets which can produce a considerable amount of SCFA, LCP diets have insufficient substrates to produce SCFA in weaned pigs, thus, synergistic effects can occur for SCFA production when LCP diets are provided with RS supplementation. Unlike SCFA, BCFA and ammonia are harmful metabolites for the intestinal mucosa and thus, have been considered as predisposing factors for post-weaning diarrhea (Heo et al., 2014; Gao et al., 2019). When pigs were fed LCP diets, the total BCFA and ammonia N concentrations in ileal digesta decreased compared to those fed HCP diets. Nyachoti et al. (2006) demonstrated that BCFA concentrations in ileal digesta of weaned pigs linearly decreased with dietary CP reduction. The work of Htoo et al. (2007) also demonstrated decreased concentrations of isobutyric and isovaleric acids and ammonia N in cecal digesta of weaned pigs by lowering dietary CP content.

5.5. CONCLUSIONS

In conclusion, feeding LCP diets reduced feed efficiency during the overall period, because of deficiencies in indispensable AA in LCP diets. However, lowering dietary CP improved gut morphology in weaned pigs. Resistant starch supplementation increased the production of beneficial microbial metabolites in the small and large intestines of weaned pigs fed the LCP diet. Taken together, feeding a LCP diet with RS supplementation improves gut health by modulating concentrations of microbial metabolites in weaned pigs.

TRANSITION STATEMENT

Experiment 2 evaluated the effects of low protein diet given together with dietary factor, which was supplementation of resistant starch, on growth performance and gut health in weaned pigs. Subsequent Experiment 3 was conducted to investigate the effects of low protein diet and environmental factor, which was different environmental conditions, on growth performance and gut health in weaned pigs.

6. MANUSCRIPT III

Effects of dietary protein content and crystalline amino acid supplementation patterns on growth performance, intestinal histomorphology, and immune response in weaned pigs raised under different sanitary conditions

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6.1. ABSTRACT

The aim of this experiment was to investigate the effects of dietary crude protein (**CP**) contents and crystalline amino acids (**CAA**) supplementation patterns on growth performance, intestinal histomorphology, and immune response in weaned pigs under clean (**CSC**) or unclean sanitary conditions (**USC**). A total of 144 weaned pigs (6.35 ± 0.63 kg body weight) were assigned to 6 treatments in a 3 × 2 factorial arrangement based on CP content and sanitary conditions using a randomized complete block design, giving 8 replicates per treatment with 3 pigs per pen. Pigs were fed 1 of 3 diets for 21 days: one high CP (**HCP**; 22%) and two low CP (**LCP**; 19%) diets supplemented with 9 indispensable AA or only 6 AA (Lys, Met, Thr, Trp, Val, and Ile) as CAA. The CSC room was washed weekly, whereas the USC room had sow manure spread in the pens and was not washed throughout the experiment. Body weight and feed disappearance were recorded weekly. Blood were sampled from one pig per pen weekly, and the same pig was euthanized for jejunal tissues sampling on day 21. Pigs raised under USC had reduced ($P < 0.05$) average daily gain and gain to feed ratio (**G:F**) in week 2, but contrary results that greater ($P < 0.05$) ADG and G:F were found in pigs under USC in week 3. Overall, there was an interaction where G:F did not differ between HCP and LCP under CSC, however, LCP decreased ($P < 0.05$) G:F compared to HCP under USC. Pigs fed the HCP diet had higher ($P < 0.05$) fecal scores than those fed the LCP diets throughout the experiment. Pigs fed the LCP had higher ($P < 0.05$) villus height to crypt depth ratio than those fed the HCP. An interaction was observed where goblet cell density in the jejunum was higher ($P < 0.05$) in pigs fed LCP than HCP under CSC, but no difference was found between HCP and LCP under USC. Different CAA supplementation patterns did not influence both growth performance and histomorphology. Pigs raised under USC had greater ($P < 0.05$) plasma interleukin (**IL**)-10 and IL-6 concentrations and reduced ($P < 0.05$)

plasma tumor necrosis factor-alpha concentration. Also, the LCP diets resulted in a greater (P < 0.05) plasma IL-10 concentration. In conclusion, overall growth performance did not differ between HCP and LCP under CSC, but LCP diets reduced G:F under USC. Feeding LCP diets to weaned pigs improved gut morphology under USC and ameliorated systemic inflammation induced by USC, whereas CAA supplementation patterns did not affect growth performance and gut morphology.

Key words: crystalline amino acids, low protein diet, sanitation, piglets

6.2. INTRODUCTION

Dietary crude protein (**CP**) content plays an important role in young piglet health. Reduced feed intake and weight gain are typically observed after weaning, and to resolve this poor performance, weaned pigs are typically fed with high CP (**HCP**) diets. However, a diet containing high CP level increases the availability of fermentable protein, thereby increasing the proliferation of pathogenic bacteria, which is a predisposing factor for post-weaning diarrhea (Opapeju et al., 2008). Therefore, feeding low CP (**LCP**) diets with supplementation of crystalline amino acids (**CAA**) has been investigated as a strategy to improve growth performance and enhance gut health in weaned piglets (Opapeju et al., 2008, 2009a). The most limiting amino acids (**AA**) in cereal–soybean meal–based swine diets, such as Lys, Thr, Met, Trp, and Val, can be supplemented as CAA, thus allowing the reduction of dietary CP contents by approximately 4% units in diets (Gloaguen et al., 2014). Previous studies demonstrated that reducing dietary CP contents by 4% units using CAA supplementation decreases undigested protein, thereby improving the efficiency of N utilization and gut health of piglets, as well as reducing the incidence of diarrhea in weaned pigs (Heo et al., 2008, 2010b; Opapeju et al., 2008, 2009a).

Reduced CP content in swine diets necessitates the provision of indispensable CAA in the diets to meet AA requirements. Although some AA, including Lys, Thr, Met, Trp, Val, and Ile, are commercially available in the feed-grade form, others, such as His and Phe, must be supplemented into the LCP diets to meet indispensable AA requirements, are not yet commonly available in feed- or food-grade forms. Low CP diets using CAA in pharmaceutical-grade cannot be practically used in commercial pork production. Therefore, two different LCP diets were prepared in this study to test the difference between LCP diets supplemented with 9 indispensable

AA as CAA (including His and Phe as pharmaceutical-grade CAA) and supplemented with 6 indispensable CAA (Lys, Met, Thr, Trp, Val, and Ile).

The NRC (2012) recommends requirements of indispensable AA for pigs in different phases, however, those requirements could be changed depending on pig genotype (Ferreira and Schinckel, 2013) or immune challenged conditions (Le Floc'h et al., 2009; Jayaraman et al., 2015, 2017; van der Meer et al., 2016). Low-grade immune activation caused by poor sanitary housing conditions increases the maintenance energy expenditure of pigs (Jayaraman et al., 2015; van der Meer et al., 2020). Previous studies reported that unclean environmental conditions reduced growth performance and caused immune activation in weaned pigs, thus, pigs raised under unclean sanitary conditions (**USC**) had higher AA requirements to maximize growth compared to those raised under clean sanitary conditions (**CSC**) (Le Floc'h et al., 2009; Jayaraman et al., 2015, 2017; van der Meer et al., 2016). However, limited information is available on the effects of LCP diets on the gut health of weaned pigs under different sanitary conditions.

Thus, the objective of this study was to investigate the effects of dietary CP content and CAA supplementation patterns on growth performance, intestinal histomorphology, and immune response in weaned pigs raised under different sanitary conditions. We hypothesized that 1) pigs raised under USC would have poorer growth performance and gut health outcomes compared to those housed under CSC, and that feeding LCP diets would improve gut health and alleviate immune responses under the USC, and 2) growth performance of pigs fed LCP diet providing 6 CAA would be poorer than those fed LCP diet providing all indispensable CAA.

6.3. MATERIALS AND METHODS

The experimental protocol was approved by the University of Manitoba Animal Care Committee (AC11406). Pigs were cared for according to the guidelines of the Canadian Council on Animal Care (2009).

6.3.1 ANIMALS, HOUSING, AND EXPERIMENTAL DESIGN

A total of 144 piglets (TN70 × TN Tempo; Topigs Norsvin, Winnipeg, MB, Canada) with average initial body weight (**BW**) of 6.35 ± 0.63 kg were obtained from Glenlea Research Station at the University of Manitoba (Winnipeg, MB, Canada). Pigs were assigned to 1 of 6 treatments in a 3 × 2 factorial arrangement based on CP content and sanitary conditions in a randomized complete block design to give 8 replications with 3 pigs per pen (1.8 m × 1.2 m). The sex of pigs was balanced within treatment (4 replicates for each male and female).

The different sanitary conditions were established as described by Jayaraman et al. (2017) and van der Meer et al. (2020). The CSC room was cleaned and sterilized before the experiment using Virkon™ S disinfectant (Lanxess, Wilmington, DE, USA) and washed once a week, whereas the USC room was maintained without disinfection after a previous batch of nursery pigs and kept without washing the pens throughout the experiment. At the beginning of the study, swine manure (5 kg per pen) from the healthy sow herd was spread in the USC room to mimic the sanitary challenge of a commercial environment. Nylon bags containing dried pig manure from the sow herd were mounted on pens in the USC room for pigs to play with and therefore served as a source of environmental dust. Room temperature was maintained at 29 ± 1ºC during first week and reduced by 1ºC for each following week.

Table 6.1 Composition of experimental diets (as-fed basis)

	Diets[1]		
	HCP	LCP 1	LCP 2
Ingredients, %			
Corn	32.88	40.93	40.60
Wheat	25.00	25.00	25.00
Soybean meal	23.64	17.56	18.00
Whey powder	10.00	10.00	10.00
Spray dried animal plasma	2.24	-	-
Vegetable oil	2.81	1.77	1.88
Limestone	1.29	1.29	1.29
Monocalcium phosphate	0.85	0.98	0.97
Salt	0.40	0.40	0.40
Vitamin-mineral premix[2]	0.15	0.15	0.15
$_{L}$-Lysine HCl	0.39	0.76	0.74
$_{DL}$-Methionine	0.18	0.30	0.30
$_{L}$-Threonine	0.11	0.29	0.28
$_{L}$-Tryptophan	0.06	0.12	0.12
$_{L}$-Valine	-	0.21	0.20
$_{L}$-Isoleucine	-	0.08	0.07
$_{L}$-Leucine	-	0.05	-
$_{L}$-Histidine	-	0.04	-
$_{L}$-Phenylalanine	-	0.08	-

[1]HCP, high crude protein; LCP 1, low crude protein supplemented with all indispensable crystalline amino acids except Arg; LCP 2, low crude protein supplemented with 6 indispensable crystalline amino acids (Lys, Met, Thr, Trp, Val, and Ile).

[2]Provided the following nutrients (per kg of diet): Vitamins: A, 2,200 IU; D_3, 220 IU; E, 16 IU; K, 0.5 mg; B_1, 1.0 mg; B_2, 3.5 mg; B_6, 7 mg; B_{12}, 17.5 µg; calcium pantothenate, 10 mg; folic acid, 0.3 mg; niacin, 30 mg; biotin, 0.05 mg. Minerals: Cu, 6 mg (as copper sulphate); iodine, 0.14 mg (as calcium iodate); iron, 100 mg (as ferrous sulphate); Mn, 4 mg (as manganese oxide); Se, 0.3 mg (as sodium selenite); Zn, 100 mg (as zinc oxide).

Table 6.2 Calculated and analyzed nutrient composition of experimental diets (as-fed basis)

	Diet[1]		
Item, %	HCP	LCP 1	LCP 2
Calculated nutrient composition			
Net energy, kcal/kg	2,560	2,560	2,560
Crude protein	22.3	19.3	19.3
Total amino acids			
Arg	1.33	1.04	1.05
His	0.56	0.48	0.44
Ile	0.92	0.82	0.83
Leu	1.78	1.51	1.48
Lys	1.49	1.47	1.47
Met	0.49	0.57	0.57
Met + Cys	0.90	0.89	0.89
Phe	1.05	0.91	0.85
Thr	0.98	0.96	0.96
Trp	0.32	0.32	0.32
Val	1.05	1.03	1.03
SID[2] amino acids			
Arg	1.23	0.96	0.97
His	0.50	0.43	0.39
Ile	0.82	0.74	0.74
Leu	1.59	1.35	1.32
Lys	1.35	1.35	1.35
Met	0.46	0.54	0.54
Met + Cys	0.81	0.81	0.81
Phe	0.93	0.81	0.74
Thr	0.85	0.85	0.85
Trp	0.30	0.30	0.30
Val	0.92	0.92	0.92
Analyzed nutrient composition			
Dry matter	89.35	88.42	88.30
Crude protein	21.42	18.48	18.12
Total amino acids			
Arg	1.25	1.01	0.98
His	0.52	0.47	0.41
Ile	0.86	0.80	0.78
Leu	1.67	1.45	1.37

Lys	1.40	1.41	1.43
Met	0.47	0.52	0.55
Met + Cys	0.85	0.82	0.85
Phe	0.99	0.89	0.79
Thr	0.95	0.87	0.94
Trp	0.32	0.32	0.32
Val	0.99	0.99	0.98

[1]HCP, high crude protein; LCP 1, low crude protein supplemented with all indispensable crystalline amino acids except Arg; LCP 2, low crude protein supplemented with 6 indispensable crystalline amino acids (Lys, Met, Thr, Trp, Val, and Ile).
[2]SID, standardized ileal digestible.

6.3.2. EXPERIMENTAL DIETS

Three experimental diets were formulated based on corn, wheat, and soybean meal, and CAA were supplemented to meet the indispensable AA to Lys ratio recommended by AMINOPig® 1.0 (2011; Evonik Industries, Hanau-Wolfgang, Hessen, Germany) for weaned pigs from 5 to 10 kg of BW (Table 6.1). The AA concentrations in corn, wheat, and soybean meal were analyzed before diet mixing, and analyzed AA values were used in diet formulation. Diets consisted of one HCP (22%) and two LCP (19%) diets. The two LCP diets were supplemented either with 1) 9 CAA (indispensable AA except for Arg) to meet all indispensable AA requirements (LCP 1) or 2) only 6 CAA (Lys, Met, Thr, Trp, Val, and Ile) to meet indispensable AA requirements except for Leu, His, and Phe which were not met their requirements (LCP 2). Arg was not included in LCP 1 because it was already above the required level without CAA supplementation. All experimental diets were formulated to meet or exceed the NRC (2012) requirements for minerals and vitamins for 7 to 11 kg BW pigs. Pigs were fed the assigned diets for 3 weeks and had free access to feed and water throughout the experiment. The calculated and analyzed nutrient composition of experimental diets is shown in Table 6.2.

6.3.3. SAMPLE PREPARATION AND MEASUREMENTS

Body weight and feed disappearance were recorded weekly to determine average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain to feed ratio (**G:F**). Feces was scored daily at 0830 throughout the experiment by two trained individuals without information on the treatment allocation to determine fecal consistency scores (0 = normal, 1 = soft feces, 2 = mild diarrhea, and 3 = severe diarrhea) as described by Nyachoti et al. (2006). The average score derived from the two individuals per week was used for statistical analysis.

Room air quality was measured three times per week by collecting air samples from 3 different places in the rooms at pig's height, and the average values of the three measurements of each week were used for statistical analysis. Hydrogen sulfide (H_2S) concentrations were determined using a JEROME-631-X instrument (Arizona Instrument Corporation, Phoenix, AZ, USA), and the ammonia (NH_3) concentrations were measured using NH_3 gas detector tubes (RAE Systems, San Jose, CA, USA). Total volatile organic compounds (**TVOC**) and formaldehyde (**HCHO**) were also measured using the air quality monitoring device (WP6912; VSON Technology Co., Ltd., China).

One pig with the closest BW to the mean BW of each pen was selected at the beginning of the experiment, and blood samples were collected weekly from the pig via jugular vein puncture into 10 mL heparinized vacutainers tubes (BD Vacutainer, Franklin Lakes, NJ, USA) after 10 hours of fasting to analyze plasma urea nitrogen (**PUN**) and plasma free AA during the post-absorptive phase. Blood samples were centrifuged at 3,600 × g at 4˚C for 10 min to recover plasma, which was kept at –80˚C until analysis. The plasma concentration of free AA was determined by ion-exchange chromatography using a Biochrom 20 amino acid analyzer lithium column (Biochrom Ltd., Cambridge, UK) and lithium buffers. The AA were determined after dissolving the freeze-dried plasma samples and conducting protein precipitation with sulfosalicylic acid and centrifugation (30 min at 10,000 rpm; temperature 20-25°C). The AA were quantified using the internal standard norleucine by measuring the absorption of reaction products with ninhydrin at 570 and 440 nm. Also, blood samples from days 14 and 21 were used for plasma cytokine analyses. The tumor necrosis factor-alpha (**TNF-α**), interleukin (**IL**)-6, and IL-10 were determined using commercially available porcine ELISA Kits (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol.

One pig (the same pig used for the blood collection) from each pen was euthanized on day 21 to collect jejunal tissue samples. One 10-cm jejunum sample (2 m away from the ileocecal junction) was collected for gut permeability, and two approximately 3-cm jejunum samples from the same site were collected to determine histomorphology and mRNA gene expression. The jejunum sample for *ex vivo* Ussing chamber analysis was rinsed with Krebs Ringer buffer (**KRB**), immersed in KRB, and then immediately transported to the laboratory. The jejunum samples for histomorphology analysis were immediately stored in 10% buffered formalin, and samples for mRNA gene expression were immediately snap-frozen in liquid nitrogen after washing with phosphate-buffered saline.

A modified Ussing chamber (VCC-MC6; Physiologic Instruments Inc., San Diego, CA, USA) was used to study gut permeability across the jejunal epithelial tissues as described by Koo et al. (2020). Briefly, the epithelial tissues were placed in the tissue holder after removing serosal and muscle layers. The holders were mounted in a chamber containing current and voltage electrode pairs. The samples were run within 15 min postmortem for Ussing chamber analysis to ensure the retention of the electrophysiological properties of the intestinal samples. The flux of 4 kDa fluorescein isothiocyanate-dextran (**FD4**) was measured by adding 0.1 mg/mL of FD4 (Sigma-Aldrich, St. Louis, MO) to the mucosal side chamber and 2 ml of the buffer from the serosal side was sampled at 60 min and transferred to an amber (light protection) tube. The mucosal-to-serosal FD4 flux was determined by a fluorescence microplate reader as the optical density of the samples was read at 450 nm with the emission wavelength set at 540 nm.

After the fixation process in 10% buffered formalin solution, samples were sent to the Histology Laboratory (University of Manitoba, Winnipeg, MB, Canada) for further processing, as described by Koo et al. (2020). Villus heights (**VH**) and crypt depths (**CD**) were measured using

an Axio Scope A1 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Lower Saxony, Germany) and Image J software (National Institutes of Health, Bethesda, MD, USA) in all distinguishable villi and crypts. Goblet cells (**GC**) were manually counted for each villus.

Table 6.3 Sequences of primers for qPCR analysis

Gene symbol[1]	Genbank Accession No.	Primer	Sequence (5′ to 3′)
GAPDH	NM_001206359.1	Forward	GTGAACGGATTTGGCCGC
		Reverse	AAGGGGTCATTGATGGCGAC
HPRT	NM_001032376	Forward	GGACTTGAATCATGTTTGTG
		Reverse	CAGATGTTTCCAAACTCAAC
IL-6	M86722	Forward	AAGGTGATGCCACCTCAGAC
		Reverse	TCTGCCAGTACCTCCTTGCT
IL-8	NM_213867.1	Forward	CACCTGTCTGTCCACGTTGT
		Reverse	AGAGGTCTGCCTGGACCCCA
IL-10	NM_214041	Forward	CATCCACTTCCCAACCAGCC
		Reverse	CTCCCCATCACTCTCTGCCTTC
TNF-α	NM_214022.1	Forward	ATGGATGGGTGGATGAGAAA
		Reverse	TGGAAACTGTTGGGGAGAAG
CLDN3	NM_001160075.1	Forward	CTACGACCGCAAGGACTACG
		Reverse	TAGCATCTGGGTGGACTGGT
OCLN	NM_001163647.2	Forward	GAGAGAGTGGACAGCCCCAT
		Reverse	TGCTGCTGTAATGAGGCTGC
ZO-1	XM003353439.2	Forward	GATCCTGACCCGGTGTCTGA
		Reverse	TTGGTGGGTTTGGTGGGTT
MUC2	XM_021082584.1	Forward	CCAGGTCGAGTACATCCTGC
		Reverse	GTGCTGACCATGGCCCC

[1]GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPRT1, Hypoxanthine-guanine phosphoribosyltransferase 1; IL, interleukin; TNF-α, tumor necrosis factor-alpha; CLDN3, claudin 3; OCLN, occludin; ZO-1, zonula occludens-1; MUC2, mucin 2.

Determination of gene expression of mRNA in jejunum tissues has also followed the method described by Koo et al. (2020). Snap-frozen samples for gene expression were ground using a pestle by immersing the tissue in liquid nitrogen. Total RNA was extracted using a TRIzol Plus RNA Purification Kit (Invitrogen Canada Inc., Burlington, ON, Canada) following the manufacturer's protocol. Both RNA quality and yield were measured using a Nanodrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE, USA). Also, agarose gel electrophoresis was conducted to confirm the integrity of total RNA. After confirmation, cDNA was synthesized using a high-capacity complementary DNA synthesis kit (Applied Biosystems, Burlington, ON, Canada), and then qPCR was performed in duplicate reactions. Primers used for qPCR analysis were designed based on the gene bank of the National Center for Biotechnology Information (Table 6.3). Results of qPCR were normalized based on two housekeeping genes, GAPDH and HPRT, by using the geometric mean of the two housekeeping genes based on the $2^{-\Delta\Delta CT}$ method to calculate the relative fold change of the target gene (Livak and Schmittgen, 2001).

6.3.4. CHEMICAL ANALYSES

Ingredients and diet samples were finely ground and analyzed for dry matter, CP, and AA composition. The dry matter content was measured according to AOAC (2006) (method 934.01; 2006), and nitrogen content was determined by the combustion method (method 990.03; AOAC, 2006) using the LECO N analyzer (model CNS-2000; LECO Corp., St. Joseph, MI) to calculate CP (nitrogen × 6.25). Amino acid contents in the diets were determined by ion-exchange chromatography with post-column derivatization with ninhydrin. Amino acids were hydrolyzed with 6 N HCl for 24 hours at 110°C, and sulfur-containing AA had an additional oxidization step before hydrolyzation using performic acid (Llames and Fontaine, 1994; Commission Directive, 1998). Amino acids were quantified with the standard internal method by measuring the absorption

of reaction products with ninhydrin at 570 nm. Tryptophan was determined using HPLC with fluorescence detection (extinction 280 nm, emission 356 nm) after alkaline hydrolysis with barium hydroxide octahydrate for 20 hours at 110°C (Commission Directive, 2000). Tyrosine was not determined.

6.3.5. STATISTICAL ANALYSES

Data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). The model included the main effects of different CP contents and sanitary conditions and their interaction. There was no sex effect, therefore, sex was removed from the model. Means were separated using specific orthogonal contrasts to compare HCP with the combination of LCP 1 and LCP 2 (HCP vs. LCP 1 and LCP 2) for the main effect of CP contents and to compare LCP 1 with LCP 2 (LCP 1 vs. LCP 2) for the main effect of CAA supplementation patterns. The pen was considered as the experimental unit for growth performance and fecal score, whereas an individual pig representing its pen was used as the experimental unit for blood, histomorphology, and gene expression data. Statistical significance was set at $P < 0.05$.

Table 6.4 Effect of sanitary conditions on room air quality[1]

	Clean sanitary conditions			Unclean sanitary conditions				*P*-values[3]		
Item[2]	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	SEM	San	Week	San × Week
H_2S, ppm	0.01^{c}	0.01^{c}	0.02^{bc}	0.01^{c}	0.03^{ab}	0.03^{a}	0.004	0.001	0.029	0.015
NH_3, ppm	1.12^{bc}	0.66^{c}	1.06^{bc}	1.25^{bc}	1.76^{ab}	2.20^{a}	0.200	0.001	0.085	0.041
TVOC, mg/m^3	0.66	1.78	1.86	1.05	3.13	4.28	0.898	0.001	0.237	0.077
HCHO, mg/m^3	0.02^{c}	0.05^{bc}	0.06^{bc}	0.03^{c}	0.09^{b}	0.20^{a}	0.022	0.001	0.036	0.001

[1]Air quality status in the rooms was analyzed three times per week for each clean and unclean sanitary condition (n = 3).
[2]H_2S, hydrogen sulfide; NH_3, ammonia; TVOC, total volatile organic compounds; HCHO, formaldehyde.
[3]San, main effect of sanitation; Week, main effect of time difference; San × Week, interactive effect of sanitation and time difference.
[a-c]Within a row, means with different superscripts differ ($P < 0.05$).

6.4. RESULTS AND DISCUSSION

6.4.1. ROOM AIR QUALITY

The H_2S, NH_3, TVOC, and HCHO concentrations in the air were higher ($P < 0.05$) in USC compared to the CSC in the overall period (Table 6.4). The H_2S and HCHO concentrations under USC were higher ($P < 0.05$) in week 3 than in week 1. Interaction effects between sanitation and time difference were found where H_2S, NH_3, and HCHO concentrations at weeks 1, 2, and 3 did not differ under CSC, but under USC, those concentrations increased ($P < 0.05$) with time. Pigs are exposed to various stressors when they are raised on conventional swine farms, which may affect their growth performance by shifting the use of AA from animal growth toward inflammatory and immune responses (Le Floc'h et al., 2006). Moreover, deteriorated air quality of the housing facility could reduce feed intake, thereby affecting the growth performance of pigs (Lee et al., 2005). Unclean sanitary conditions are used as a challenge model to provoke immune stimulation in piglets, which mimics the environment of conventional production facilities (Le Floc'h et al., 2006; Adewole et al., 2016). Hydrogen sulfide and NH_3 are among the most representative gaseous compounds in swine barns, generated either by the animals themselves or through manure fermentation (Kim et al., 2008b). Greater than 100 ppm of H_2S is considered to be immediately dangerous to the life and health of animals, and the common H_2S level in an environmentally controlled swine barn is around 5 ppm (Barker et al., 1986). A review paper by Barker et al. (1986) mentioned that when pigs are exposed to 20 ppm H_2S continuously, pigs may have a loss of appetite and nervousness. Also, typical NH_3 concentrations in swine buildings are 10 to 20 ppm for liquid manure systems and 50 ppm for barns with solid floors, and greater than 50 ppm of NH_3 can reduce pigs' growth and affect their health (Barker et al., 1986). According to those reference values of H_2S and NH_3, all pigs under CSC and USC were raised without critical

damage to their health. Volatile organic compounds consist of compounds that easily volatilize at room temperature, and some of them, such as formaldehyde, benzene, or toluene, cause adverse effects in the respiratory system of animals when inhaled (Kim et al., 2019). Results observed in the current study show poor air quality under USC and higher concentrations of toxic compounds under USC as time went by, as seen by the increase in H_2S, NH_3, TVOC, and HCHO concentrations in USC. Our results are consistent with the findings of previous works by Lee et al. (2005) and Jayaraman et al. (2017), which indicated that environmental adjustment for the USC room effectively changed the air quality.

Table 6.5 Effects of dietary crude protein content and sanitary conditions on plasma urea nitrogen (**PUN**) and plasma-free amino acid concentrations during the post-absorptive phase in weaned pigs[1]

	Clean conditions			Unclean conditions				*P*-values[2]			
Item	HCP	LCP 1	LCP 2	HCP	LCP 1	LCP 2	SEM	San	CP	AA	San × CP
PUN, mmol/L											
day 1	2.89	3.51	3.89	3.66	3.64	3.16	0.444	0.854	0.414	0.897	0.115
day 7	3.58	1.75	1.71	3.25	2.41	1.40	0.452	0.981	0.001	0.230	0.506
day 14	3.34	1.21	0.46	3.85	1.19	0.86	0.382	0.323	0.001	0.146	0.607
day 21	3.54	1.58	1.08	3.98	1.36	1.73	0.367	0.335	0.001	0.852	0.732
Amino acids, mg/100 ml											
Arg											
day 1	1.41	1.32	1.34	1.68	1.57	1.55	0.135	0.029	0.381	0.988	0.871
day 7	1.40	1.22	1.36	2.09	1.63	1.01	0.190	0.111	0.012	0.203	0.053
day 14	2.00	1.16	0.99	2.19	1.44	0.86	0.164	0.407	0.001	0.030	0.661
day 21	2.36	1.72	1.42	2.38	1.95	1.71	0.232	0.348	0.002	0.261	0.567
His											
day 1	1.19	1.11	1.07	1.20	1.04	1.27	0.076	0.423	0.271	0.224	0.717
day 7	1.02	0.82	0.86	1.28	1.06	0.82	0.085	0.028	0.001	0.227	0.288
day 14	0.88	0.42	0.25	0.99	0.77	0.22	0.070	0.008	0.001	0.001	0.689
day 21	1.00	0.50	0.32	1.12	0.56	0.25	0.074	0.535	0.001	0.001	0.314
Ile											
day 1	1.77	1.60	1.81	1.77	1.78	1.70	0.141	0.821	0.657	0.598	0.892
day 7	1.85	2.53	2.60	2.05	2.42	2.59	0.168	0.834	0.001	0.484	0.367
day 14	2.60	2.68	3.20	2.14	2.65	2.83	0.220	0.119	0.020	0.114	0.515
day 21	2.16	2.18	2.81	2.24	2.70	2.82	0.181	0.177	0.009	0.047	0.553
Leu											
day 1	1.92	1.90	2.09	2.21	2.21	2.17	0.166	0.067	0.839	0.613	0.724
day 7	1.98	1.63	1.22	2.62	1.68	1.29	0.177	0.083	0.001	0.028	0.065

day 14	2.51	1.85	1.26	2.39	2.25	1.02	0.187	0.930	0.001	0.001	0.499
day 21	2.64	2.19	2.20	2.77	2.29	1.86	0.210	0.821	0.002	0.302	0.478
Lys											
day 1	1.62	1.70	1.64	1.51	1.76	1.72	0.157	0.907	0.256	0.706	0.459
day 7	1.13	2.07	2.54	0.90	2.28	2.32	0.395	0.817	0.001	0.517	0.743
day 14	0.59	1.62	3.02	0.92	1.55	1.91	0.437	0.434	0.002	0.048	0.239
day 21	1.11	1.84	1.60	0.94	1.79	2.96	0.402	0.245	0.006	0.241	0.242
Met											
day 1	0.64	0.63	0.58	0.60	0.67	0.63	0.036	0.556	0.755	0.152	0.105
day 7	0.54^{c}	1.01^{a}	1.07^{a}	0.68^{bc}	0.92^{ab}	0.85^{ab}	0.067	0.291	0.001	0.993	0.009
day 14	0.65	0.82	1.09	0.76	0.64	0.62	0.137	0.130	0.457	0.367	0.070
day 21	0.69	0.76	0.81	0.72	0.85	1.04	0.137	0.275	0.156	0.359	0.544
Cys											
day 1	0.54	0.49	0.49	0.40	0.50	0.46	0.059	0.230	0.764	0.719	0.177
day 7	0.32	0.50	0.40	0.50	0.48	0.55	0.044	0.007	0.067	0.754	0.130
day 14	0.56	0.48	0.37	0.48	0.45	0.45	0.043	0.801	0.019	0.157	0.150
day 21	0.47	0.35	0.33	0.44	0.41	0.38	0.039	0.265	0.004	0.477	0.141
Phe											
day 1	1.05	1.18	1.19	1.25	1.22	1.29	0.073	0.045	0.218	0.576	0.299
day 7	1.15	0.93	0.70	1.19	0.86	0.63	0.074	0.535	0.001	0.001	0.345
day 14	1.09	0.77	0.51	1.20	0.81	0.45	0.070	0.606	0.001	0.001	0.322
day 21	1.20	0.86	0.76	1.18	0.94	0.75	0.080	0.774	0.001	0.070	0.717
Thr											
day 1	1.72	2.11	1.97	1.82	2.07	2.01	0.228	0.851	0.172	0.655	0.810
day 7	0.90	2.73	1.84	1.93	2.18	2.48	0.410	0.271	0.015	0.489	0.170
day 14	3.31	4.74	3.67	3.80	4.79	5.18	0.613	0.180	0.056	0.578	0.782
day 21	4.15	3.80	3.07	4.02	4.53	5.32	0.634	0.073	0.864	0.961	0.146
Val											

day 1	1.67	1.70	2.62	1.99	1.60	1.93	0.260	0.458	0.544	0.019	0.112
day 7	1.97	4.43	5.08	2.72	4.52	5.40	0.397	0.237	0.001	0.062	0.428
day 14	2.70	4.21	5.71	2.79	4.42	5.01	0.396	0.675	0.001	0.011	0.619
day 21	2.90	3.88	5.52	3.22	4.75	5.37	0.312	0.179	0.001	0.001	0.947

[1]HCP, high crude protein; LCP 1, low crude protein supplemented with all indispensable crystalline amino acids except Arg; LCP 2, low crude protein supplemented with 6 indispensable crystalline amino acids (Lys, Met, Thr, Trp, Val, and Ile).
[2]San, main effect of sanitation; CP, main effect of crude protein contents (HCP vs. LCP 1 and LCP 2); AA, main effect of crystalline amino acids supplementation patterns (LCP 1 vs. LCP 2); San × CP, interactive effect of sanitation and crude protein contents (HCP vs. LCP 1 and LCP 2).
[a-c]Within a row, means with different superscripts differ ($P < 0.05$).

6.4.2. POST-ABSORPTIVE PLASMA UREA NITROGEN AND PLASMA FREE AMINO ACIDS

Sanitary conditions did not influence the PUN concentration, however, LCP diets decreased ($P < 0.05$) the PUN concentrations throughout the experiment (Table 6.5). Plasma urea nitrogen is an indicator of protein utilization. Increased PUN is observed when AA provided in diets does not meet the ideal ratio for protein synthesis or when AA are mobilized from body reserves due to dietary AA deficiency, and excess AA are degraded into urea (Waguespack et al., 2011). Higher PUN in pigs fed HCP diet might be due to increased AA breakdown from excessive AA in the diet. Decreased PUN in pigs fed LCP diets in the current study agrees with the results from the previous studies of Figueroa et al. (2008), Heo et al. (2008), and Yu et al. (2019).

Sanitary conditions influenced plasma free concentrations of only a few AA in the current study. Higher concentrations of plasma free His on days 7 and 14 and plasma free Cys on day 7 were obtained ($P < 0.05$) in pigs raised under USC than CSC, and similar results were observed in the work of Le Floc'h et al. (2006), who found a higher proportion of His and Gly in pigs kept under an unsanitary environment. Melchior et al. (2004) also found higher plasma His in pigs with chronic lung inflammation than that in healthy controls. Increased plasma His concentration could be due to increased muscle catabolism during inflammation (Wannemacher, 1977). We found a higher proportion of plasma free Lys, Met, Thr, Ile, and Val in pigs fed LCP diets, whereas the reverse was observed for plasma free Leu, His, Phe, Arg, and Cys. Compared to the HCP diet, LCP diets increased ($P < 0.05$) the plasma free Lys, Ile, and Val concentrations throughout the experiment and Met and Thr concentrations on day 7, whereas plasma free Leu, His, Phe, Arg, and Cys (days 14 and 21) concentrations were higher ($P < 0.05$) in pigs fed the HCP diet than those fed the LCP diets throughout the experiment. Pigs fed the LCP 1 diet showed higher ($P < 0.05$)

plasma free Leu (days 7 and 14), His (days 14 and 21), Phe (days 7 and 14), and Arg (day 14) concentrations compared to those fed the LCP 2 diet, whereas higher ($P < 0.05$) plasma free Lys (day 14), Ile (day 21), and Val (days 14 and 21) concentrations were found in piglets fed the LCP 2 diet. A significant interaction effect was found only for plasma free Met concentration on day 7, where higher ($P < 0.05$) plasma free Met concentration was observed in pigs fed the LCP diets than those fed the HCP diet under CSC, however, no difference was found under USC. Higher plasma Lys, Met, Thr, Ile, and Val concentrations in LCP diets could be explained by lower utilization of these AA for pig growth and muscle synthesis due to an imbalance of ideal protein in LCP diets. This raises the possibility that Leu, His, or Phe may not be provided sufficiently in LCP diets. The total basis of His, Leu and Phe recommendations by NRC (2012) for 7-11 kg of pigs are 0.53, 1.54, and 0.91%, respectively, and the analyzed values of those total AA in HCP and LCP diets were: His (HCP: 0.52% vs. LCP: 0.47 and 0.41%), Leu (HCP: 1.67% vs. LCP: 1.45 and 1.37%), and Phe (HCP: 0.99% vs. LCP: 0.89 and 0.79%). Thus, it is speculated that His, Leu, and Phe were not provided sufficiently in LCP diets. Also, analyzed AA values in the current study indicate that total His, Leu, Met + Cys, Thr, and Phe to Lys ratios were lower than ideal AA to Lys ratios of calculated values (optimal ratios) in LCP diets. This temporary imbalance of AA may lead to a greater plasma level of these AA during post-absorptive phase, thereby leading to the degradation of other AA, including Lys. Lys is the first limiting AA in cereal-soybean meal-based diets, therefore, it has to be mainly used for protein synthesis in young animals if other AA are provided in sufficient amounts. The higher plasma Leu, His, and Phe concentrations observed for pigs fed the LCP 1 diet compared to those fed the LCP 2 diet are also explained by the fact that the LCP 1 diet contained more Leu, His, and Phe than LCP 2 diet, thus, more AA were degraded in LCP 1 diet.

Table 6.6 Effects of dietary crude protein content and sanitary conditions on growth performance in weaned pigs[1]

	Clean conditions			Unclean conditions				*P*-values[3]			
Item[2]	HCP	LCP 1	LCP 2	HCP	LCP 1	LCP 2	SEM	San	CP	AA	San × CP
BW, kg											
Initial	6.36	6.35	6.34	6.35	6.34	6.34	0.251	0.755	0.388	0.741	0.895
Week 1	7.77	7.70	7.52	7.80	7.62	7.77	0.280	0.367	0.093	0.850	0.731
Week 2	10.98	10.80	10.66	10.62	10.48	10.33	0.363	0.024	0.145	0.410	0.909
Week 3	14.61	14.61	14.24	14.66	14.75	14.50	0.539	0.536	0.659	0.308	0.776
ADG, g/d											
Week 1	202	193	168	212	190	209	12.7	0.142	0.130	0.813	0.687
Week 2	442	443	450	411	425	373	21.8	0.023	0.843	0.299	0.671
Week 3	519	544	511	589	648	588	31.3	0.001	0.402	0.081	0.637
Overall	393	393	376	396	407	376	16.1	0.623	0.590	0.089	0.869
ADFI, g/d											
Week 1	252	230	207	239	248	253	15.1	0.136	0.336	0.526	0.061
Week 2	542	543	541	520	567	531	22.3	0.874	0.361	0.296	0.364
Week 3	757	787	740	789	903	803	37.0	0.006	0.169	0.018	0.271
Overall	495	484	459	484	540	497	30.2	0.191	0.794	0.177	0.203
G:F, g/g											
Week 1	0.82	0.84	0.80	0.89	0.78	0.83	0.043	0.626	0.240	0.997	0.212
Week 2	0.80	0.79	0.84	0.76	0.75	0.70	0.026	0.001	0.632	0.936	0.228
Week 3	0.68	0.69	0.69	0.74	0.72	0.73	0.016	0.001	0.565	0.571	0.315
Overall	0.79^{ab}	0.79^{ab}	0.83^{a}	0.83^{a}	0.74^{b}	0.76^{ab}	0.022	0.055	0.026	0.130	0.001

[1]HCP, high crude protein; LCP 1, low crude protein supplemented with all indispensable crystalline amino acids except Arg; LCP 2, low crude protein supplemented with 6 indispensable crystalline amino acids (Lys, Met, Thr, Trp, Val, and Ile).
[2]BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed ratio.
[3]San, main effect of sanitation; CP, main effect of crude protein contents (HCP vs. LCP 1 and LCP 2); AA, main effect of crystalline amino acids supplementation patterns (LCP 1 vs. LCP 2); San × CP, interactive effect of sanitation and crude protein contents (HCP vs. LCP 1 and LCP 2).

[a,b]Within a row, means with different superscripts differ ($P < 0.05$).

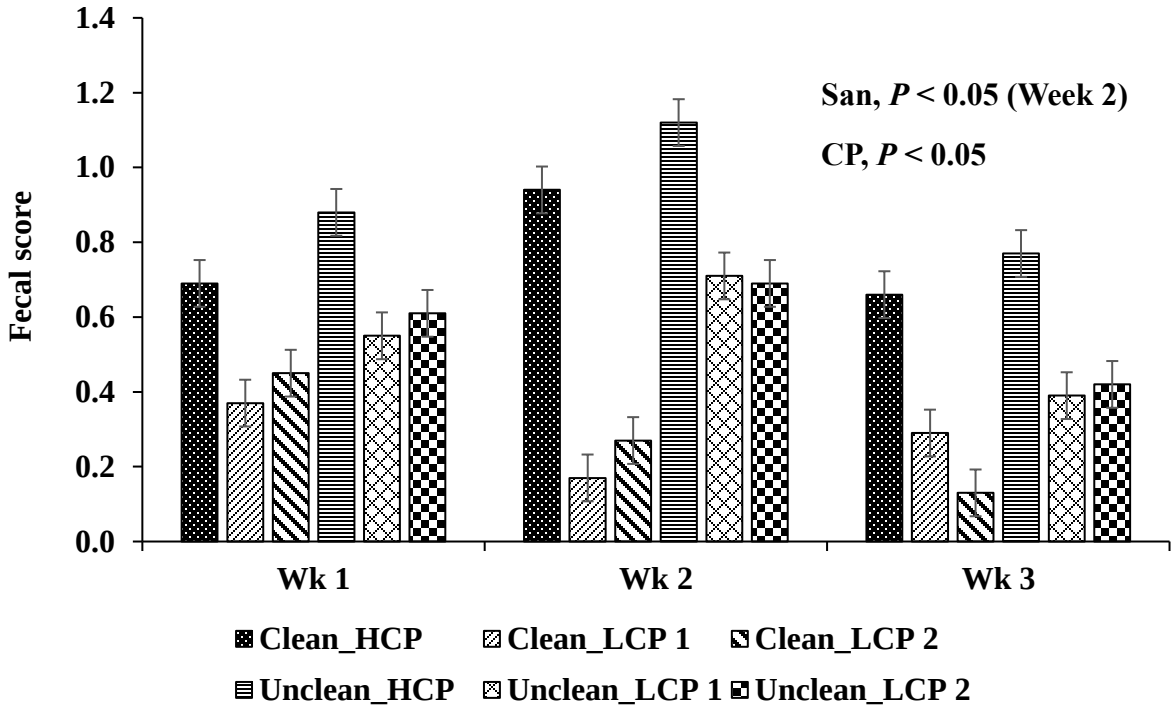

Figure 6.1 Effect of dietary crude protein content and sanitary conditions on fecal score of weaned pigs (0 = normal; 1 = soft feces; 2 = mild diarrhea; and 3 = severe diarrhea). Clean_HCP, high crude protein under clean sanitary conditions; Clean_LCP 1, low crude protein supplemented with all indispensable amino acids except Arg under clean sanitary conditions; Clean_LCP 2, low crude protein supplemented with 6 essential amino acids (Lys, Met, Thr, Trp, Val, and Ile) under clean sanitary conditions; Unclean_HCP, high crude protein under unclean sanitary conditions; Unclean_LCP 1, low crude protein supplemented with all indispensable amino acids except Arg under unclean sanitary conditions; Unclean_LCP 2, low crude protein supplemented with 6 essential amino acids (Lys, Met, Thr, Trp, Val, and Ile) under unclean sanitary conditions. Unclean sanitary condition increased ($P < 0.05$) the incidence of diarrhea of weaned pigs in week 2 compared to clean sanitary condition, and pigs fed the HCP diet had higher ($P < 0.05$) fecal scores throughout the experiment than those provided LCP diets regardless of sanitary conditions.

6.4.3. GROWTH PERFORMANCE AND FECAL SCORE

Pigs raised under USC had reduced ($P < 0.05$) ADG and G:F in week 2, but no difference was found between sanitary conditions over the overall period (Table 6.6). Increased ADFI was observed ($P < 0.05$) in pigs kept under USC in week 3 compared to those under CSC. Overall, there was an interaction effect on G:F, whereby G:F did not differ between HCP and LCP diets under CSC, but feeding the LCP diets decreased ($P < 0.05$) G:F under USC. The CAA supplementation patterns did not influence pig growth except that ADFI in pigs fed LCP 2 diet was reduced ($P < 0.05$) compared to that of pigs fed the LCP 1 diet in week 3.

This study hypothesized that pigs raised under USC would have poorer growth performance and gut health outcomes compared to those housed in CSC, and that feeding LCP diets would improve indices of gut health and alleviate immune responses under the USC. Reduced growth performance is typically expected when piglets are subjected to poor sanitary conditions because of immune system stimulation (Adewole et al., 2016), which was also found in the current study in week 2 as indicated by the decrease in ADG and G:F. However, the results of the current study show that piglets raised under USC could compensate for lost growth in the subsequent week. This might be interpreted as a beneficial effect of fecal microbiota transplantation (**FMT**) in USC. Studies in pigs showed that exogenous FMT from adult pigs to newborn piglets increased ADG in piglets (Qi et al., 2021), and FMT in sows and their offspring resulted in improved feed efficiency and greater microbial diversity and abundances of beneficial bacteria in the offspring (McCormack et al., 2019). Pigs were exposed to feces via environmental conditions such as manure spread on pen floors and hung in the bags in the current study, whereas previous studies referred to above used oral inoculation of feces. Although there was a difference in the way pigs were exposed to feces between this and previous studies, the current research has the potential that

pigs were possibly coprophagous, which could derive similar results. Also, FMT beneficial outcomes may be able to explain the jejunal histomorphology and gut permeability results of the current study. Although Hu et al. (2018) reported no significant difference in villus height of jejunum in FMT recipient piglets compared to control pigs, Yu et al. (2021) showed that VH and VH:CD in the jejunum of birds increased in the FMT group than the control group, which was also observed in the current study with pigs raised under USC having longer villi and higher VH:CD. Moreover, the result of the current study showing a higher number of GC per jejunal villus under USC is consistent with the finding of McCormack et al. (2018), which showed a higher number of ileal GC/μm of VH in FMT piglets. Cheng et al. (2019) demonstrated that maternal FMT in suckling pigs decreases intestinal permeability and improves piglet growth performance, which can relate to the result of reduced gut permeability under USC in this study. An increased number of GC is related to increased MUC2 secretion as MUC2 is the major secretory mucin that is synthesized and secreted by goblet cells in the small and large intestines (Faderl et al., 2015). The major function of the mucus layer is to serve as a physical barrier between the luminal contents and gut epithelia by protecting the gastrointestinal tract from harmful pathogens (Qin et al., 2011). Tight junction proteins, including CLDN, OCLN, and ZO-1, also play critical roles in gut barrier function by maintaining cellular polarity and establishing a permeability barrier for paracellular transport in epithelial and endothelial cells (Rangel et al., 2003; Pummi et al., 2004). Increased expressions of CLDN3, OCLN, ZO-1, and MUC2 were expected in pigs fed LCP diets due to improved gut function derived from feeding LCP diets. Nonetheless, the current study showed no significant difference in relative mRNA gene expression of gut barrier function genes in jejunal tissue.

Previous works demonstrated that feeding a LCP diet with CAA supplementation to achieve the recommended ideal AA pattern did not compromise growth performance of pigs (Htoo et al., 2007; Heo et al., 2008, 2009), however, in the current study, feed efficiency was reduced in weaned pigs fed LCP diets than those fed the HCP diet under USC in the overall experimental period. A possible reason for this observation is lower ideal AA to Lys ratios in LCP diets. Based on the analyzed AA in the diets, LCP 1 diet had lower ideal ratios for Met + Cys and Thr and LCP 2 diet had lowered His, Leu, Met + Cys, and Phe to Lys ratios compared to optimal AA ratios for weaned pigs recommended by AMINOPig. Thus, pigs fed LCP diets might experience shortage of some AA for optimal growth, and this effect could be more obvious under USC because more AA could be allocated to the immune system rather than growth under USC. Another possible reason for greater feed efficiency in pigs fed a HCP diet is that the HCP diet contained spray-dried animal plasma (**SDAP**). Plasma products are widely used to improve feed intake and efficiency in the pre-starter phase because they are known to reduce pro-inflammatory cytokines and lower antigen load (Torrallardona, 2010). Although a review by Torrallardona (2010) summarized that the optimal inclusion level of SDAP as an alternative to antibiotics is 4 to 8% to obtain significant improvement, SDAP could be a factor showing comparatively lower feed efficiency in LCP diets than the HCP diet under USC, which contained only 2% SDAP in the present study. Moreover, Zhao et al. (2007) reported that SDAP has greater efficacy in weaned pigs raised under USC, but no response in CSC, which is also in agreement with this study that G:F in HCP diet (containing SDAP) was greater than LCP diet under USC. The HCP diet in this study contained about 10% more Ile and about 19% more Leu compared to LCP diets. The work of Zhang et al. (2013) confirmed that branched-chain AA supplementation to a LCP diet could enhance intestinal AA transporter expression, thereby improving growth performance in weanling pigs. Dietary Leu

supplementation plays a significant role in the upregulation of AA transporter genes such as SLC6A19 and SLC6A14 in piglets, which is related to the efficient absorption and utilization of dietary protein in the gut (Sun et al., 2015). Excessive Leu intake due to feeding a HCP diet, which is also proved by the high plasma Leu concentration in the HCP diet in the current study, could improve intestinal AA transporters, leading to increased feed efficiency in pigs raised under USC.

It was expected that the growth performance of pigs fed a LCP diet with 6 CAA (LCP 2) would be poorer than those fed a LCP diet with all indispensable CAA (LCP 1). However, the CAA supplementation patterns in the present study did not affect overall growth performance and gut morphology, which indicates that shortage of His and Phe may have less significant impacts on pig growth. A previous study showed similar findings that there was no difference in growth performance between a LCP diet with Ile and Val supplementation and a LCP diet with Ile, Val, and His supplementation in growing pigs (Powell et al., 2011). Also, Kang et al. (2020) demonstrated that addition of dietary His in the LCP diet could not improve growth performance and intestinal morphology in weaned pigs.

Unclean sanitary conditions increased ($P < 0.05$) the incidence of diarrhea of weaned pigs in week 2, and pigs fed the HCP diet had higher ($P < 0.05$) fecal scores throughout the experiment than those fed the LCP diets regardless of sanitary conditions (Figure 6.1). The fecal score data obtained in the current study agrees with previous studies reporting reduced incidence of diarrhea in weaned pigs fed LCP diets (Heo et al., 2008, 2009; Lynegaard et al., 2021).

Table 6.7 Effects of dietary crude protein content and sanitary conditions on histomorphology of jejunum in weaned pigs[1]

Item[2]	Clean conditions			Unclean conditions			SEM	*P*-values[3]			
	HCP	LCP 1	LCP 2	HCP	LCP 1	LCP 2		San	CP	AA	San × CP
VH, µm	385	436	405	407	476	483	28.3	0.030	0.018	0.626	0.395
CD, µm	242	227	231	207	194	202	8.8	0.001	0.116	0.483	0.809
VH:CD	1.7	2.0	1.8	2.1	2.6	2.6	0.18	0.001	0.017	0.533	0.347
GC/100 µm villus	2.8^{b}	4.5^{a}	3.9^{ab}	5.2^{a}	3.4^{ab}	4.4^{a}	0.48	0.142	0.921	0.703	0.002
GC/villus	10.6^{b}	18.7^{a}	15.3^{ab}	20.5^{a}	15.7^{ab}	21.0^{a}	2.10	0.012	0.223	0.628	0.015

[1]HCP, high crude protein; LCP 1, low crude protein supplemented with all indispensable crystalline amino acids except Arg; LCP 2, low crude protein supplemented with 6 indispensable crystalline amino acids (Lys, Met, Thr, Trp, Val, and Ile).
[2]VH, villus height; CD, crypt depth; GC, goblet cell counts.
[3]San, main effect of sanitation; CP, main effect of crude protein contents (HCP vs. LCP 1 and LCP 2); AA, main effect of crystalline amino acids supplementation patterns (LCP 1 vs. LCP 2); San × CP, interactive effect of sanitation and crude protein contents (HCP vs. LCP 1 and LCP 2).
[a,b]Within a row, means with different superscripts differ ($P < 0.05$).

6.4.4. JEJUNAL HISTOMORPHOLOGY

Pigs raised under USC had higher ($P < 0.05$) VH and VH:CD ratio but decreased ($P < 0.05$) CD than pigs under CSC (Table 6.7). Pigs fed the LCP diets had longer ($P < 0.05$) jejunal villi and higher VH:CD ratio than those fed the HCP diet. No significant difference was found in jejunal histomorphology between pigs fed LCP diets with different CAA supplementation patterns. Interactions between sanitation and dietary CP content were observed for GC density in the jejunum, where goblet cell density in the villus of jejunum was higher ($P < 0.05$) in pigs fed LCP than HCP under CSC, but no difference was found between HCP and LCP under USC. The digestion and absorption of nutrients mainly happen in the stomach and small intestine, and the gut is the largest immune organ in the animal. Thus, well-developed gut morphology is essential not only for nutrient digestibility and optimal pig growth (Jha et al., 2019) but also for its immune function (Choct, 2009). Higher VH indicates an increased absorptive capacity of the small intestine, and shallow CD is considered to have a decreased metabolic cost for epithelial turnover, therefore, both are components of a healthy gut (Yang and Liao, 2019). Faster tissue turnover happens in a deeper crypt to renew the villi in enterocytes, which may be required under inflammation caused by pathogens (Xue et al., 2018). Longer villi and higher VH:CD were observed in weaned pigs fed LCP diets in the present study, which is in agreement with the findings of Opapeju et al. (2009a). However, some previous studies reported that dietary CP content did not affect or even impaired intestinal morphology in the jejunum of weaned pigs, which may be due to the fact that pigs cannot maintain intestinal epithelium structure due to protein deficiency in LCP diets (Li et al., 2019; Yu et al., 2019).

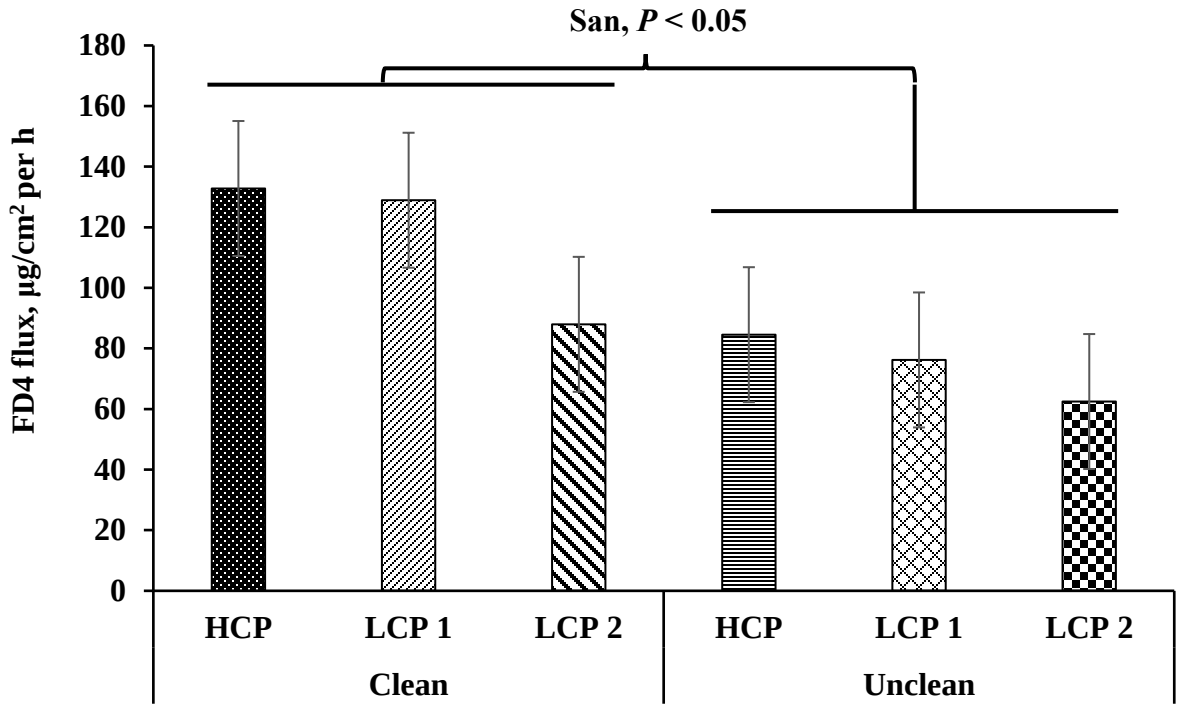

Figure 6.2 Effect of dietary crude protein content and sanitary conditions on the flux of fluorescein isothiocyanate dextran 4 kDa (**FD4**) flux in the jejunum. HCP = high crude protein, LCP 1 = low crude protein supplemented with all indispensable crystalline amino acids except Arg, LCP 2 = low crude protein supplemented with 6 indispensable crystalline amino acids (Lys, Met, Thr, Trp, Val, and Ile). Unclean sanitary conditions decreased ($P < 0.05$) *ex vivo* gut permeability measured by using FD4 flux, however, no interaction between protein contents and sanitary conditions was observed.

Table 6.8 Effects of dietary crude protein content and sanitary conditions on relative mRNA gene expression ($2^{-\Delta\Delta Ct}$) in jejunum of weaned pigs[1,2]

	Clean conditions			Unclean conditions				*P*-values[4]			
Item[3]	HCP	LCP 1	LCP 2	HCP	LCP 1	LCP 2	SEM	San	CP	AA	San × CP
IL-6	1.00	1.52	0.92	1.07	1.20	1.03	0.213	0.757	0.511	0.071	0.675
IL-8	1.00	1.00	1.21	0.82	0.66	1.09	0.186	0.165	0.612	0.098	0.883
IL-10	1.00	0.80	0.89	1.25	1.09	0.89	0.150	0.149	0.118	0.758	0.686
TNF-α	1.00	0.94	0.75	0.92	1.06	0.84	0.123	0.677	0.537	0.100	0.402
CLDN3	1.00	1.09	1.36	1.49	0.90	1.22	0.223	0.785	0.603	0.190	0.100
OCLN	1.00	0.79	0.80	1.11	0.82	1.20	0.146	0.135	0.229	0.184	0.688
ZO-1	1.00	1.18	0.95	1.24	0.96	1.18	0.124	0.428	0.647	0.948	0.273
MUC2	1.00	1.15	1.15	1.39	0.82	1.17	0.188	0.861	0.453	0.361	0.102

[1]HCP, high crude protein; LCP 1, low crude protein supplemented with all indispensable crystalline amino acids except Arg; LCP 2, low crude protein supplemented with 6 indispensable crystalline amino acids (Lys, Met, Thr, Trp, Val, and Ile).

[2]The relative data were expressed as a ratio of the target gene to the Clean conditions (HCP) gene, using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001), where $\Delta\Delta Ct = (Ct_{target} - Ct_{GAPDH/HPRT1})_{treatment} - (Ct_{target} - Ct_{GAPDH/HPRT1})_{Clean\ conditions\ (HCP)}$.

[3]IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; TNF-α, tumor necrosis factor-alpha; CLDN3, claudin 3; OCLN, occludin; ZO-1, zonula occludens-1; MUC2, mucin 2.

[4]San, main effect of sanitation; CP, main effect of crude protein contents (HCP vs. LCP 1 and LCP 2); AA, main effect of crystalline amino acids supplementation patterns (LCP 1 vs. LCP 2); San × CP, interactive effect of sanitation and crude protein contents (HCP vs. LCP 1 and LCP 2).

6.4.5. GUT PERMEABILITY AND RNA GENE EXPRESSION IN JEJUNUM

Unclean sanitary conditions decreased ($P < 0.05$) *ex vivo* gut permeability measured by using FD4 flux, however, no interaction effect between dietary protein content and sanitary conditions was found (Figure 6.2). Dietary CP content and sanitation did not affect relative mRNA gene expression of IL-6, IL-8, IL-10, TNF-α, claudin 3 (**CLDN3**), occludin (**OCLN**), zonula occludens (**ZO**)-1, and mucin (**MUC**) 2 in the jejunum of weaned pigs (Table 6.8).

Table 6.9 Effects of dietary crude protein content and sanitary conditions on plasma cytokine in weaned pigs[1]

	Clean conditions			Unclean conditions				*P*-values[3]			
Item[2]	HCP	LCP 1	LCP 2	HCP	LCP 1	LCP 2	SEM	San	CP	AA	San × CP
IL-6, pg/mL											
day 14	556	613	1049	1108	1632	1231	261	0.009	0.190	0.949	0.914
day 21	669	815	1263	1474	1663	1632	342	0.020	0.362	0.548	0.741
IL-10, pg/mL											
day 14	22.5	28.7	47.3	27.8	96.3	48.4	9.83	0.004	0.001	0.141	0.093
day 21	19.3	27.5	38.0	30.0	78.9	31.4	8.96	0.016	0.016	0.051	0.447
TNF-α, pg/mL											
day 14	33.1	25.3	30.8	5.5	10.4	18.3	5.33	0.001	0.680	0.222	0.137
day 21	18.3	14.1	14.1	16.1	21.7	23.4	4.30	0.175	0.750	0.854	0.143

[1]HCP, high crude protein; LCP 1, low crude protein supplemented with all indispensable crystalline amino acids except Arg; LCP 2, low crude protein supplemented with 6 indispensable crystalline amino acids (Lys, Met, Thr, Trp, Val, and Ile).
[2]IL-6, interleukin-6; IL-10, interleukin-10; TNF-α, tumor necrosis factor-alpha.
[3]San, main effect of sanitation; CP, main effect of crude protein contents (HCP vs. LCP 1 and LCP 2); AA, main effect of crystalline amino acids supplementation patterns (LCP 1 vs. LCP 2); San × CP, interactive effect of sanitation and crude protein contents (HCP vs. LCP 1 and LCP 2).

6.4.6. PLASMA CYTOKINES

Compared to CSC, pigs raised under USC had greater ($P < 0.05$) plasma IL-10 and IL-6 concentrations on days 14 and 21 and decreased ($P < 0.05$) plasma TNF-α concentration on day 14 (Table 6.9). Also, LCP diets resulted in a greater ($P < 0.05$) plasma IL-10 concentration in weaned pigs on days 14 and 21. Different CAA supplementation patterns did not affect the plasma cytokine in weaned pigs during the whole experimental period.

It was hypothesized that LCP diets would ameliorate the inflammatory responses of pigs, especially under USC, by decreasing pro-inflammatory cytokines such as TNF-α and increasing anti-inflammatory cytokines such as IL-10. The toxins secreted by *Escherichia coli* can affect the immune system of weaned pigs because their lipopolysaccharide is a highly pro-inflammatory molecule which elicits up-regulation of cytokines and induces endothelial cell death (Bannerman and Goldblum, 2003). Many of pathogenic bacteria, including *Escherichia coli*, are protein fermenters, therefore, feeding a LCP diet can decrease proliferation of *Escherichia coli* by reducing substrates for proteolytic fermentation, thereby decreasing inflammation in weaned pigs. Blood cytokine levels are generally considered an indicator of systemic inflammation. IL-6 is typically considered a pro-inflammatory cytokine produced in the early phase of inflammation, and its blood concentration is increased in a systemic inflammatory status (Oda et al., 2005). However, many studies have shown that IL-6 also coordinates anti-inflammatory activities to control local or systemic acute inflammatory responses (Xing et al., 1998; Hunter and Jones, 2015). IL-10 is an anti-inflammatory cytokine, which suppresses the action of pro-inflammatory cytokines, whereas TNF-α is a pro-inflammatory cytokine and principal mediator of inflammation (Harawa et al., 2018). Increased concentrations of IL-10 in the blood plasma of weaned pigs raised under USC could also be explained by FMT application. Burrello et al. (2018) demonstrated

reduced colonic inflammation and intestinal homeostasis maintenance by applying therapeutic FMT because FMT stimulates immune pathways, including both innate and adaptive immune cells, and results in IL-10 production by T cells. As discussed above, sow manure in USC could function as FMT to weaned pigs, thereby possibly increasing plasma IL-10 concentrations in weaned pigs. Contrary results between IL-6 and TNF-α concentrations that increased IL-6 but decreased TNF-α under USC could be explained by the work of Schindler et al. (1990), who reported that IL-6 suppresses TNF-α in human peripheral blood mononuclear cells after incubation with several stimuli. Moreover, TNF-α and IL-10 play opposite roles during infection (Rojas et al., 1999). For example, TNF-α increases nitric oxide synthesis related to induction of DNA damage and apoptosis, however, IL-10 inhibits the function of macrophage and nitric oxide production. Therefore, the contrary effects of sanitation on plasma TNF-α and IL-10 on d 14 were found in this study as expected. Increased plasma IL-10 concentration in pigs fed LCP diets in this study could be partially explained by the down-regulation effect of LCP diets on gene expression of pro-inflammatory cytokines (Zhang et al., 2017), which may increase anti-inflammatory cytokines like IL-10. Although sanitary conditions and CP contents affected cytokines concentration in blood, the mRNA gene expression of IL-6, IL-8, IL-10, and TNF-α in jejunum did not differ among treatments. This discrepancy might be because each cytokine gene shows different expression rates in various organs (Gourbeyre et al., 2015). For example, Gourbeyre et al. (2015) investigated that IL-10 and TNF-α genes showed higher expression in lymphoid organs such as ileal Peyer's patches or mesenteric lymph nodes than in the jejunum. Thus, the sample collection location should be taken into consideration for gene expression analysis.

6.5. CONCLUSIONS

Feeding low crude protein diets improved gut histomorphology in the jejunum and increased plasma anti-inflammatory cytokine profile in weaned pigs. However, growth performance decreased when pigs were fed low crude protein diets due to some amino acids deficiencies. Poor sanitation resulted in reduced growth performance in the second week of the trial, however, pigs could compensate for lost growth in the subsequent week. These positive effects on growth might be caused by being exposed to a beneficial microbiome through fecal microbiota transplantation. Under these conditions crystalline amino acids supplementation patterns did not affect growth performance, gut histomorphology, or immune response in weaned pigs.

TRANSITION STATEMENT

Manuscript III and IV were written based on results obtained from Experiment 3. Manuscript III tested the effects of low protein diet in association with different environmental conditions on growth performance, gut histomorphology, and immune response in weaned pigs. Samples obtained from Experiment 3 were further analyzed to investigate the effects of low protein diet in association with different environmental conditions on microbial composition and their metabolism in the intestine of weaned pigs, which was reported in Manuscript IV.

7. MANUSCRIPT IV

Effects of dietary protein content and crystalline amino acid supplementation patterns on intestinal bacteria and their metabolites in weaned pigs raised under different sanitary conditions

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John Kyaw Htoo: conceptualization, methodology, writing–review & editing.

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Jolie Caroline González-Vega: conceptualization, writing–review & editing.

Charles Martin Nyachoti: conceptualization, supervision, project administration, writing–review & editing.

7.1. ABSTRACT

The objective of this experiment was to investigate the effects of dietary crude protein (**CP**) content and crystalline amino acids (**CAA**) supplementation patterns on the bacteria and their metabolites in the intestine of weaned pigs raised under clean (**CSC**) or unclean sanitary conditions (**USC**). One hundred forty-four piglets (6.35 ± 0.63 kg BW) were assigned to 1 of 6 treatments in a 3 × 2 factorial arrangement based on CP content and sanitary conditions in a randomized complete block design to give 8 replications with 3 pigs per pen. Diets consisted of a high CP (**HCP**; 21%) and two low CP (**LCP**; 18%) diets supplemented with 9 indispensable AA (except Arg) as CAA or only 6 CAA (Lys, Met, Thr, Trp, Val, and Ile). The CSC room was washed weekly, whereas the USC room had sow manure spread in the pens from the beginning of the study and was not washed throughout the experiment. Digesta samples were collected on day 21. Digesta from jejunum and colon were analyzed for ammonia N, short-chain fatty acids, and biogenic amines, but only colonic digesta was analyzed for microbiome composition (16s rRNA sequencing on MiSeq). Data were analyzed using R software for 16S rRNA and the MIXED procedure of SAS for microbial metabolites. Sanitation, CP content, and CAA supplementation patterns did not affect the diversity of colonic bacterial composition in weaned pigs. Pigs raised under USC had greater ($P < 0.05$) jejunal ammonia N than those under CSC. Pigs fed LCP diets had reduced ($P < 0.05$) jejunal ammonia N compared to those fed the HCP diet. Interactions between sanitation and dietary CP content were observed ($P < 0.05$) for 1) jejunal acetate and 2) colonic spermidine and spermine, whereby 1) acetate concentrations decreased from NCP to LCP in pigs raised under the CSC but those concentrations increased under the USC, and 2) spermidine and spermine concentrations increased in LCP diets compared to HCP diet under USC, unlike CSC which did not show any difference between HCP and LCP. In conclusion, reducing dietary CP lowered ammonia nitrogen

content regardless of the sanitation and increased microbial metabolites in weaned pigs raised under USC. However, LCP diets with different CAA supplementation patterns did not affect bacterial diversity in weaned pigs, regardless of the hygienic conditions where the animals were housed.

Key words: crystalline amino acids, low protein diet, microbiome, sanitation

7.2. INTRODUCTION

Since in-feed antibiotics have been banned for growth promotion purposes, the importance of alternative strategies to promote growth has increased considerably. High crude protein (**HCP**) concentration in the diet for weaned piglets stimulates protein fermentation as well as the proliferation of pathogenic bacteria in the gut. Thus, lowering dietary crude protein (**CP**) concentration by 3 to 4 percentage units has been used as one of the strategies to improve gut health and growth performance of weanling pigs (Nyachoti et al., 2006; Opapeju et al., 2009a). This is because feeding a low CP (**LCP**) diet with crystalline amino acids (**CAA**) supplementation can reduce the amount of undigested protein which becomes available for fermentation. Reducing protein fermentation decreases the production of harmful metabolites such as biogenic amines and ammonia, leading to decreased incidence of diarrhea (Heo et al., 2008; Opapeju et al., 2009a). The metabolites derived from protein fermentation are detrimental not only to gut health of host animals but also to the environment (Le et al., 2007, 2009). Because of the urease activity of the fecal microbiome, urea is easily converted to ammonia, which causes a concern such as aerial pollution and acidification of the soil. For example, feeding higher concentrations of nutrients especially high CP concentrations in diets has a greater potential for environmental contamination than low concentrations of nutrients in the diets due to increased aerial pollutants such as ammonia nitrogen derived from undigested nutrients (Hernández et al., 2011). Moreover, excessive ammonia negatively affects the immune system. Qin et al. (2022) and Li et al. (2023) found disrupted immune homeostasis and increased oxidative stress and lung injuries when pigs were exposed to aerial ammonia for 30 days.

The use of CAA is necessary for LCP diets to meet the indispensable amino acids (**AA**) requirements. However, the LCP diets using CAA in pharmaceutical grades, such as histidine or

phenylalanine, cannot be adapted in the industry due to current high cost. Therefore, the difference between LCP providing all indispensable AA as CAA and 6 indispensable CAA (Lys, Met, Thr, Trp, Val, and Ile) was investigated current study. Williams et al. (2018) demonstrated that different CAA supplementation patterns alter the fecal microbial community. Moreover, previous studies showed that sanitary conditions change the gut microbiome in nursery pigs (Cho et al., 2020; te Pas et al., 2020). Unclean sanitary conditions (**USC**) may have detrimental effects on growth performance, which may be due to differences in nutrient metabolism in the gut (te Pas et al., 2020). te Pas et al. (2020) reported that pigs raised under clean sanitary conditions (**CSC**) showed higher digestibility and a higher abundance of bacteria in the colon. Gut bacteria are one of the major regulatory factors underlying nutrient digestion and fermentation, mostly placed at the hindgut of animals (Pluske et al., 2018). A clean environment can modify gut health of animals for both gut microbial composition and metabolism in a positive way, which in turn affects higher nutrient digestibility, and leads to improved growth performance of pigs (van der Meer et al., 2016; te Pas et al., 2020). However, there is a scarcity of information about the effects of sanitary conditions and CAA supplementation patterns on the gut microbial composition and their metabolites in weaned pigs. Thus, the current study was conducted to investigate the effects of dietary CP content and CAA supplementation patterns on bacteria composition and their metabolites in the intestine of weaned pigs raised under CSC or USC.

This was a series of studies with the effects of dietary protein content and CAA supplementation patterns on growth performance, intestinal histomorphology, and immune response in weaned pigs raised under different sanitary conditions (Lee et al., 2022).

7.3. MATERIALS AND METHODS

Experimental diets, experimental design, and the management of animals and housing were the same as Manuscript III.

7.3.1. SAMPLE PREPARATION

One pig with the closest BW to the mean BW of each pen was selected at the beginning of the experiment and euthanized on day 21 to collect jejunum and colon digesta samples for ammonia-N, volatile fatty acids (**VFA**), and biogenic amines analyses. Also, colonic digesta was sampled for microbial analysis. Samples were immediately snap-frozen in liquid nitrogen and transferred to a –80°C freezer. The ammonia-N concentrations in jejunal and colon digesta were determined using the method described by Novozamsky et al. (1974). A 1.5 mL of a reagent was obtained from a mixture of 200 mL of 0.05% sodium nitroprusside and 10 mL of 4% ethylenediaminetetraacetic acid and added to 50 µL of digesta fluid. A 2.5 mL of a solution containing 10% sodium hypochlorite was then added to the previous mixture. Test tubes containing the final mixture were incubated in the dark for 30 min, and then the optical density of the mixture was immediately read at 630 nm using a spectrophotometer (SoftMax Pro Software; Molecular Devices, San Jose, CA, USA). The VFA and branched-chain fatty acids (**BCFA**) concentrations were determined using the method described by Erwin et al. (1961) using a gas chromatography (Varian Chromatography System, model Star 3400; Varian Medical Systems, Palo Alto, CA). Briefly, 1 mL of 25% metaphosphoric acid was mixed with 5 mL of digesta fluid. The mixture was neutralized with 0.4 mL of 25% NaOH and then 0.65 mL of 0.3 M oxalic acid was added. The samples were then centrifuged for 20 min at 3,000 × g at 4°C, and 2 mL of the supernatant was transferred to a gas chromatography vial. The biogenic amines were analyzed by liquid chromatography according to the method described by Smělá et al. (2003).

7.3.2. DNA EXTRACTION AND SEQUENCING

To assess the diversity of the microbial community, DNA was extracted from colon digesta using QIAamp PowerFecal Pro DNA Kit (Cat. No. / ID: 51804; Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The extracted DNA samples were sent to LGC Genomics GmbH (Berlin, Germany) for microbial sequencing using the 16S rRNA technique. The V4 region of the bacterial 16S rRNA gene was amplified from the total extracted DNA. DNA library was demultiplexed for each sequencing lane using the Illumina's bcl2fastq version 2.20 software (Illumina; San Diego, CA, USA), and the reads were sorted by amplicon inline barcodes.

7.3.3. MICROBIAL COMMUNITY ANALYSIS

The obtained raw sequences were pre-processed with the Mothur version 1.35.1 software program for the operational taxonomic unit (**OTU**) cluster analysis (Schloss et al., 2009) as follows: 1) the sequences containing ambiguous bases were removed with homopolymer stretches of more than 8 bases or with an average Phred quality score below 33; 2) sequence alignments were performed against the 16S Mothur-Silva SEED r119 reference alignment; 3) short alignments and chimera were filtered, and sequencing error was reduced by pre-clustering; 4) taxonomical classification of the sequences was performed against the Silva reference classification and sequences from other domains of life were removed; 5) OTU was picked by clustering at the 97% identity level, and 6) OTU consensus taxonomical calling, integrating the taxonomical classification of the cluster member sequences was performed.

The OTU diversity was analyzed by using Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.0 (Caporaso et al., 2010). Microbial diversity was assessed within samples (alpha-diversity) or between samples (beta-diversity). Alpha-diversity (observed OTUs) was calculated through rarefaction with ten iterations. Beta-diversity was calculated on the sequence

reads based on weighted and unweighted UniFrac distance matrices, and principal coordinate analysis (**PCoA**) plots were used for visualization of the results for beta-diversity.

7.3.4. CHEMICAL ANALYSES

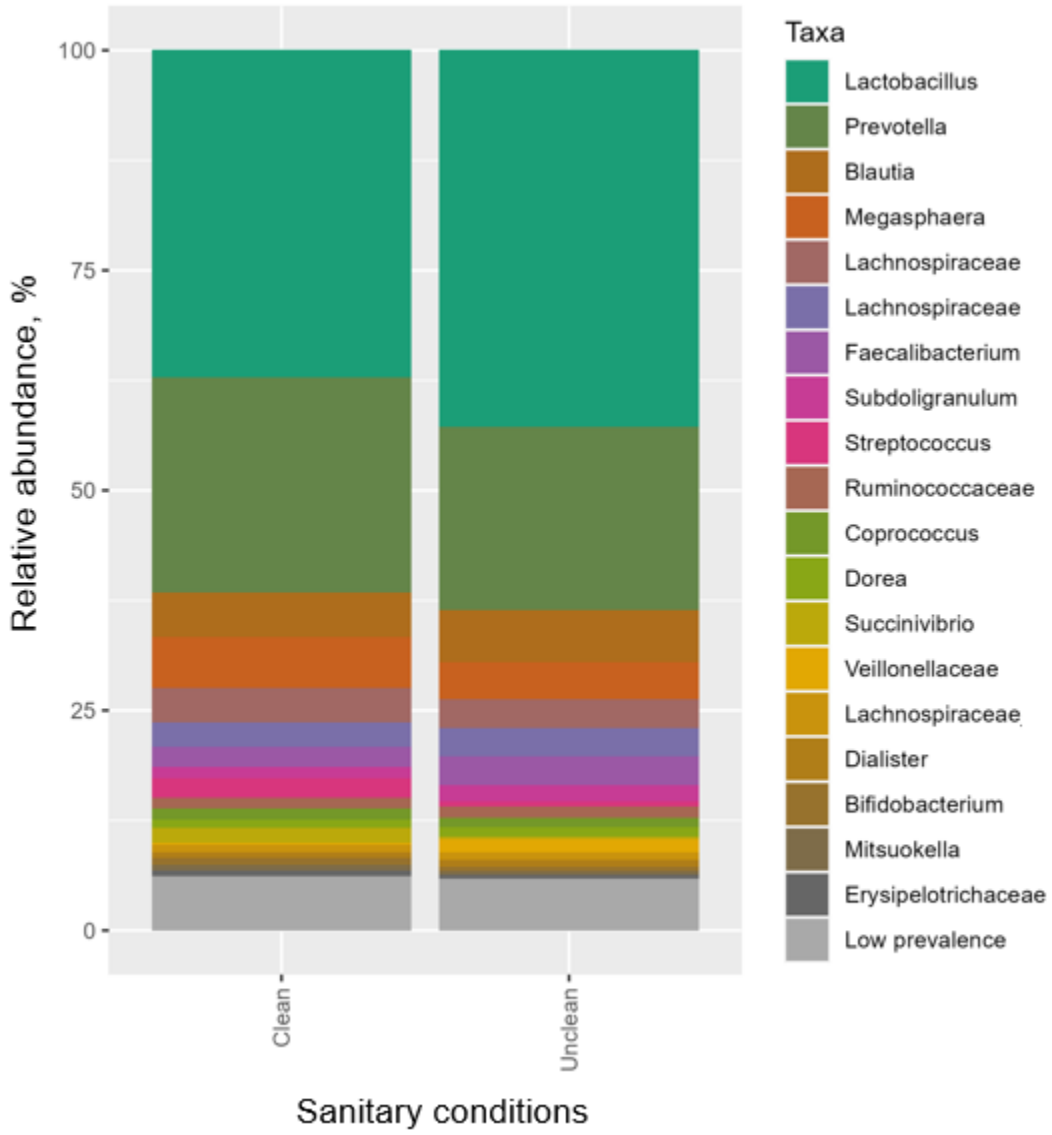
Diets were analyzed for dry matter, CP, and AA compositions. The dry matter content was measured according to AOAC (2006) (method 934.01; 2006), and nitrogen content was determined by the combustion method (method 990.03; AOAC, 2006) using the LECO N analyzer (model CNS-2000; LECO Corp., St. Joseph, MI, USA) to calculate CP (nitrogen × 6.25). Amino acid contents in the diets were determined by ion-exchange chromatography with post-column derivatization with ninhydrin. Amino acids were hydrolyzed with 6 N HCL for 24 hours at 110°C, and samples for sulfur-containing AA analysis had an additional oxidization step before hydrolyzation using performic acid (Llames and Fontaine, 1994; Commission Directive, 1998). Amino acids were quantified with the standard internal method by measuring the absorption of reaction products with ninhydrin at 570 nm. Tryptophan was determined using HPLC with fluorescence detection (extinction 280 nm, emission 356 nm) after alkaline hydrolysis with barium hydroxide octahydrate for 20 hours at 110°C (Commission Directive, 2000). Tyrosine was not determined.

7.3.5. STATISTICAL ANALYSES

Data for microbial metabolites (volatile fatty acids, biogenic amines, and ammonia N) were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). The model included the main effects of different CP contents and sanitary conditions and their interaction, and replicate was included as random effects. There was no sex effect, therefore, sex was removed from the model. Means were separated using specific orthogonal contrasts to compare HCP with the combination of LCP 1 and LCP 2 (HCP vs. LCP 1 and LCP 2) for the main

effect of CP contents and to compare LCP 1 with LCP 2 (LCP 1 vs. LCP 2) for the main effect of CAA supplementation patterns. Microbiome data were analyzed using R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria). Analyses were based on packages "vegan" and "phyloseq". If not otherwise indicated, data were analyzed with the Kruskal-Wallis test and Dunn's test for post-hoc analysis. An individual pig was used as the experimental unit, and significant differences were considered at $P < 0.05$.

(a)


Taxa
Lactobacillus
Prevotella
Blautia
Megasphaera
Lachnospiraceae
Lachnospiraceae
Faecalibacterium
Subdoligranulum
Streptococcus
Ruminococcaceae
Coprococcus
Dorea
Succinivibrio
Veillonellaceae
Lachnospiraceae
Dialister
Bifidobacterium
Mitsuokella
Erysipelotrichaceae
Low prevalence
Relative abundance, %
100
75
50
25
0
Clean
Unclean
Sanitary conditions

(b)

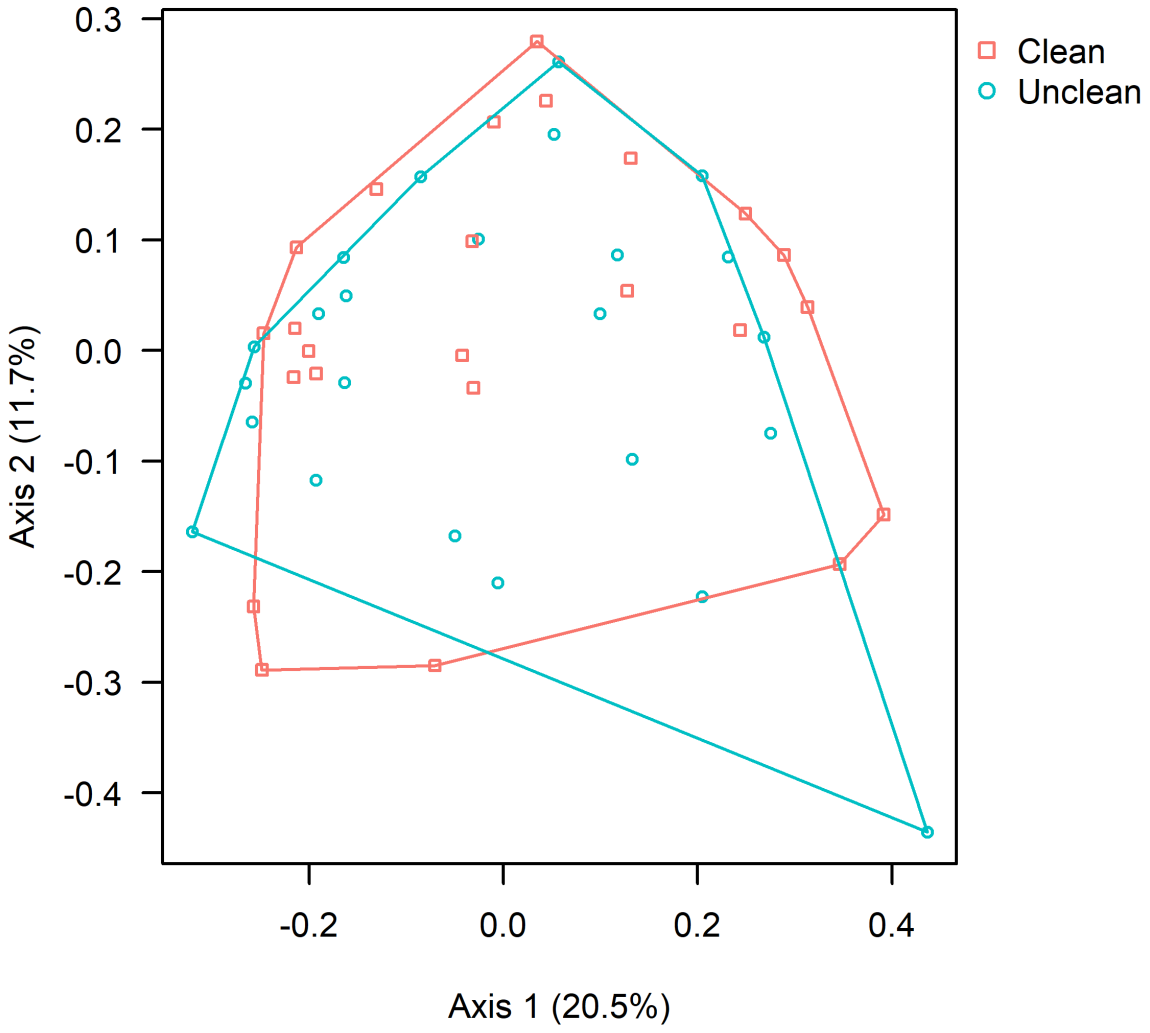
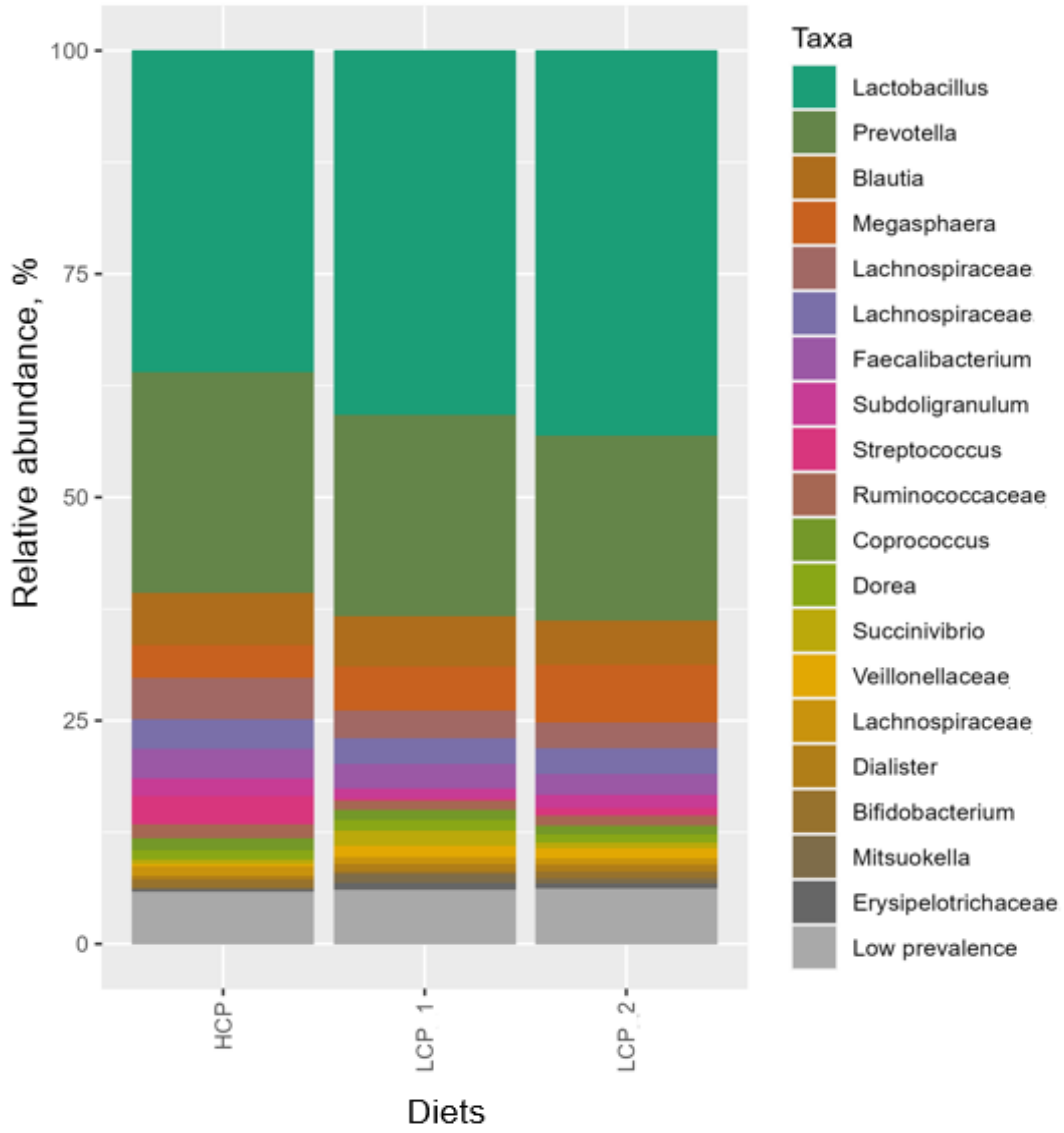


Figure 7.1 The effect of sanitary conditions on colonic bacterial diversity in weaned pigs (*n* = 8). (a) The relative abundance of bacterial composition at genus level in colon digesta of weaned pigs raised under clean or unclean sanitary conditions. (b) PCoA plots of the microbial communities in colon digesta of weaned pigs raised under clean or unclean sanitary conditions. Clean, clean sanitary conditions; Unclean, unclean sanitary conditions.

(a)


Relative abundance, %
100
75
50
25
0
HCP
LCP_1
LCP_2
Diets
Taxa
Lactobacillus
Prevotella
Blautia
Megasphaera
Lachnospiraceae
Lachnospiraceae
Faecalibacterium
Subdoligranulum
Streptococcus
Ruminococcaceae
Coprococcus
Dorea
Succinivibrio
Veillonellaceae
Lachnospiraceae
Dialister
Bifidobacterium
Mitsuokella
Erysipelotrichaceae
Low prevalence

(b)

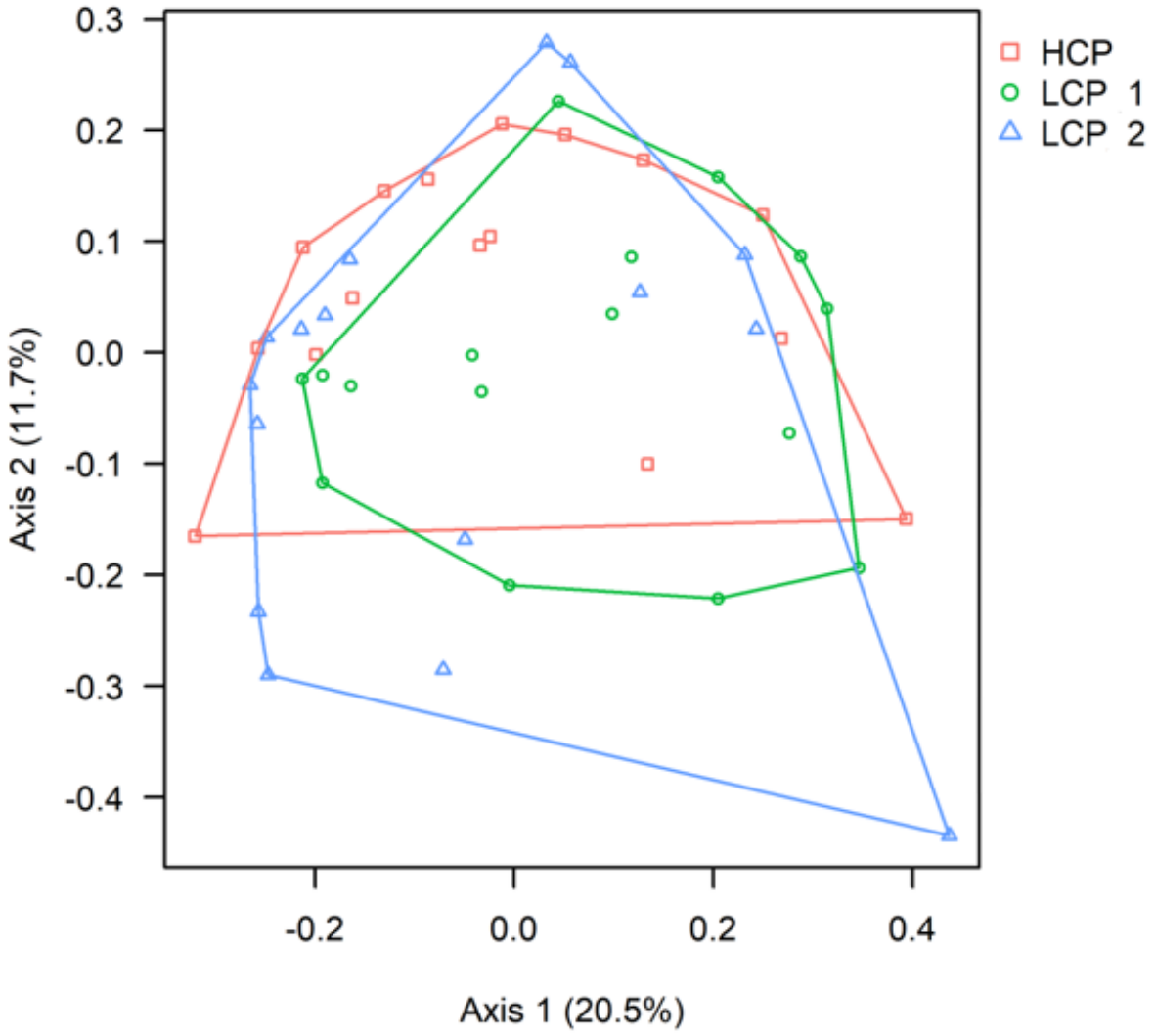
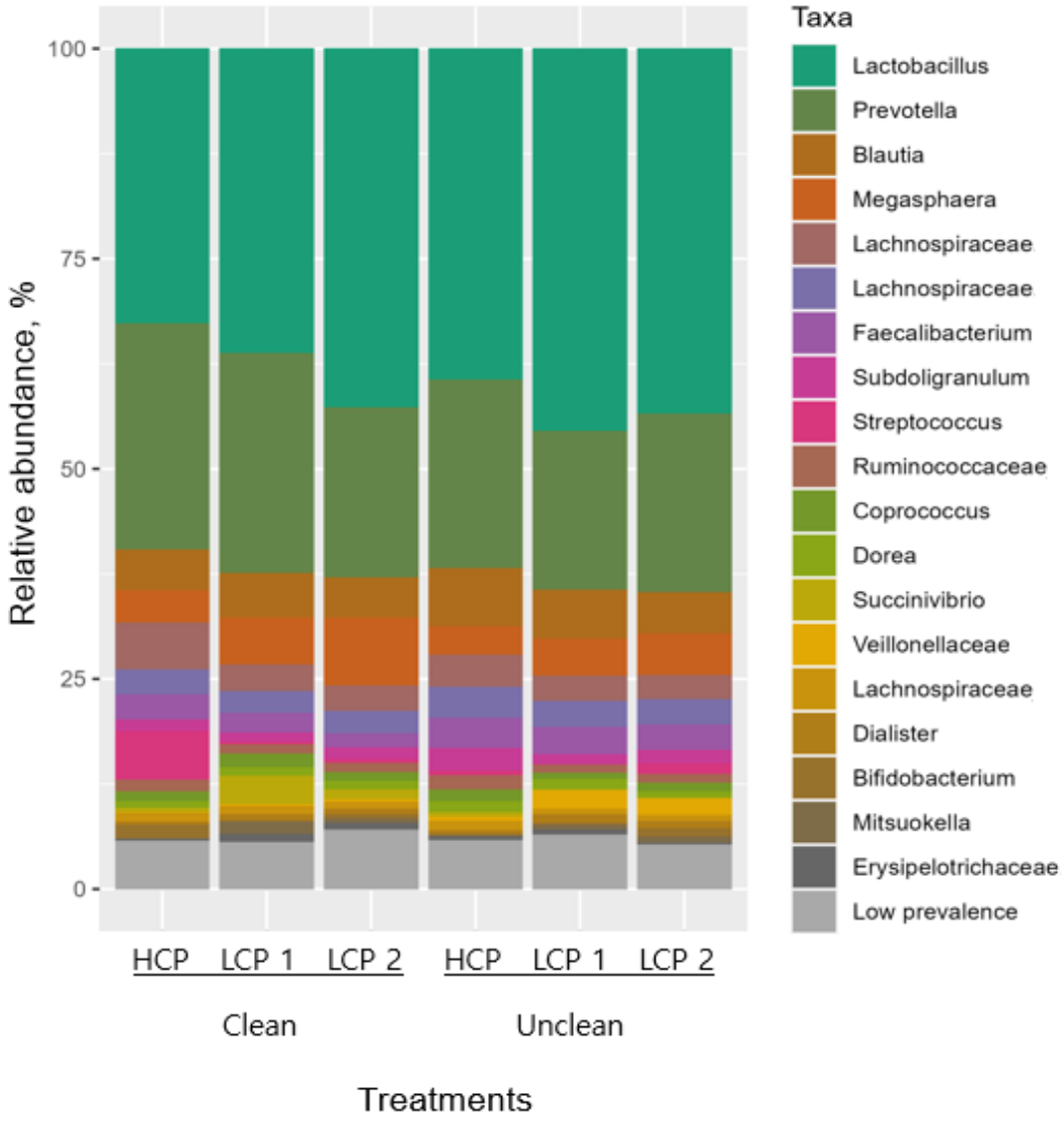


Figure 7.2 The effect of protein content on colonic bacterial diversity in weaned pigs (n = 8). (a) The relative abundance of bacterial composition at genus level in colon digesta of weaned pigs fed the diets contained different protein contents. (b) PCoA plots of the microbial communities in colon digesta of weaned pigs fed the diets contained different protein contents. HCP, high crude protein; LCP 1, low crude protein using all indispensable amino acids except Arg; LCP 2, low crude protein using 6 indispensable amino acids (Lys, Met, Thr, Trp, Val, and Ile).

(a)


Taxa
Lactobacillus
Prevotella
Blautia
Megasphaera
Lachnospiraceae
Lachnospiraceae
Faecalibacterium
Subdoligranulum
Streptococcus
Ruminococcaceae
Coprococcus
Dorea
Succinivibrio
Veillonellaceae
Lachnospiraceae
Dialister
Bifidobacterium
Mitsuokella
Erysipelotrichaceae
Low prevalence
Relative abundance, %
100
75
50
25
0
HCP
LCP 1
LCP 2
HCP
LCP 1
LCP 2
Clean
Unclean
Treatments

(b)

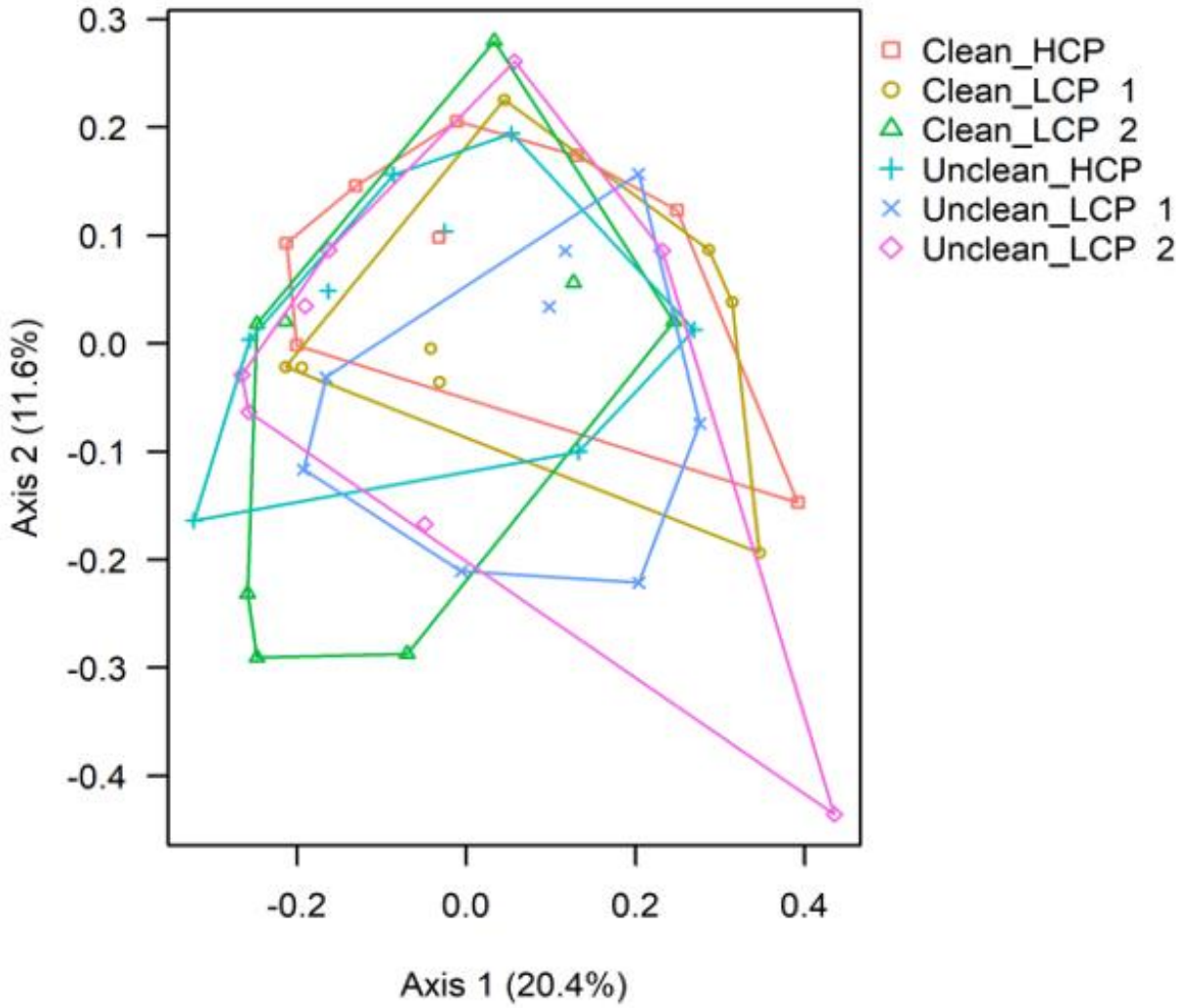


Figure 7.3 Colonic bacterial diversity among treatments groups evaluated at the genus level (n = 8). (a) The relative abundance of bacterial composition at genus level in colon digesta of weaned pigs. (b) PCoA plots of the microbial communities in colon digesta of weaned pigs. Clean_HCP, high crude protein under clean sanitary conditions; Clean_LCP 1, low crude protein using all indispensable amino acids except Arg under clean sanitary conditions; Clean_LCP 2, low crude protein using 6 indispensable amino acids (Lys, Met, Thr, Trp, Val, and Ile) under clean sanitary conditions; Unclean_HCP, high crude protein under unclean sanitary conditions; Unclean_LCP 1, low crude protein using all indispensable amino acids except Arg under unclean sanitary conditions; Unclean_LCP 2, low crude protein using 6 indispensable amino acids (Lys, Met, Thr, Trp, Val, and Ile) under unclean sanitary conditions.

7.4. RESULTS AND DISCUSSION

A contrast in sanitary conditions was generated by differences in hygiene with housing and management of the rooms. All pigs were clinically healthy throughout the trial.

7.4.1. DIVERSITY OF MICROBIAL COMMUNITY IN THE COLON

Generally, no difference was found ($P > 0.05$) in both alpha- and beta-diversity of the microbial community in colon digesta between the different sanitary conditions (Figure 7.1), CP levels (Figure 7.2), or both dietary and environmental treatments (Figure 7.3). A sanitary status model was applied in this study to stimulate a low-grade immune response (compared to other immune challenge models such as enterotoxigenic *Escherichia coli* or Salmonella) and measure aspects of microbial diversity and microbial metabolites observed in pigs under different sanitary conditions. It was expected that sanitary conditions might affect the microbiota diversity in colon digesta based on results of previous studies (Kubasova et al., 2018; Cho et al., 2020; te Pas et al., 2020). High diversity of microbial community is often found in pigs raised in CSC (te Pas et al., 2020). However, there was no difference in microbial diversity between the different sanitary conditions in the present study. The sow manure seemed to have a low effect on microbiota modulation in the USC group in this study. One important thing to be considered is the time of sampling (duration of the experiment) for microbial analysis. The exposure to different sanitary conditions might not be enough to change the microbial composition in the gut of pigs in this study. Kubasova et al. (2018) showed the microbiota composition did not differ during the nursery phase (from weaning until 4 wk after weaning), however, the complexity of pig fecal microbiota increased with age (up to 25 wk of age). In contrast, pigs used in Cho et al. (2020) showed a difference in microbial diversity on d 7 of the trial. Another possible explanation for this result together with the growth

performance data in the previous publication (Lee et al., 2022) could be that microbial compositions could be changed by transplanting fecal microbiota from sow feces to weaned pigs raised under USC. Several studies showed that fecal microbiota transplantation has beneficial effects on growth performance and immune function in young piglets (Hu et al., 2018; Niederwerder et al., 2018; Wan et al., 2019). Hu et al. (2018) reported that *Prevotella* and *Oscillospira* were increased on day 12 in piglets that received daily oral inoculation from day 1 to 11, which are beneficial bacteria associated with the digestion of carbohydrates (Wu et al., 2011), and butyrate production (Konikoff and Gophna, 2016), respectively. It was possible that the sow feces spread in USC beneficially affected pig health, including growth performance in this study. Indeed, pigs raised under USC showed reduced daily gain and feed efficiency in week 2, however, the growth performance of pigs raised in different sanitary conditions was not different in week 3, which is time colon digesta was collected for microbial diversity analysis (Lee et al., 2022). Intestinal microbial shifts in pigs are correlated with the growth performance of pigs (Kim and Isaacson, 2015), and the body weights of weaned pigs on day 21 did not differ between different sanitary conditions in this research, thus, no significant difference was observed in microbial diversity in this study.

Differences in diversity of gut microbiota were also expected between pigs fed the diets containing different CP levels because a major reason for the complexity of microbial community in the gut could be the amount of available substrates for bacteria fermentation (Blaut and Clavel, 2007). Dietary carbohydrates and protein can be used as primary substrates for bacteria fermentation. These substrates include indigestible dietary components and endogenous components, such as mucus and sloughed epithelial cells (Blaut and Clavel, 2007). Although diet is the primary factor that can modulate the gut microbiota, dietary crude protein content had no

significant effects on microbial diversity in the current study. Zhang et al. (2016) demonstrated that feeding a LCP diet caused significant modulation on some of the bacterial groups in pigs, including reducing *Escherichia coli* and increasing *Clostridium* cluster XIVa and *Clostridium* cluster IV groups. Also, *Escherichia coli* is one of the major proteolytic fermenters, and its abundance is decreased by lowering CP levels in the diet, whereas *Clostridium* cluster XIVa and *Clostridium* cluster IV groups, butyrate producers, increased as carbohydrase fermentation increased. Peng et al. (2017) also reported decreased *Escherichia coli* counts in growing pigs when CP level was reduced from 20.0 to 15.3%. According to a review paper by Zhang et al. (2020a), however, the diversity of microbial population was not affected by dietary CP levels in most of the studies, although dietary protein content sometimes modulated protein fermentation by bacteria. A similar result was revealed by Yu et al. (2019), who reported that the colonic bacterial community was not changed in weaned pigs fed diets containing 20, 17, and 14% CP, which was probably due to a lack of fermentable carbohydrate sources in the diets. Thus, results of the current study are showing that no differences in colonic bacterial community between pigs fed HCP and LCP diets could be explained by insufficient fermentable carbohydrate sources in these diets.

Different bacterial community structures were observed when different CAA supplementation patterns were used in piglet diets (Williams et al., 2018; Spring et al., 2020), however, the diversity of the microbial community in the current study did not differ between LCP 1 (all indispensable CAA) and LCP 2 (only 6 CAA). Williams et al. (2018) demonstrated that pigs fed diets with fewer CAA inclusion showed an increased abundance of *Paraprevotellaceae*, *Lactobacilliaceae*, and *Ruminococcaceae* than those fed diets with more CAA inclusion. Diets with fewer CAA supplementation had more intact protein sources than those with more CAA supplementation, which might affect the microbial composition due to the different amounts of

available substrates for microbial fermentation. However, the amounts of intact protein and free AA were similar between LCP 1 and LCP 2 diets in the current study, which might explain the lack of significant difference in the obtained results. The results of the present study were also supported by Zhao et al. (2020), who observed no differences in alpha-diversity of colonic microbiota in fattening pigs fed diets with different dietary CP levels or CAA balanced patterns. In addition, considering the relatively unstable structure of the bacterial community in young piglets, another factor that can influence microbial composition is weaning stress (Kim and Isaacson, 2015), therefore, a higher number of observations might be required for such dynamic changes in gut microbiota in weaned pigs (Yu et al., 2019).

Table 7.1 Effects of crude protein level and sanitary conditions on ammonia N and volatile fatty acids (VFA) in jejunal and colonic digesta in weaned pigs[1]

	Clean conditions			Unclean conditions				*P*-values[2]			
Item	HCP	LCP 1	LCP 2	HCP	LCP 1	LCP 2	SEM	San	CP	AA	San × CP
Ammonia N, mg/L											
Jejunum	50.0	24.8	22.4	64.4	33.5	31.0	5.9	0.034	< 0.001	0.676	0.583
Colon	63.8	61.6	94.6	75.4	85.0	122.8	27.7	0.348	0.366	0.196	0.762
Jejunal VFA, mmol/L											
Acetic acid	4.60	2.71	3.21	3.83	5.43	3.95	0.675	0.113	0.513	0.467	0.041
Propionic acid	0.14	0.16	0.20	0.22	0.16	0.38	0.054	0.032	0.241	0.010	0.894
Butyric acid	0.36	0.31	0.33	0.26	0.27	0.27	0.030	0.006	0.437	0.669	0.298
Isobutyric acid	0.72	0.58	0.49	0.83	0.78	0.93	0.073	< 0.001	0.190	0.672	0.077
Isovaleric acid	0.02	0.01	0.03	0.12	0.10	0.14	0.023	< 0.001	0.869	0.226	0.858
Valeric acid	0.07	0.06	0.05	0.01	0.02	0.02	0.009	< 0.001	0.676	0.625	0.305
Total BCFA[3]	0.80	0.65	0.57	1.02	0.87	1.09	0.087	< 0.001	0.110	0.401	0.335
Total VFA	5.08	2.89	3.75	4.29	5.85	4.61	0.723	0.097	0.521	0.792	0.041
Colonic VFA, mmol/L											
Acetic acid	60.0	50.2	57.3	56.7	52.1	53.3	2.91	0.450	0.043	0.170	0.649
Propionic acid	28.3	31.6	30.1	29.3	31.8	35.9	2.30	0.217	0.072	0.577	0.590
Butyric acid	21.6	18.6	29.0	18.1	21.4	22.2	4.32	0.483	0.427	0.211	0.844
Isobutyric acid	0.94	0.89	1.56	0.70	0.67	0.72	0.160	0.002	0.291	0.031	0.287
Isovaleric acid	1.14	1.13	1.95	0.79	0.82	0.92	0.209	0.002	0.191	0.038	0.376
Valeric acid	6.10	7.83	9.65	4.80	8.15	8.63	1.662	0.627	0.036	0.496	0.741
Total BCFA	8.25	9.85	13.06	6.29	9.64	10.28	1.756	0.256	0.029	0.285	0.881
Total VFA	109	99	107	104	105	112	5.8	0.719	0.917	0.242	0.331

[1]HCP, high crude protein; LCP 1, low crude protein using all essential amino acids except Arg; LCP 2, low crude protein using 6 essential amino acids (Lys, Met, Thr, Trp, Val, and Ile).

[2]San, main effect of sanitation; CP, main effect of crude protein levels (HCP vs. LCP 1 and LCP 2); AA, main effect of crystalline amino acids supplementation patterns (LCP 1 vs. LCP 2); San × CP, interactive effect of sanitation and crude protein levels (HCP vs. LCP 1 and LCP 2).
[3]Branched chain fatty acids = isobutyrate + isovalerate + valerate.

7.4.2. MICROBIAL METABOLITES IN JEJUNUM AND COLON

A greater ($P < 0.05$) jejunal ammonia N concentration was measured in pigs housed in USC than those in CSC (Table 7.1). Pigs fed LCP diets had reduced ($P < 0.05$) jejunal ammonia N compared to those fed the HCP diet, however, no difference was found in colonic ammonia N in weaned pigs. Decreased ammonia N concentrations in the pig intestine as a result of reducing dietary protein level have been demonstrated in previous studies (Nyachoti et al., 2006; Heo et al., 2008; Opapeju et al., 2009a). The relatively high SEM might explain the lack of differences in colonic ammonia N in the present study.

Both dietary origin carbohydrates and proteins and host-derived proteins are utilized for microbial fermentation (Egert et al., 2006). Carbohydrates are the preferred substrates for bacterial fermentation, and protein fermentation occurs when fermentable carbohydrates have been used up (Ouwehand et al., 2005). Carbohydrate fermentation yields health-promoting metabolites such as acetate, propionate, and butyrate, whereas protein fermentation produces toxic components, including ammonia and phenolic and indolic compounds (Scheppach, 1994; Egert et al., 2006). The BCFA, which includes isobutyrate, isovalerate, and valerate, are metabolites produced by the deamination of branched-chain AA (**BCAA**) such as leucine, valine, and isoleucine (Rasmussen et al., 1988). Although there were no differences in diversity of microbial composition, VFA and biogenic amine concentrations were changed by different sanitary conditions, CP content, or CAA supplementation patterns in the current study. Similar results were found in previous work by van Zanten et al. (2014) in which dietary intervention of symbiotics in humans did not affect microbiota diversity but increased BCFA concentrations in fecal samples. However, the reason for this result has not been clearly identified. The ratio of acetate:propionate:butyrate is reported to be 3:1:1 (Cummings, 1981; Scott et al., 2013), which is similar to the ratio found in jejunal and colonic

digesta in this study. Different sanitary conditions affect jejunal VFA concentrations, whereas CP content and CAA supplementation patterns did not influence VFA concentrations in jejunal digesta in weaned pigs, except that propionic acid concentration was higher ($P < 0.05$) in pigs fed the LCP 2 diet than those fed LCP 1 diet. Higher ($P < 0.05$) propionic acid concentrations were found in pigs raised under USC, whereas pigs raised under CSC showed higher ($P < 0.05$) butyric acid concentrations in jejunal digesta of weaned pigs. Cho et al. (2020) and te Pas et al. (2020) reported that butyrate concentrations decreased in the colon digesta or feces when pigs were raised in low sanitary conditions, and this is consistent with the current result showing a higher butyric acid concentration in the pigs housed under the CSC. However, contrasting results were observed for propionic acid concentration of pigs housed under the USC, which could be explained by the so-called "hygiene hypothesis" (Strachan, 1989). The "hygiene hypothesis" is a concept that lack of exposure to infectious agents may be the culprit for the increased immune-mediated disease prevalence, and microbial exposure helps animals to develop healthy immune systems and intestinal microbiota (Stiemsma et al., 2015). Montagne et al. (2012) postulated poor sanitary conditions led to ecosystem modification which could be considered more beneficial such as more *Lactobacillus* or increased VFA in the intestine of pigs. Higher ($P < 0.05$) BCFA concentrations were obtained in piglets housed in USC compared to those in CSC, which was in accordance with a previous study by Cho et al. (2020). Intestinal concentrations of BCFA are used as indicators for the extent of protein fermentation (Macfarlane et al., 1992). The BCFA is considered one of the harmful metabolites derived from proteolytic fermentation together with ammonia, indoles, and phenols, thus, has been considered a predisposing factor for post-weaning diarrhea (Gao et al., 2019). Therefore, greater BCFA concentrations indicate an adverse effect on the gut health of pigs. Interactions between sanitation and dietary CP content were detected ($P < 0.05$) for jejunal acetate

as well as total VFA, whereby the acetate and total VFA concentrations decreased from HCP to LCP in pigs raised under the CSC but those increased under the USC, indicating that LCP diets were effective to increase acetic acid concentration under USC. The SCFA including acetate, propionate, and butyrate, play an important role in colonocytes as an energy source (Roediger, 1982), inflammatory reactions and immune parameters (Sweeney et al., 2012; Xu et al., 2016), and gut cell proliferation of the host (Daly and Shirazi-Beechey, 2006). For instance, Sweeney et al., (2012) observed a positive relationship between acetic acid concentration and pro-inflammatory cytokine in the colon of pigs fed β-glucan diets. Liu et al. (2017) reported that orally administrated acetic acid suppresses gastric apoptosis and promotes mucin production in mice. Acetic acid takes the highest concentrations among the SCFA, thus, the increased acetic acid concentrations of pigs especially those raised under the USC is more important to ameliorate the immune response derived from the USC.

Although feeding LCP diets increased acetic acid concentrations in the jejunum of pigs housed in USC, higher ($P < 0.05$) acetic acid concentrations in colon digesta were observed in pigs fed the HCP diet regardless of the sanitary conditions, which is in agreement with the previous studies by Hobbs et al. (1996) and Nyachoti et al. (2006). However, this finding contrasts with the previous report by Qiu et al. (2018) that LCP diets had higher acetate and butyrate concentrations in ileal digesta of growing pigs because of increased amounts of digestible carbohydrates in LCP diets. Compared to the differences in the amount of corn (increased 12% from HCP to LCP) and soybean meal (decreased 12% from HCP to LCP) in the previous study by Qiu et al. (2018), the differences in the amount of corn (increased 8% from HCP to LCP diets) and soybean meal (decreased 6% from HCP to LCP diets) were less in the current study, thereby resulting in a less difference in digestible carbohydrates between HCP and LCP diets and no increase in VFA

concentrations in pigs fed the LCP diets. One possible reason for the increased acetic acid concentration in pigs fed the HCP diet might be the inclusion of spray-dried animal plasma in the HCP diet. Che et al. (2020) reported increased acetate, propionate, and butyrate concentrations, along with an improved bacterial diversity in the colon of pigs fed a diet containing spray-dried animal plasma. Contrary to the results in jejunal digesta, the isobutyric and isovaleric acid concentrations were higher ($P < 0.05$) in CSC than USC in colonic digesta. The reason for this discrepancy is not clear. Moreover, increased ($P < 0.05$) isobutyric and isovaleric acid concentrations were measured in the LCP 2 diet compared to the LCP 1 diet. Suppressed utilization of BCAA for microbial fermentation is expected in the LCP diets, thereby reducing BCFA concentrations (Yu et al., 2019). The BCFA concentrations were reduced in the colonic digesta or feces in pigs fed LCP diets compared to HCP diets in the previous studies (Hobbs et al., 1996; Yu et al., 2019), however, increased ($P < 0.05$) valeric acid as well as total BCFA concentrations were observed in the colon of pigs fed the LCP diets than the HCP diet in the current study. Isobutyric acid, isovaleric acid, and valeric acid are the end products of deamination of leucine, valine, and isoleucine, respectively (Poston, 1976; Langer et al., 2000). The discrepancy between the results of this and previous studies might be due to an imbalance of AA in the LCP diets, which is supported by the plasma AA results of this study (Lee et al., 2022). Higher plasma lysine, methionine, threonine, isoleucine, and valine concentrations were found in pigs fed the LCP diets compared to those of the HCP diet, which could be resulting from the imbalance of ideal protein in LCP diets, leading to more deamination of AA not used for growth and muscle synthesis.

Table 7.2 Effects of crude protein level and sanitary conditions on biogenic amines in jejunal and colonic digesta in weaned pigs[1]

	Clean conditions			Unclean conditions				*P*-values[2]			
Item, mg/g	HCP	LCP 1	LCP 2	HCP	LCP 1	LCP 2	SEM	San	CP	AA	San × CP
Jejunum											
Putrescine	0.10	0.06	0.05	0.12	0.09	0.06	0.031	0.420	0.112	0.554	0.935
Histamine	0.05	0.12	0.04	0.08	0.12	0.03	0.020	0.608	0.419	< 0.001	0.360
Cadaverine	0.41	0.39	0.41	0.34	0.51	0.43	0.128	0.833	0.571	0.796	0.493
Spermidine	0.10	0.08	0.06	0.09	0.10	0.13	0.023	0.176	0.872	0.780	0.152
Tyramine	0.03	0.02	0.00	0.01	0.03	0.03	0.017	0.447	0.922	0.786	0.158
Spermine	0.05	0.05	0.07	0.04	0.05	0.06	0.009	0.528	0.274	0.291	0.637
Tryptamine	0.01	0.01	0.02	0.01	0.02	0.02	0.004	0.395	0.067	0.851	0.402
Colon											
Putrescine	0.13	0.14	0.14	0.14	0.19	0.22	0.034	0.135	0.170	0.732	0.383
Histamine	0.04	0.07	0.04	0.02	0.11	0.05	0.017	0.339	0.026	0.008	0.112
Cadaverine	0.81	1.09	1.31	0.65	1.45	0.97	0.218	0.784	0.016	0.564	0.651
Spermidine	0.29^{ab}	0.24^{ab}	0.20^{b}	0.31^{ab}	0.37^{a}	0.39^{a}	0.035	< 0.001	0.952	0.774	0.031
Tyramine	0.09	0.05	0.02	0.04	0.05	0.03	0.027	0.522	0.300	0.306	0.210
Spermine	0.03^{bc}	0.03^{c}	0.03^{c}	0.04^{bc}	0.05^{ab}	0.06^{a}	0.005	< 0.001	0.085	0.221	0.007
Tryptamine	0.03	0.04	0.03	0.03	0.04	0.03	0.005	0.675	0.240	0.005	0.554

[1]HCP, high crude protein; LCP 1, low crude protein using all essential amino acids except Arg; LCP 2, low crude protein using 6 essential amino acids (Lys, Met, Thr, Trp, Val, and Ile).

[2]San, main effect of sanitation; CP, main effect of crude protein levels (HCP vs. LCP 1 and LCP 2); AA, main effect of crystalline amino acids supplementation patterns (LCP 1 vs. LCP 2); San × CP, interactive effect of sanitation and crude protein levels (HCP vs. LCP 1 and LCP 2).

[a,b]Within a row, means with different superscripts differ ($P < 0.05$).

No significant differences were seen in the biogenic amine concentrations in jejunal digesta in weaned pigs, except for lower ($P < 0.05$) histamine in the LCP 2 diet compared to the LCP 1 diet, which is due to the lower inclusion rate of total histidine in LCP 2 diet (0.41%) than LCP 1 diet (0.47%) (Table 7.2). The same result was found in colonic digesta, where lower ($P < 0.05$) histamine concentration was measured in LCP 2 diet compared to LCP 1 diet. However, higher ($P < 0.05$) histamine concentrations were observed in colonic digesta in pigs fed LCP diets compared to the HCP diet. Histamine is produced from histidine by decarboxylation (Jarisch et al., 2015). One possible reason for this result could be that histidine in LCP diets might not have been fully used for protein synthesis due to the imbalance of ideal protein in LCP diets. Interactions were present ($P < 0.05$) for sanitation and dietary CP content in spermidine and spermine concentrations in colonic digesta, whereby there was increased spermidine and spermine concentrations in pigs fed LCP diets than HCP diet under the USC, whereas those concentrations did not differ between LCP and HCP diets under the CSC. Spermidine and spermine are polyamines derived from the decarboxylation of ornithine and N-rich AA such as glutamine, asparagine, and arginine (Ramani et al., 2014). The first step of polyamine biosynthesis is the decarboxylation of ornithine into putrescine, then putrescine is turned into spermidine by the addition of an aminopropyl group via spermidine synthase, and then spermine is derived from spermidine by spermine synthase (Ramani et al., 2014). Therefore, similar results were observed between spermidine and spermine concentrations. Polyamines, including spermidine and spermine, play an essential role in rapidly dividing immune cells such as the proliferation and differentiation of lymphocytes (Li et al., 2007a) or promotion of T-cell development (Carriche et al., 2021). Increased spermidine and spermine concentrations in pigs housed in the USC room in this research might be due to activation of the immune system. However, studies by Cao et al. (2017) and Liu et al. (2020a) showed that spermine

supplementation alleviates inflammatory response, enhances the immune and ileal barrier function, and maintains large intestinal microbial homeostasis in piglets. Thus, an increment of those polyamines in pigs fed LCP diets under USC indicates that feeding LCP diets to weaned pigs may ameliorate inflammation induced by USC, which is supported by the results showing greater plasma anti-inflammatory cytokine (interleukin-10) concentrations in pigs fed the LCP diets than those fed the HCP diet under USC (Lee et al., 2022).

7.5. CONCLUSIONS

In conclusion, feeding LCP diets reduced proteolytic fermentation regardless of the sanitation conditions, as indicated by lower ammonia N content in the jejunal digesta compared to those fed the HCP diet. Increased acetic acid concentrations and spermidine and spermine concentrations in piglets fed LCP diets under USC indicate that LCP diets might be effective in increasing beneficial VFA and alleviating inflammatory response under USC. However, LCP diets with different CAA supplementation patterns did not affect bacterial diversity in weaned pigs, regardless of the hygienic conditions where the animals were housed.

8. GENERALL DISCUSSION

Various estimates of SID Lys requirements could be obtained depending on the response criteria, statistical method, and experimental diet used. The SID Lys requirement for maximum growth of 7- to 15-kg weanling pigs ranged from 1.27 to 1.30% for ADG and 1.27 to 1.43% for G:F using linear and quadratic broken-lines, respectively, thus giving an average value of 1.32% in Manuscript I. Higher Lys requirement values were commonly obtained based on feed efficiency compared to those based on weight gain (Baker et al., 2002; Nemechek et al., 2012; Zhou et al., 2019), which was also found in the current study. This is because once dietary Lys concentration exceeds the requirement point, the growth rate remains constant, however, feed consumption decreases, resulting in a higher Lys requirement estimate for feed efficiency than for weight gain (Baker et al., 2002). One-slope and quadratic broken-lines are the most widely used statistical models in nutrient requirement studies. The requirement using one-slope broken-line analysis typically shows a lower estimate than the requirement derived from the quadratic broken-line model. The limitation of the current SID Lys requirement study is that while SID Lys concentration increased, contents of CP and other AA remained constant in the experimental diets. Growth performance in pigs fed the 1.64 and 1.80% SID Lys diets could be limited in the current study because of deficient nitrogen content for non-essential AA synthesis. Thus, the SID Lys requirements could be higher when enough nitrogen was provided in the diets to synthesize non-essential AA. Unlike the present study, Kahindi et al. (2017a) provided sufficient nitrogen in the experimental diets, thus, this could be a reason why the SID Lys requirements from the current study were slightly lower than the values obtained by Kahindi et al. (2017a). Another factor affecting the Lys requirement values is the pig genotypes. Most of AA requirement studies were conducted with crossbred pigs [e.g., (Yorkshire × Landrace) × Duroc], however, a verification of

requirement values for recent genotypes is necessary due to continuous genetic development in pigs. Piglets used for the current study were offspring of TN Tempo boars mated to TN70 females (Topigs Norsvin). The TN Tempo boar is developed as a synthetic breed of Yorkshire and Piétrain and the TN 70 is an F1 sow which is a crossbred between Landrace and Large White (Yorkshire), and these genotypes were developed about 10 years ago which could be considered as a recent breed. Different growth rates and Lys requirement values were previously observed depending on pig genotypes due to different genetic potential for protein deposition in pigs (Bikker et al., 1994; Schneider et al., 2010). However, the SID Lys requirements determined in this study were similar to the estimates from a previous study in which 3-way crossbred pigs were used (Kahindi et al., 2017). One possible reason why there was no significant difference between the current and previous studies might be because the growth performance of nursery pigs might not be different between the piglets from Duroc or Piétrain boars, which was the main difference in genotypes. This can be supported by the work of Edwards et al. (2006) that there was no difference between Duroc- and Piétrain-sired pigs in BW before 10 weeks of age.

Weaned pigs are typically fed HCP diets to resolve poor performance after weaning. However, providing a diet with high CP causes protein maldigestion and increases the amount of undigested protein used for protein fermentation, thus increasing the proliferation of pathogenic bacteria, which is a predisposing factor for post-weaning diarrhea (Opapeju et al., 2008). For this reason, LCP diets with supplementation of CAA have been utilized to enhance gut health without growth retardation in weaned pigs (Opapeju et al., 2008, 2009a). Previous studies proved that dietary CP contents could be reduced by approximately 4% units in diets to maintain a healthy gut by reducing PWD without impaired growth performance in weaned pigs (Nyachoti et al., 2006; Heo et al., 2008, 2009).

It was hypothesized that feeding a LCP diet with RS supplementation would have synergistic effects to show better gut health and thereby improve growth performance in weaned pigs in Manuscript II. However, there was no interaction between CP content and RS supplementation on growth performance in Manuscript II, and the major limitation of this result was the use of whey permeate in experimental diets instead of whey powder. In all experimental diets, AA and CP contents were reduced by using whey permeate, and especially a more severe AA deficiency was found in the LCP diets. Therefore, the LCP diets could not meet the requirements of significant AA, including lysine, methionine, and threonine, and poor growth performance in pigs fed LCP diets was observed in week 3 and the overall period than those fed HCP diets. For this reason, it would be interesting to repeat this study using accurate ingredient composition in diet formulation, and the diet should meet the requirements for all indispensable AA to carefully formulate LCP diets. Unlike the growth performance data, LCP diets and RS supplementation had beneficial effects on gut health in weaned pigs. Increased ileal VH and VH:CD ratios were observed in pigs fed LCP diets in Manuscript II. The increased ileal VH could be due to reduced ammonia concentrations in ileal digesta derived from feeding LCP diets compared to HCP diets. Moreover, pigs fed RS-supplemented diets showed increased GC density in the ileum of weaned pigs. A similar result was found in a previous study, where by providing a raw potato starch diet tended to increase the number of GC in the jejunum of growing pigs (Nofrarías et al., 2007). These results could be due to increased expression of mucin genes in pigs fed a raw potato starch diet (Zhou et al., 2017). Both LCP and RS-supplemented diets decreased fecal scores in Manuscript II. Feeding LCP diets decrease the fermentation of undigested protein and proliferation of pathogenic bacteria, thereby reducing toxic products such as ammonia and biogenic amines in the intestine of weaned pigs (Pieper et al., 2012; Gilbert et al., 2018). Resistant

starch supplementation in nursery diets is also known to mitigate diarrhea because RS can produce SCFA in the colon, and these SCFA facilitate salt and water absorption via stimulation of sodium-dependent fluid absorption (Yang et al., 2017). There were interaction effects in that RS supplementation increased ileal propionate and colonic butyric acid concentrations in the LCP diets, whereas the HCP-RS diet did not differ from the HCP diet. The reason RS supplementation was effective in the LCP diet but not in the HCP diet could be due to the lack of nutrient substrates for microbial metabolism in the LCP diet. Because protein fermentation produces not only toxic metabolites but also SCFA, feeding HCP diets has the potential to produce more SCFA compared to LCP diets (Macfarlane and Macfarlane, 2012).

The hypothesis in Manuscript III that pigs raised under USC would have poorer growth performance and gut health outcomes compared to those housed under CSC and feeding LCP diets would improve gut health and alleviate immune responses under the USC, was partially accepted. Decreased ADG and G:F were found in pigs raised under USC in week 2, however, compensation was observed for growth in week 3 in the same group. Therefore, there was no difference between CSC and USC in growth performance over the entire experimental period. This might be explained by the beneficial effects of FMT in pigs raised under USC. Previous studies showed exogenous FMT from adult pigs to newborn piglets increased ADG or G:F in piglets (McCormack et al., 2019; Qi et al., 2021). However, there was a difference in how pigs were exposed to feces between this (sanitary conditions) and previous studies (oral inoculation of feces). More studies are needed to investigate the effects of FMT on growth performance and gut health in weaned pigs using different sanitation models. Feeding LCP diets would have improved gut health and alleviated immune responses in pigs raised under the USC in Manuscript III. For intestinal morphology, pigs fed the LCP diet had longer jejunal villi and a higher VH:CD ratio than those fed the HCP diet,

which agreed with the findings of Opapeju et al. (2009a). However, there were no interaction effects of protein content and sanitary conditions on jejunal histomorphology in weaned pigs. In terms of the immune response, it was hypothesized that LCP diets would ameliorate the inflammatory responses of pigs, especially under USC, by decreasing pro-inflammatory cytokines such as TNF-α and increasing anti-inflammatory cytokines such as IL-10. Pigs raised under USC had greater plasma IL-10 and IL-6 concentrations on days 14 and 21 and decreased plasma TNF-α concentration on day 14. Also, LCP diets resulted in a greater plasma IL-10 concentration in weaned pigs on days 14 and 21, which could be partially explained by the down-regulation effect of LCP diets on gene expression of pro-inflammatory cytokines (Zhang et al., 2017), thus increasing anti-inflammatory cytokines like IL-10. Unlike the observation on cytokine concentrations in blood, no differences were observed among treatments in the mRNA gene expression of IL-6, IL-8, IL-10, and TNF-α in the jejunum. This discrepancy could be due to different expression rates of each cytokine gene in various organs (Gourbeyre et al., 2015). For example, Gourbeyre et al. (2015) reported that IL-10 and TNF-α genes showed higher expression in lymphoid organs than in the jejunum. Thus, the sample collection location for gene expression analysis should be thoughtfully considered in future studies. Different CAA supplementation patterns did not influence the growth performance or gut health in weaned pigs in the present investigation.

High diversity for microbial structure is often observed in pigs raised in CSC (te Pas et al., 2020), however, different sanitary conditions did not modulate colonic bacterial composition or diversity in weaned pigs in Manuscript IV. One possible reason would be that intestinal microbial shifts in pigs are correlated with the growth performance of pigs (Kim and Isaacson, 2015), and the body weights of weaned pigs on day 21 did not differ between different sanitary conditions,

thus, no significant difference was observed in microbial diversity in Manuscript IV. Although previous studies demonstrated that the primary factor that can modulate the gut microbiota could be the amounts of available substrates for bacteria fermentation (Blaut and Clavel, 2007), dietary CP content also did not influence the microbial composition or diversity in the colon of weaned pigs. A previous study reported a similar result that the colonic bacterial community was not changed in weaned pigs fed diets containing 20, 17, and 14% CP and explained that this might have been due to a lack of fermentable carbohydrate sources (Yu et al., 2019). Feeding LCP diets reduced proteolytic fermentation regardless of the sanitary conditions, which was shown by reduced jejunal ammonia N in pigs fed LCP diets compared to those fed the HCP diet. Increased acetic acid concentrations and spermidine and spermine concentrations in piglets fed LCP diets under USC indicate that LCP diets might effectively increase beneficial VFA and ameliorate inflammation response under USC. Different CAA supplementation patterns did not affect the intestinal bacteria composition and metabolites in weaned pigs.

9. CONCLUSIONS AND FUTURE STUDIES

9.1. CONCLUSIONS

The following conclusions can be drawn from the results of the present research:

1. The SID Lys requirement for optimal growth performance of 7- to 15-kg weanling pigs ranged from 1.27 to 1.30% for daily weight gain and 1.27 to 1.43% for feed efficiency using linear and quadratic broken-lines, respectively, thus giving an average value of 1.32%.
2. Feeding LCP diets reduced feed efficiency during a 3-week experimental period because LCP diets caused deficiencies of indispensable or dispensable AA. LCP diets should be carefully formulated to meet all indispensable AA requirements and to provide enough nitrogen content.
3. Lowering dietary protein content decreased the incidence of diarrhea, enhanced development of small intestinal morphology, and improved immune response by increasing expression of plasma anti-inflammatory cytokine in weaned pigs.
4. Resistant starch supplementation in combination with the LCP diets increased the concentrations of the beneficial microbial metabolites, including propionate and butyric acid, in the intestines of weaned pigs.
5. Poor sanitary conditions impaired growth performance in week 2 of the experimental period, however, weaned pigs could compensate for the lost growth in the subsequent week. These positive effects on growth might be due to exposure to a beneficial microbiome through fecal microbiota transplantation under unclean conditions.
6. Crystalline amino acids supplementation patterns did not affect growth performance, gut histomorphology, or immune response in weaned pigs.

7. Increased acetic acid concentrations and spermidine and spermine concentrations in piglets fed LCP diets under poor sanitary conditions may indicate that LCP diets might be effective in increasing beneficial microbial metabolites and ameliorating inflammatory responses in pigs under poor sanitation.
8. Dietary protein content, sanitary conditions, or crystalline amino acid supplementation patterns did not change the colonic bacterial composition and diversity in weaned pigs.

9.2. FUTURE STUDIES

Low protein diets have been applied to alleviate diarrhea in young pigs after weaning. The present research demonstrates that LCP diets might offer a means of supporting a healthy gut in weaned pigs. However, future studies are required to:

1. Determine the effects of transplantation of fecal microbiota on growth performance and gut health in weaned pigs using clean and dirty sanitation models instead of previously used oral inoculation.
2. Examine the optimal time point of digesta or blood collection for intestinal microbiota or immune response analyses in pigs.
3. Expand the size and duration of the growth performance study to test how LCP can be applied on commercial farms.
4. Examine the economic cost and effects of different crystalline amino acid supplementation patterns related to the productivity of nursery pigs in large-scale swine production.

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