Nutritional Strategies to Promote the Health of Weaned Piglets: An Evaluation of Dietary Supplementation with Functional Amino Acids

by

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ABSTRACT

Functional AA (FAA), the need for which dramatically increases in response to immune response, are mobilized through the degradation of muscle protein. Dietary FAA supplementation has been hypothesized to enhance the immune system or fortify the health of weaned piglets, thereby improving protein accretion and growth performance. The objective of this study was to evaluate the efficacy of dietary FAA supplementation in weaned pigs in relation to different weaning stressors. In Experiments 1 and 2, dietary Thr supplementation was evaluated in relation with diet complexity. Dietary $_{L}$-Thr supplementation enhanced the intestinal morphology (villus height and number of goblet cells) and microbial metabolites in the gut (short-chain fatty acid concentration), as well as body protein mass. The benefits of supplementation were greater with a simple diet than with a complex diet. However, the inflammation (the increase in interleukin-6 and interleukin-10) caused by feeding a simple diet could not be modulated with $_{L}$-Thr supplementation. In Experiment 3, dietary sulfur amino acids supplementation was investigated in pigs under oxidative and inflammatory status that were induced by a lipopolysaccharide challenge. Dietary $_{DL}$-Met supplementation at 0.1% failed to restore the intestinal morphology, permeability, and redox status impaired by a lipopolysaccharide (LPS) challenge, but it improved the hepatic glutathione levels and inflammatory responses. By contrast, dietary $_{L}$-Cys supplementation at 0.1% improved intestinal morphology, permeability, and redox status of LPS-challenged pigs, restoring them to a level comparable to the sham-challenged control. Supplementation with combined $_{DL}$-Met and $_{L}$-Cys showed antagonistic effects on the inflammatory response, intestinal morphology, and redox status. Experiment 4 explored the effect of dietary Val supplementation on inflammatory status and intestinal microbial composition in weaned pigs raised in low or high

sanitary condition. Dietary L-Val supplementation beneficially modulated intestinal bacteria composition and microbial metabolites in the gut and suppressed a systemic inflammatory response, regardless of the sanitary condition. In Experiment 5, the supplementation of an AA blend, including Met, Thr, Trp, Val, and Ile, throughout a five-week nursery production period enhanced growth performance for phases I and III. However, the withdrawal of supplementation after phase I led to a lower gain to feed ratio compared with the continuous supplementation. Overall, dietary FAA supplementation enhanced the immune system or fortified the health of weaned piglets, thereby improving protein accretion and growth performance. Dietary FAA supplementation to a weaner pig diet can be a beneficial nutritional strategy to cope with post-weaning stress (e.g., dietary stress, oxidative stress, inflammation, and poor sanitary condition) by enhancing intestinal integrity and barrier function, antioxidant defense system, and N utilization and by modulating the microbial profile and metabolites in the gut.

DEDICATION

This thesis is dedicated to my pillars of support: my parents, Jawon Koo and Jongmi Kim; my older brother, Bonseok; my late sister, Bonhee, who will remain forever in my heart;

and especially

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This thesis was prepared following a manuscript format and is composed of separate journal manuscripts. All manuscripts were written according to the guidelines of the *Journal of Animal Science* for manuscript preparation. Manuscripts I and II were published in the *Journal of Animal Science*; Manuscript III was submitted to *Amino Acids* journal; and Manuscripts IV and V were submitted to *Translational Animal Science* and the *Journal of Animal Science*, respectively.

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4EBP-1	Eukaryotic translation initiation factor 4E-binding protein 1
ADF	Acid detergent fiber
AA	Amino acid(s)
ADFI	Average daily feed intake
ADG	Average daily gain
Akt	Protein kinase B
APP	Acute-phase protein
ATTD	Apparent total tract digestibility
BCAA	Branched chain amino acid(s)
BW	Body weight
CAT1	Cationic amino acid transporter 1
CBS	Cystathionine β-synthase
CC	Challenged control
CD	Crypt depth
CSE	Cystathionine gamma-lyase
CYS	CC + 0.1% $_{L}$-Cys
CySS	Cystine
DE	Digestible energy
$_{DL}$-HMTBA	$_{DL}$-2-hydroxy-(4-methylthio)butanoic acid
DM	Dry matter
EAA	Essential amino acid(s)
EE	Ether extracts
ETEC	Enterotoxigenic *Escherichia coli*
FAA	Functional amino acid(s)
FD4	Fluorescein isothiocyanate dextran – 4kDa
G:F	Gain to feed ratio
GAPDH	Glyceraldehyde 3-phophate dehydrogenase
GC	Goblet cell
GE	Gross energy
GIT	Gastrointestinal tract
GSH	Glutathione
GSR	Glutathione-disulfide reductase
GSSG	Oxidized glutathione
HS	High sanitary
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist

ISS	Immune system stimulation
KRB	Krebs Ringer buffer
LAT1A	L-type AA transporter 1A
LNAA	Large neutral amino acids
LPS	Lipopolysaccharides
LS	Low sanitary
MAT	Methionine adenosyltransferase
ME	Metabolizable energy
MEF2A	Myocyte enhancer factor 2A
MET	Challenged control + 0.1% $_{DL}$-Met
MET+CYS	CC + 0.1% $_{DL}$-Met + 0.1% $_{L}$-Cys
mTOR	Mammalian target of rapamycin
MyoD1	Myoblast determination protein 1
MyoD88	Myeloid differentiation primary response 88
MyoG	Myogenin
NDF	Neutral detergent fiber
NE	Net energy
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthetase
Nrf2	Nuclear factor erythroid 2-related factor 2
NSP	Non-starch polysaccharides
P70	Ribosomal protein S6 kinase
PBS	Phosphate buffered saline
PM	Particle matter
PR39	Proline-arginine-rich 39-amino acid peptide
PUN	Plasma urea nitrogen
SAA	Sulfur-containing amino acids
SAM	S-adenosylmethionine
SCC	Sham-challenged control
SCFA	Short chain fatty acids
SID	Standardized ileal digestible
sIgA	Secretary immunoglobulin A
SOD	Superoxide dismutase
ST	Salmonella enterica serovar typhimurium
STD	A diet containing standard levels of amino acids
STD Thr	100% of NRC Thr requirement
STD Val	A diet containing standard level of valine
STD35	Feeding of STD diet for 35 days
SUP	A diet supplemented with amino acid blend
SUP Thr	115% of NRC Thr requirement

SUP Val	A diet supplemented with L-Val
SUP21	Feeding of SUP diet for phase II
SUP35	Feeding of SUP diet for phase III
SUP7	Feeding of SUP diet for phase I
TEAC	Trolox equivalent antioxidant capacity
TEER	Transepithelial electrical resistance
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
UPS	Ubiquitin proteasome system
VFA	Volatile fatty acid(s)
VH	Villus height
WBC	White blood cells
ZO	Zonula occludens

1. GENERAL INTRODUCTION

Amino acids (**AA**) are one of the most actively researched topics in swine nutrition because of their nutritional and economical significance (Stein et al., 2007; Wu, 2018). AA are the building blocks of body proteins that are mostly reserved in the skeletal muscle which eventually becomes pork, the final product of the pig industry (D'Mello, 2003). Feed cost accounts for the largest proportion of the total cost in pork production and dietary AA is the second most expensive component in swine diet (Manitoba Agriculture, 2016). Thus, many efforts have been made to optimize dietary AA content for the maximum performance of pigs. However, the metabolism of AA is not always constant but changes depending on the physiological state, which suggests that the standard AA requirement is not applicable to pigs under an abnormal physiological state such as an inflammatory state (Obled, 2003; Le Floc'h et al., 2004). This is relevant to weaned pigs because their immune system is likely to be stimulated by various weaning stressors (Heo et al., 2013; Moeser et al., 2017). Previous studies have found that sanitary degradation and enterotoxigenic *Escherichia coli* (**ETEC**) infection, which are common in commercial nursery pig production, can increase the AA requirement for optimal growth and immune response (Jayaraman et al., 2015; Jayaraman et al., 2017b; Kahindi et al., 2017a). Immune system stimulation (**ISS**) diverts AA metabolism from protein anabolism (growth) to specific protein synthesis (e.g., acute-phase protein [**APP**]) and AA metabolites (e.g., nitric oxide [**NO**] and kynurenine) that are associated with immune response (Obled, 2003; de Ridder et al., 2012; McGilvray et al., 2019; Rakhshandeh et al., 2019). AA with a metabolism that dramatically increases in response to immune response are commonly called functional amino acids (**FAA**) (Wu, 2010). The increased need for FAA is supplied through the degradation of skeletal muscle, which is an AA reservoir (Attaix et al., 2005). In this regard, there is growing interest in the use of FAA for weaned pigs to

enhance their immune system and to minimize the loss of skeletal muscle (Le Floc'h et al., 2018). According to the documented biological roles of FAA, Arg, Gln, Trp, Thr, branched chain amino acids (**BCAA**), and sulfur-containing amino acids (**SAA**) can be considered FAA for weaned pigs because they modulate inflammation, oxidative stress, and gastrointestinal health (Wu, 2009, 2010; Le Floc'h et al., 2018). However, nursery pig studies seem to remain stagnant because they are usually limited to research on Arg and Gln. Furthermore, little information is available on the efficacy of FAA against different post-weaning stressors. There are no studies available on whether dietary FAA can improve or maintain growth performance during the nursery production period, although this is a major interest in the swine industry. Therefore, previous literature was reviewed to understand the standard requirements and its limitation for nursery pigs and the biological roles of FAA. FAA were carefully chosen for the post-weaning stressor and experiments were designed with the main hypothesis that dietary FAA supplementation would enhance the immune system or fortify the health of weaned piglets, thereby improving protein accretion and growth performance. The following specific hypotheses were formulated in each study:

i) Dietary Thr supplementation ameliorates the negative effects of a simple diet on the intestinal health and inflammatory state of weaned pigs.

ii) Dietary SAA supplementation attenuates the oxidative stress and inflammation caused by lipopolysaccharides (**LPS**) injection in weaned pigs.

iii) Dietary Val supplementation alleviates pathogen loading and inflammation in weaned pigs housed under poor sanitary conditions.

iv) Dietary supplementation with an FAA blend improves AA utilization and growth performance of weaned pigs

The main objective of this study was to evaluate the efficacy of dietary FAA supplementation in weaned pigs in relation to different weaning stressors. To achieve the main objective, the following specific objectives were formulated:

i) To investigate the effects of diet complexity and Thr supplementation on N utilization, immune response, and gut health.

ii) To evaluate the interactive effects of dietary Met and Cys supplementation in response to LPS injection.

iii) To examine the effects of dietary Val supplementation on intestinal bacteria composition, microbial metabolites, and antimicrobial peptide expression in response to poor sanitary condition.

iv) To investigate the effects of FAA blend supplementation with different feeding regimens on growth performance and N utilization.

2. LITERATURE REVIEW

Amino Acids Requirement and Functional Amino Acids for Nursery Pigs: A Review

2.1. ABSTRACT

Optimizing dietary AA contents for nursery pigs is economically and nutritionally essential. Models have been established to simulate the essential AA requirement curve for the lifetime of pigs in a dynamic way. In principle, the models use the factorial method to sum up the components of AA utilization, including maintenance, growth, and AA metabolic efficiency. However, the AA requirement for nursery pigs is estimated differently because of a lack of information to predict equations of AA utilization. Standardized ileal digestible (**SID**) Lys requirement for nursery pigs is averaged based on estimates from empirical studies. The SID Lys requirement is partitioned into maintenance and growth, and each AA requirement is calculated using the ratios relative to Lys. The AA requirements provided by such a model can be inaccurate in certain conditions where fibrous ingredients are fed, or pigs are immunologically challenged. Immune system stimulation can change the metabolic pathways of dietary AA to cope with the immune system demands by increasing the synthesis of acute phase protein, immune-related proteins, signaling molecules, and immune cells. In an inflammatory state, skeletal muscle degradation is typically activated to provide additional AA for these processes. Thus, a supply of additional AA that possesses specific biological functions associated with the immune system can be a nutritional strategy to maintain the health status of nursery pigs while minimizing muscle protein degradation and improving growth performance. This review discusses the biological functions of Arg, Glu + Gln, Thr, sulfur-

containing AA, Trp, and branched-chain AA, as they are closely associated with weaning stress and pathological status of nursery pigs, and provides future direction toward the successful use of functional AA in coping with common weaning stress.

Key words: amino acid requirement, functional amino acids, immune system stimulation, nursery pigs

2.2. INTRODUCTION

Dietary AA have been an important research topic in swine nutrition for a long time. The content of dietary AA is directly related to profit and loss in the pig industry because it is the second most expensive component in swine diets and the building blocks of proteins in the body, which in turn become pork. Thus, numerous studies have been conducted to estimate AA requirements and evaluate feedstuffs for digestible AA contents. When the AA requirement is estimated for nursery-finishing pigs, growth performance or N retention is traditionally evaluated as the response criterion (NRC, 2012; Kahindi et al., 2017b). However, in the last decade, the functionality of specific AA has received great attention in swine nutrition. Apart from being the building block of proteins, AA are directly and indirectly involved in the immune system and health maintenance (Wu, 2010). Thus, ISS shifts traditional AA requirement estimates based on growth responses (Jayaraman et al., 2017b; Kahindi et al., 2017a; Lee et al., 2021), which supports the hypothesis that the supplementation of specific AA beyond traditional requirements can fortify the immune system or help recover from an inflammatory state. AA supplementation would be more relevant in nursery pigs whose immune system is often compromised by various weaning stressors (Heo et al., 2013; Pluske et al., 2018a). The use of antimicrobial growth promoters for nursery pigs has been banned in many countries due to concerns over the zoonotic transmission of antimicrobial resistance to humans (Vidovic and Vidovic, 2020). Synthetic feed-grade AA at a reasonable price has become more popular, driving the use of FAA in nursery pig diets as a nutritional strategy to enhance the immune system (Le Floc'h et al., 2018). Therefore, this review focuses on the current AA requirements for nursery pigs, ISS in relation with AA metabolism, and comprehensively discusses current knowledge on FAA for nursery pigs.

2.3. AMINO ACID REQUIREMENT FOR NURSERY PIGS

To properly use the values of suggested requirements for diet formulation, it is critical to understand how AA requirements are modeled and the limitations of traditional models. However, despite the active discussion on the AA requirement for growing pigs and sows, the literature discussing AA requirement models for nursery pigs is scant.

AA requirement reflects the amounts of AA that must be included in complete diets for optimal growth and healthy pigs (Wu, 2018). Determining the precise AA requirement is nutritionally and economically important because dietary AA content determines feed cost unit and governs the pigs' overall performance. The AA requirements for pigs continuously change as pigs grow. It is practically impossible to conduct individual experiments to empirically determine AA requirements for the lifetime of pigs. Thus, NRC and InraPorc have established models that use the factorial method with functions that are associated with AA utilization and growth, providing AA requirements in a dynamic way (van Milgen et al., 2008; NRC, 2012). The factorial method adds up all components that contribute to the AA requirement, including protein deposition, integument losses, and basal endogenous gastrointestinal tract (**GIT**) losses, which are estimated in functions (NRC, 2012; van Milgen and Dourmad, 2015). To be specific, daily protein deposition at a specific body weight (**BW**) can be computed from different curves, including standard gender-specific curves, the Gompertz function, and the generalized Michaelis-Menten kinetics function (NRC, 2012). Daily integument losses that indicate AA losses from the skin and hair are estimated as a function of $BW^{0.75}$ (van Milgen et al., 2008; NRC, 2012). Basal endogenous GIT losses indicate proteins secreted in the lumen of GIT but not reabsorbed, including digestive enzymes, mucins, and albumin, as well as sloughed epithelial cells (Nyachoti et al., 1997; Adeola et al., 2016). The amount of basal endogenous GIT losses can be calculated in proportion to the dry

matter (**DM**) intake. When the function is computed in the NRC model, it is assumed that AA losses in the large intestine are 10% of AA losses determined at the ileum (NRC, 2012). Daily AA requirement (g/day) that is computed from the functions can be converted to dietary AA (%) requirement based on the metabolizable energy (**ME**) intake curve (van Milgen et al., 2008; NRC, 2012). The models consider the inefficiency of AA utilization for maintenance and protein deposition, which indicates the minimum and inevitable AA catabolism and "between-animal variation" (Pomar et al., 2003; NRC, 2012; van Milgen and Dourmad, 2015).

Due to insufficient empirical data on the biological relationship between AA utilization and growth, available models do not incorporate nursery pigs. Thus, NRC (2012) refers to empirical studies that estimated Lys requirements at 1.50%, 1.35%, and 1.23% standardized ileal digestible (**SID**) Lys for 6 kg, 9 kg, and 18 kg BW, respectively. The NRC model predicts a regression equation that represents a best-fitting line for SID Lys requirement (% of diet): 1.871 - 0.22 × ln(BW). Using the equations for basal endogenous GIT losses and integument losses predicted for growing pigs, the SID Lys requirement is partitioned into requirements for maintenance and growth (total requirement - maintenance requirement). The requirements for other essential amino acid (**EAA**) and N are then calculated based on ratios relative to the Lys content determined for maintenance and growth (NRC, 2012).

It is worthwhile to note that the AA requirement for nursery pigs estimated by the abovementioned models can differ depending on dietary composition. There is a growing interest in the use of a simplified nursery diet or the use of various alternative fibrous feed ingredients for nursery pigs (Koo et al., 2017). High inclusion of dietary fiber in nursery pig diet can change the traditional ME intake curve and requirement for maintenance by increasing endogenous losses, thereby changing dietary AA requirement (Montagne et al., 2003; Wellington et al., 2018).

Furthermore, although the NRC (2012) provides the AA requirements at total AA and apparent ileal digestible AA levels, these values are back-calculated from the SID AA requirement, assuming that corn-soybean meal containing 3% premix and 0.1% Lys HCl are fed to pigs. Therefore, it is recommended that future research related to FAA studies should formulate diets at the SID levels and provide formulation information to increase reproductivity among studies. Furthermore, because dietary energy content changes the ME intake curve and thus dietary AA requirement (% in diet) (van Milgen, 2002), dietary energy content should be considered together with dietary AA content supported with research results (SID Lys/kcal ME). As discussed above, the AA requirement for nursery pigs is estimated based on the functions determined from data obtained from growing-finishing pigs. Thus, although the AA required for maintenance is not quantitatively substantial, their functions should be verified to confirm that they are a good fit for nursery pigs. Further, as lower dietary protein content for nursery pigs becomes more popular, future empirical studies should verify the model-estimated AA requirement for all individual EAA as well as non-EAA or total N, and the current models should be corrected or remodeled to incorporate the empirical data. Notably, the SID His requirement suggested by NRC (2012) appears to be higher than that estimated by an empirical study by Cemin et al. (2018), who estimated the SID His:SID Lys requirement for 7–11 kg as 31%, which is lower than NRC (2012) requirement. An NRC (2012) requirement for His limits lowering dietary protein content below 21% because feed-grade of L-His is commercially unavailable at the moment. Lastly, it is of utmost relevance that the pathological and immunological state can change AA requirements for nursery pigs whose immune systems are vulnerable to exogenous stressors, which will be discussed below.

2.4. WEANING STRESS AND IMMUNE SYSTEM STIMULATION IN NURSERY PIGS

Piglets are weaned at 18–28 days old, and this process is inevitable in pig production. Weaning is the most stressful event during their lifetime, and physiological changes are inevitable. To be specific, energy and nutrients are supplied by a plant-based diet after weaning. Compared with sow milk, plant-based diets are richer in antinutritional factors such as dietary fiber (Bach Knudsen, 2001). Pigs are separated from sows and their littermates, and they are mingled with new piglets, which leads to rearrangement of hierarchy accompanied by drastic fights for a few days (Campbell et al., 2013). Piglets get also stressed from handling (e.g., transportation) and the new environment (e.g., feeder, rooms, nipple) (Campbell et al., 2013). Thus, weaning usually accompanies anorexia, which results in intestinal dysfunction (Pluske et al., 1997; McCracken et al., 1999). Compromised intestinal barrier function increases the possibility of pathogen loading (Wijtten et al., 2011). Numerous studies revealed that weaning stress often leads to oxidative stress in nursery pigs (Buchet et al., 2017).

The above-mentioned stressors can activate the immune system of weaned pigs. To be specific, dietary antigen consumption and pathogen infection are the typical causes of local inflammation in pigs (Li et al., 1990; Heo et al., 2013; Koo et al., 2017). Soybean meal contain antigenic compounds, including glycinin and β-conglycinin, which activate the immune system by increasing immunoglobulin (**Ig**) production and the lymphocyte proliferations (Sun et al., 2008; Hao et al., 2009). Pathogenic infection is another typical cause of intestinal inflammation. The most common pathogenic infections in nursery pigs are salmonellosis and swine enteric colibacillosis characterized by watery diarrhea (Foley et al., 2008; Luppi, 2017). The respective causal pathogens of these diseases are *Escherichia coli* and *Salmonella* spp. F4+ and F18+ ETEC are the most predominant pathotypes in post-weaning diarrhea (Luppi, 2017). Although

colibacillosis is a major concern in nursery production, salmonellosis caused by infection of *Salmonella enterica*, particularly the Typhimurium (ST) serotype, is widely observed throughout pork production. This is a critical issue in food safety because salmonellosis is zoonotic, suggesting that contaminated pork can cause salmonellosis in humans (Foley et al., 2008). Although the enteric pathogens have different pathotypes and pathogenicity, they all infect intestinal epithelial cells, trigger the host's immune system, and generally lead to watery diarrhea (Luppi, 2017). An adhesin, called EtpA, located on the tip of flagella initiates adherence to host cells. The ETEC then secretes EatA, a serine protease autotransporter of Enterobacteriaceae, hydrolyze the EtpA, and the intimate adherence is created by Tia (outer membrane protein) and TibB (glycosylated autotransporter) (Clements et al., 2012). The main pathology is generated by the influx of heat stable and heat labile toxins. The heat stable and heat labile toxins increase cyclic AMP and GMP, which disrupt the Cl channel and modulate other ion channels and eventually lead to osmotic diarrhea. (Clements et al., 2012).

Similarly, ingested ST attaches to the intestinal epithelial cells and delivers a specialized set of effectors. For example, ST injects salmonella invasion protein into the intestinal epithelial cells, leading to cytoskeletal rearrangement in host cells and disruption of the normal epithelial brush border (Fàbrega and Vila, 2013). The transformed membranes result in epithelial membrane ruffles, which engulf adherent ST in large vesicles, often called Salmonella-containing vacuoles in which ST replicates. After release from the submucosa, the ST are internalized within phagocytes and disseminated to other tissues through the lymph and the bloodstream (Fàbrega and Vila, 2013).

It has been well documented that antigen presenting cells, including macrophage and dendritic cells, recognize exogenous pathogens and initiate the immune response via phagocytosis

(Abbas et al., 2017). To be specific, the model of pathogen-associated molecular pattern recognition is generally accepted to explain the mechanism for microbial detection (Akira and Hemmi, 2003). The antigen presenting cells express numerous surface molecules, which allows them to recognize both endogenous (host derived) and exogenous ligands before engulfment (Abbas et al., 2017). Various receptors that are present on the antigenic presenting cells are able to sensitize the conserved molecules derived from microbes and initiate an inflammatory cascade (Brubaker et al., 2015). For example, CD14 and toll-like receptor (**TLR**)-4 recognize LPS, and CD14 detects LPS-binding protein and mediates transcriptional factors by recruiting myeloid differentiation primary response 88 (**MyoD88**) (Takeuchi and Akira, 2010). Afterwards, MyoD88 recruits interleukin (**IL**)-1 receptor-associated kinase, which eventually activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Takeuchi and Akira, 2010). NF-κB as a transcription factor acts as a central mediator of inflammatory response by inducing various inflammatory proteins, including pro-inflammatory cytokines, adhesion molecules, and associated enzymes (Abbas et al., 2017).

Oxidative stress is generally defined as the imbalance between antioxidants and the production of free radicals (Bhattacharyya et al., 2014a). In a study by Buchet et al. (2017), weaned piglets, regardless of weaning age (21-d or 28-d), showed higher plasma concentrations of hydroperoxide, an oxidative product, and lower antioxidant capacity. The authors also found that the extent of weaning-induced oxidative stress depends on management and conditions at weaning, such as animal density, hygiene, and temperature. Oxidative stress commonly accompanies systemic inflammation (Gessner et al., 2017). The relationship between oxidative stress and the inflammation pathway is poorly understood. So far, it is believed that the TLR recognize damage-

associated molecular pattern molecules that are produced by oxidative stress, which activate the NF-κB signaling pathway and eventually the inflammatory response (Gill et al., 2010).

2.5. IMMUNE SYSTEM STIMULATION AND AMINO ACIDS METABOLISM

Pigs as mammalian animals have an evolved an immune system to cope with exogenous stressors. Once the immune system is activated, marked alteration of metabolic pathways and nutrient utilization follow. The normal growth metabolic pathways are altered to prioritize immune response, which quantitatively and qualitatively shifts the requirements for specific nutrients, particularly AA (Obled, 2003). The noticeable changes in metabolic pathways include the syntheses of APP, immune cell proliferations, and endogenous antioxidants (Obled et al., 2002).

The noticeable response to inflammation is the change in concentrations of specific circulating blood proteins, which are commonly called APP (Parra et al., 2006). The liver is the main organ that synthesizes and releases APP into plasma, and APP synthesis is systemically regulated by inflammatory mediators, such as IL-1, IL-6, glucocorticoids, and growth factors (Parra et al., 2006). The pro-inflammatory cytokines, such as Il-1 and IL-6, upregulate the expression of APP-encoding genes, whereas glucocorticoids and growth factors regulate APP synthesis by modulating cytokine activity (Ceciliani et al., 2002). The synthesized APP contributes to acute phase reaction by being involved in the complement system and coagulation and fibrinolytic system, serving as transport or scavenger proteins, protease inhibitors, and participating in inflammatory response (Ceciliani et al., 2002). APP that quantitatively increase during inflammation are called positive APP, and include C-reactive protein, serum amyloid A, haptoglobin, and pig-MAP. By contrast, APP that quantitatively decrease are called negative APP, including albumin and transferrin (Parra et al., 2006). The immunological roles of down-regulating negative APP are not clearly understood. However, it is commonly believed that the abrupt AA

supplies for positive APP synthesis are in part derived from the degradation of negative APP that are not necessary in immunological defense (Parra et al., 2006).

The other pathway that draws free circulating AA during inflammation is increased immune cell proliferation (e.g., clonal lymphocyte and monocyte differentiation), various molecules secreted by immune cells (e.g., cytokines, Ig), and lymphoid tissue hyperplasia (Le Floc'h et al., 2004). Although the quantity of AA required for these is not substantial for whole-body protein turnover, specific AA (e.g., glutamate) plays a crucial role in immune cell proliferation (Reeds and Jahoor, 2001; Obled, 2003). This will be further discussed later in this review.

Mucins are heavily glycosylated proteins that coat the surfaces of epithelial layers of respiratory, digestive, and urogenital tracts (Perez-Vilar and Hill, 1999). As an innate immunity component, mucins are the main structural components of the mucus layer and play crucial roles in maintaining commensal microbes and protecting the gut against enteric pathogens (Dharmani et al., 2009). Nichols and Bertolo (2008) and Rémond et al. (2009) found that mucosal and mucin protein synthesis in the proximal small intestine increases during acute ileitis. Apart from mucin proteins, intestinal inflammation (e.g., ulcerative colitis) can also upregulate the expression of antimicrobial peptides (Takahashi et al., 2001; Fahlgren et al., 2003; Campbell et al., 2012), reflecting the alteration in AA metabolism.

Another significant expenditure of AA in the inflammatory condition is the synthesis of endogenous antioxidants to cope with oxidative stress in the body. Although the mitochondrial electric transport chain is a major site of free radical production in the body, immune cells, such as phagocytes and monocytes, produce free radicals in the form of bactericidal agents (e.g., $O_2^{\bullet}$ and HOCl) or during the process of producing immune-signaling compounds (e.g., leukotriene and

NO) (Bhattacharyya et al., 2014b). For example, phagocytic cells consume large amounts of oxygen during phagocytosis and rapidly release superoxide (via the activation of NADPH oxidase) into the phagosome to kill bacteria. This process is commonly called the *respiratory burst* (Bhattacharyya et al., 2014b). A tripeptide, γ-glutamyl-cysteinyl-glycine or glutathione (**GSH**), is a key, non-enzyme antioxidant in the body, and its turnover increases in response to ISS. Isotope tracer studies confirmed that as an immune stimulant, LPS accelerates the transsulfuration pathway that converts Met to Cys and increases Cys metabolism, mainly to synthesize GSH in pigs (Stipanuk, 2004; Rakhshandeh et al., 2019).

As a means to save metabolic cost (i.e., anabolic-catabolic balance), pigs tend to source AA from internal stores rather than digesting and absorbing dietary nutrients and energy (Johnson, 1998). These stores are mainly located in skeletal muscle. Indeed, in a pro-inflammatory condition, the production of feed intake-lowering (anorexigenic) peptides is upregulated—mainly in the hypothalamus—while the secretion of orexigenic peptides is downregulated, resulting in appetite reduction (Maletínská et al., 2019). Furthermore, pro-inflammatory cytokines, such as IL-1β, IL-6, and tumor necrosis factor (**TNF**)-α, suppress the contractility of gastrointestinal smooth muscle, thereby reducing its motility and sparing energy and nutrient expenditure for digestion (Akiho et al., 2011). Instead, APP (e.g., serum amyloid A1), glucocorticoids, and pro-inflammatory cytokines, such as TNF-γ, IL-6, IL-1, interferon-γ, (**IFN-γ**), and TNF-like weak inducers of apoptosis, promote proteolysis and suppress protein synthesis in myocytes of skeletal muscle (Haberecht-Muller et al., 2021). Specifically, pro-inflammatory cytokines sensitize receptors on the membrane of myocytes and upregulate the expression of *F-box only protein 32*, thereby activating the ubiquitin proteasome system (**UPS**) (Haberecht-Muller et al., 2021). The UPS, together with proteases such as calpains and caspase, break down myofibrillar proteins into free

AA (Attaix et al., 2005). Furthermore, there is a report that IL-6 increases the activity of proteasome and cathepsin, thereby decreasing the half-life of proteins in murine myotubes (Ebisui et al., 1995). By contrast, pro-inflammatory cytokines activate the protein kinase B (**Akt**) pathway, which in turn suppresses the expression of mammalian target of rapamycin (**mTOR**) and eukaryotic translation initiation factor 4E-binding protein 1 (**4EBP**-1) phosphorylation, thereby decreasing muscle protein synthesis (Haberecht-Muller et al., 2021).

Taken together, traditional AA requirements are not applicable to pigs with activated immune systems because the requirements are determined for optimal growth (protein accretion) under normal physiological conditions, as aforementioned. The requirements for several AA, such as Thr, Trp, and sulfur-containing amino acids (SAA), in ETEC or ST-challenged pigs are elevated for optimal growth performance and immune response (Ren et al., 2014; Capozzalo et al., 2017b; Jayaraman et al., 2017b; Wellington et al., 2019).

2.6. BIOLOGICAL FUNCTION OF AMINO ACIDS AND DIETARY FUNCTIONAL AMINO ACID SUPPLEMENTATION

The AA profile in skeletal muscle substantially differs from that in APP, indicating a shift in limiting AA. For instance, the Lys content in muscle is 98 g/kg, but 6–66% lower in APP, including C-reactive protein, fibrinogen, α-acid glycoprotein, haptoglobin, and amyloid A (Reeds et al., 1994). By contrast, the Trp content in muscle is 13 g/kg, which is approximately 3 times lower than that in APP (Reeds et al., 1994). This reflects that AA supply from the proteolysis of skeletal muscle causes an excess of nonlimiting AA. It is commonly observed that concentrations of plasma urea nitrogen (**PUN**) or protein metabolites increase in the inflammatory state (Webel et al., 1997; Mao et al., 2014b).

Many nutritionists pay attention to the shift in limiting AA in an inflammatory state and hypothesize that dietary supplementation of the limiting AA can dampen inflammation and minimize muscle protein degradation. This strategy seems to be more relevant for nursery pigs, which generally experience ISS during the post-weaning period (Wijtten et al., 2011; Heo et al., 2013).

The term 'FAA' was proposed by Dr. Guoyao Wu (Wu, 2009, 2010), who defined the term as "*AA that participate in and regulate key metabolic pathways to improve health, survival, growth, development, lactation, and reproduction of the organisms.*" Based on this definition, almost all AA can be classified as "FAA" because they have their own distinguishable biologic roles and are essential in physiologic reactions in the body. However, the functional importance of some AA in a specific physiologic condition can be different. Thus, it is suggested that the term FAA be mentioned together with specific physiological conditions or diseases. Following section discusses the functional roles of AA selected based on their relevant biological roles in the immune response of weaned pigs. Table 2.1–2.6 list and describe studies on the effects of dietary FAA on immune response and gut health in nursery pigs.

2.6.1. ARGININE

Arginine can be synthesized via the urea cycle in the liver; however, due to rapid hydrolysis of Arg by arginase, there is no net synthesis of Arg (Wu, 2018). Thus, Glu, derived from the whole body, is converted into Cit in the intestine, which is then metabolized into Arg (Wu et al., 2018). This de novo synthesis is not sufficient to supply Arg for optimal growth of pigs; thus, Arg is regarded as an EAA in pig nutrition (D'Mello, 2003).

Table 2.1 Effects of dietary Arg supplementation on growth performance, metabolism, and immune response in nursery pigs

Reference	Basal diet	Supplementation level	Physiological status	Results[1]
Zhan et al. (2008)	total 0.82%	0.70%	early weaning (5.0 kg)	↑intestinal morphology ↑immunoreactive expression of CD34 ↑immunoreactive expression of vascular endothelial growth factor ↑ NO production
Liu et al. (2008)	total 1.28 %	0.5 or 1.0 %	LPS challenged pigs (5.8 kg)	↑ADG ↑protein and DNA contents in SI ↑intestinal morphology ↑*PPARγ* mRNA expression in SI ↓*IL-6* and *TNF-α* mRNA expression
Li et al. (2012)	N/A	0.50%	LPS challenged pigs (7.5 kg)	↓hepatic TNF-α level ↓mast cells in liver ↓hepatic NF-κB and TLR-4-possitive cells
Han et al. (2009)	total 1.28 %	0.50%	Cyclophosphamide -challenged pigs (9.3 kg)	↑ADG, ADFI, G:F ↑bovine serum albumin antibody ↑serum IL-2 and IFN-γ
Liu et al. (2009)	total 1.28%	0.5% or 1.0%	LPS-challenged pigs (5.8 kg)	↑lactase activity in SI ↑intestinal mucosal DAO activity ↑intestinal SOD activity ↓intestinal MDA content ↑intestinal NO content and iNOS activity
Mao et al. (2012)	total 1.24%	0.5% or 1.0%	normally weaned pigs (7.2 kg)	↑β-defensin mRNA expression in various tissues including SI and muscle
Zhu et al. (2013)	N/A	0.5% or 1.0%	LPS-challenged pigs (5.5 kg)	↑intestinal morphology ↓intestinal mast cell counts ↓intestinal IgA-secreting cell proportion ↓intestinal counts of CD4+ T lymphocytes ↓lymphocyte apoptosis of Peyer's patches

Table 2.1 Effects of dietary Arg supplementation (FAA) on growth performance, metabolism, and immune response in nursery pigs (Continued)

Reference	Basal diet	Supplementation level	Physiological status	Results[1]
Zheng et al. (2013) Zheng et al. (2017)	total 0.95%	0.8 or 1.6%	diquat-challenged pigs (8.7 kg)	↑cortisol concentrations in plasma ↑activities of GPx and SOD and total antioxidant capacity in plasma ↑activities of GPx and SOD and total antioxidants in liver ↓*IL-6* and *TNF-α* mRNA expression in liver ↑intestinal morphology ↑*CAT-1* mRNA expression
He et al. (2011)	total 1.31%	1.00%	weaned pigs (5.4 kg)	↑ADG Restored the serum metabolites that were changed by early weaning
Wu et al. (2015)	N/A	1.00%	Deoxynivalenol-consumed weaned pigs	↑intestinal morphology ↑AA concentrations in serum, jejunum and ileum ↑ *SGLT-1*, *GLUT-2*, and $y^{(+)}$*LAT-1* mRNA expression

[1]NO, nitric oxide; ADG, average daily gain; SI, small intestine; PPARr, peroxisome proliferator-activated receptor gamma; IL, interukine; TNF-α, tumor necrosis factor alpha; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IFN-γ, interferon γ; ADFI, average daily feed intake; G:F, gain to feed ratio; DAO, diamine oxidase; SOD, superoxide dismutase; MDA, malondylaldehyde; iNOS, inducible nitric oxide synthase; Ig, immunoglobulin; GPx, glutathione peroxidase; SGLT, sodium-dependent glucose cotransporter; GLUT, glucose transporter, LAT, L-type AA transporter. N/A, information is not available.

Arg is involved in the syntheses of creatinine and Pro, but the most noticeable Arg pathway is NO production (Wu, 2009). Arg is converted into NO by nitric oxide synthetase (**NOS**), and three types of NOS in different cells impart distinct physiological roles: neuronal NOS, inducible NOS, and endothelial NOS (Bogdan, 2001). NO has versatile functions, and its mechanisms are poorly understood. The most prominent function of NO is the regulation of the dilation of blood vessels as a vasodilator, the majority of which is produced by endothelial cells of blood vessels, controlling blood flows to tissue (Magierowski et al., 2015). The control of blood flow is important in terms of AA metabolism in the body because it regulates the transport of circulating AA (Wu, 2018). In this regard, NO production in nursery pigs is relevant to the maintenance of intestinal integrity in which AA metabolism is critical (Magierowski et al., 2015). Further, NO plays a protective role in the GIT by promoting the repair of damaged tissues and mucus secretion (Lanas, 2008). NO has antimicrobial activity and immunosuppressive effect (Bogdan, 2001). However, NO production can be deleterious when it acts as a cytotoxic molecule, causing oxidative stress (Tripathi et al., 2007).

Research on dietary Arg has focused on sows and lactating piglets because of its effect on mammalian AA metabolism and the lack of capacity to synthesize Arg *de novo* (Wu et al., 2013). It is widely accepted that Arg is an EAA in nursery pigs, but it seems not to be a quantitative concern in a typical corn-soybean meal-based swine diet. Although the NRC (2012) suggested 0.68% for the requirement of SID Arg (45% of SID Lys requirement), Wu (2014) proposed 1.01% (100% of SID Lys requirement). In support of Wu's suggestion, previous studies reported that 0.5–1% Arg supplementation enhances gut health and growth performance in nursery pigs (Table 2.1). Zhan et al. (2008) reported that 0.7% L-Arg supplementation can improve microvascular development in the small intestine by upregulating the expression of angiogenic factors and NO

production in early-weaned pigs with 5.0 kg of initial BW. There is a consensus that dietary Arg supplementation can enhance intestinal morphology (Liu et al., 2008; Zhan et al., 2008; Zhu et al., 2013; Wu et al., 2015; Zheng et al., 2017). Arg supplementation can beneficially modulate the intestinal inflammatory response, possibly by upregulating peroxisome proliferator-activated receptors- γ (Liu et al., 2008), increasing intestinal CD4+ T cells, inhibiting TLR-4 signaling, decreasing Peyer's patch cell apoptosis and mast cell number (Zhu et al., 2013), and enhancing redox status (Zheng et al., 2013). Dietary Arg also fortifies the antioxidant defense system by improving the activities of superoxide dismutase (**SOD**) and glutathione peroxidase in the small intestine, liver, and plasma (Liu et al., 2009; Zheng et al., 2017). Although the mechanism is not clear, the beneficial effects of dietary Arg supplementation on nutrient transporters (Wu et al., 2015; Zheng et al., 2017), antimicrobial peptide production (Mao et al., 2012), and digestive enzymes (Liu et al., 2009) have been reported. Not surprisingly, these benefits of dietary Arg supplementation seem to improve growth performance (Liu et al., 2008; Han et al., 2009; He et al., 2011). However, in a metabolomic study by He et al. (2011), dietary Arg did not restore the microbial metabolites that were altered by weaning stress. This is further supported by Yoon et al. (2020), who showed that dietary Arg supplementation did not alter intestinal bacterial composition. However, the authors found that the combination of Arg with a low dose of Zn (500 ppm) beneficially suppressed *Clostridium* spp. and enhanced growth performance and intestinal integrity to the level with a high dose of ZnO (2500 ppm) supplementation. Zheng et al. (2008) reported that a high dose of Arg supplementation (1.2%) could aggravate weaning stress and intestinal dysfunction, which reflects the importance of an optimal Arg supplementation dose. Surprisingly, no studies have indicated Arg content on an SID basis, although information on SID Arg content is available for all ingredients. Without dietary SID Arg content, it would be difficult

to estimate the optimal levels of dietary Arg. Thus, we recommend providing dietary Arg levels on an SID basis. Furthermore, considering that Arg requirements can be shifted depending on the dietary N content because of the role of Arg in the urea cycle (Milner and Visek, 1978), providing SID N will be informative.

2.6.2. GLUTAMINE AND GLUTAMATE

The vital biological roles of Glu and Gln have been well documented. Glu and Gln are involved in numerous metabolic pathways and biological functions directly or indirectly by serving as precursors of metabolites (Wu, 2018). Given that most dietary Gln is metabolized by the intestine and does not enter the portal vein, Gln is supplied via *de novo* synthesis (Ji et al., 2019). The liver lacks branched-chain amino acid aminotransferase, which allows absorbed dietary BCAA to bypass the liver. The BCAA in blood is then taken up by extrahepatic tissues, mainly in the skeletal muscle, in which the BCAA is transaminated with α-ketoglutarate into branched-chain keto acids and Glu (Zhang et al., 2017). Glu is either amidated with NH_3 by glutamine synthetase into Gln or transaminated with pyruvate and α-ketoglutarate to yield Ala and Asp, respectively. Glu cannot readily cross the cell membrane, so once Glu is converted into Gln, the latter circulates in blood vessels and is taken up by different tissue (Wu, 2018). Thus, the highest concentrations of Gln and Glu are observed outside and inside cells, respectively (Shah et al., 2020). Most of the Gln in cells undergo deamination by glutaminase into Glu and ammonia. This process is critical, particularly in the kidney, for maintaining acid-base balance because ammonia combines with H^+ to produce NH_4^+ and thus urea (Wu, 2009). Apart from this acid-base balance, Gln serves as an energy fuel and precursor for γ-aminobutyric acid, Arg, GSH, and Orn (Wu, 2009). When Glu is transaminated with pyruvate, Ala and 2-oxoglutarate are released, which are then oxidized in the tricarboxylic acid cycle, eventually producing ATP (Shah et al., 2020). Glutamate is decarboxylated by

glutamate decarboxylase into γ-aminobutyric acid, which acts as an inhibitory neurotransmitter and is found at high concentrations in the central nervous system (Diana et al., 2014). Further, γ-aminobutyric acid is produced in immune cells, including T cells and macrophages, suppressing pro-inflammatory responses by blocking Ca signaling and NF-κB activity (Hodo et al., 2020). Glu is converted into γ-glutamyl-cysteine, a precursor of GSH that is a critical intracellular antioxidant (Shah et al., 2020). The biological importance of GSH will be discussed below, along with the discussion of SAA. Despite not being quantitatively substantial, Gln serves as a precursor for proteins, including glycoproteins, purines, pyrimidines, and NAD(P), and cannot be replaced with other AA in these molecules (Wu, 2018).

The roles of Glu and Gln in the GIT have been extensively researched (Ji et al., 2019). Approximately 96% of Gln in the intestinal lumen is metabolized in the intestine (Wu, 2018). Furthermore, the intestine takes up a quarter of the plasma Gln, suggesting the significance of Gln in the intestine (Wu, 2018). Gln is quantitatively the most significant energy fuel in the intestine and is also involved in maintaining enterocyte proliferation and tight junctions by regulating mitogen-activated protein kinases and augmenting epidermal growth factor, insulin-like growth factor-I, and transforming growth factor-α (Kim and Kim, 2017). In this regard, numerous studies have focused on the efficacy of dietary Gln supplementation in intestinal health. There is general agreement that dietary Gln supplementation can enhance intestinal morphology (Wu et al., 1996; Liu et al., 2002; Lee et al., 2003; Domeneghini et al., 2006; Shan et al., 2012; Rezaei et al., 2013; Wang et al., 2015a).

Studies by Ewaschuk et al. (2011) and Wang et al. (2015a) reported that dietary Gln supplementation (4.4% or 1%) can upregulate the expressions of tight junction proteins, including occludins, claudins, and zonula occludens (**ZO**) and the genes encoding these proteins.

Table 2.2 Effects of dietary Gln supplementation on growth performance, metabolism, and immune response in nursery pigs

Reference	Basal	Supplementation level	Physiological status	Results[1]
Wu et al. (1996)	?	1.00%	Weaned pigs	↑ G:F ↑intestinal morphology
Wang et al. (2008)	total 3.36% (Glu + Gln)	1.00%	Weaned pigs (5.9 kg)	↑gene expression related with cell growth and removal of oxidants ↓gene expression related with promoting oxidative stress and immune activation ↑GSH concentrations in jejunum ↓GSSG concentration in jejunum
Liu et al. (2002)	?	1.00% $_{L}$-Glu or 1.00% $_{L}$-Gln	Weaned pigs	↑intestinal morphology ↑RNA concentration in skeletal muscle ↑$_{D}$-xylose concentration in serum
Xiao et al. (2012b)	total 3.97% (Glu + Gln)	1.00%	Weaned pigs (5.6 kg)	↑metabolism of carbohydrates, Pro, Tyr, and glycerophospholipids ↑Ala aminotransferase and hexokinase ↓pyruvate kinase activity
Ewaschuk et al. (2011)	?	4.40%	ETEC-challenged in situ Weaned pigs	↑OCLN protein expression in intestinal mucosa ↓potential difference and short-circuit current in intestinal loops
Lee et al. (2003)	total 3.70% (Glu + Gln)	0.50%	Weaned pigs (6.0 kg)	↓small intestine:empty carcass weight ↑intestinal morphology ↑IgA and protein in the bile
Zhong et al. (2012)	?	orally 1 g/kg BW per 12 h	21-d-old piglets with intrauterine growth retardation (3.8 kg)	↑ADG ↓IL-1 and IL-8 in serum and jejunum ↑IL-4 and IL-10 in serum and jejunum ↑Hsp70 and I-κB gene expression in jejunum ↓p65 gene expression in jejunum and ileum

Table 2.2 Effects of dietary Gln supplementation on growth performance, metabolism, and immune response in nursery pigs (Continued)

Reference	Basal	Supplementation level	Physiological status	Results[1]
Shan et al. (2012)	total 3.48% (Glu + Gln)	0.70% or 1.00 %	Weaned pigs (7.2 kg)	↑ADG ↑intestinal morphology ↑sucrase and maltase activities ↓diarrhea incidence
Ma et al. (2021)	?	0.2% for 2 weeks post-weaning	Weaned pigs (5.6 kg)	↑meat color stability
Domeneghini et al. (2006)	?	0.50%	Weaned pigs (5.1 kg)	↑intestinal morphology ↓ apoptosis of epithelial cells and lymphatic follicles ↑ mitosis of epithelial cells
Xiao et al. (2012a)	total 3.83% (Glu)	1.00%	Weaned pigs (5.7 kg)	↑ADG ↑ATTD of DM, OM, GE, and AA ↑AID of GE, Leu, Lys, Cys, and Pro ↑jejunal alkaline phosphatase activity ↑ *PPARγ* gene expression ↓Gln synthetase and pyruvate kinase in the SI
Wang et al. (2015a)	total 2.20% (Gln) and 2.07% (Glu)	1.00%	Weaned pigs (6.7 kg)	↑ADG ↑intestinal morphology ↓lactulose recovery ↓jejunal CRF protein and gene expression ↑jejunal OCLN, claudin-1, claudin-3, claudin-4, ZO-2, and ZO-3 expression
Johnson et al. (2006)	?	4%	Weaned pigs	↓neutrophile oxidative burst ↓IgA+ cells in mesenteric lymph nodes ↓CD45RA+ and CD4+CD45RA+ cells ↓Peyer's patch B cells ↑response to pokeweed mitogen ↑TNFα and IFN-γ/IL-4 in mesenteric lymph nodes

Table 2.2 Effects of dietary Gln supplementation on growth performance, metabolism, and immune response in nursery pigs (Continued)

Reference	Basal	Supplementation level	Physiological status	Results[1]
He et al. (2016)	total 3.57%	1.00%	Weaned pigs (9.2 kg)	↑ADG and G:F ↑ATTD of DM and N ↓serum CD8+ concentration ↑serum IgG and IgM concentration ↓jejunal Gln synthetase ↑jejunal lactase activity ↓*PPARγ* and pyruvate kinase gene expression in SI ↑*mTOR* gene expression in SI ↑SOD activity in serum ↓MDA concentration in serum
Rezaei et al. (2013)	total Glu 1.91%	1%, 2%, or 4% MSG[2]	Weaned pigs (5.5 kg)	↑ADG, G:F ↓ADFI ↑plasma concentrations of Glu, Gln, other AA ↑intestinal morphology ↑SI weight ↑jejunal DNA content and antioxidant capacity ↓diarrhea incidence

[1]G:F, gain to feed ratio; GSH, glutathione; GSSG, glutathione disulfide; OCLN, occludin; Ig, immunoglobulin; ADG, average daily gain; IL, interleukine; Hsp, heat shock protein; DM, dry matter; OM, organic matter; GE, gross energy; AA, amino acid; AID, apparent ileal digestibility; PPARγ, peroxisome proliferator-activated receptor gamma; ZO, zonula occludens; mTOR, mammalian target of rapamycin; ADFI, average daily feed intake; SOD, superoxide dismutase; MDA, malondialdehyde

[2]monosodium glutamine.

The upregulated tight junction proteins result in a lower recovery of lactulose, a marker of intestinal barrier function (Wang et al., 2015a). Furthermore, dietary Gln supplementation enhances the activities of brush border enzymes, including lactase, sucrase, maltase, and alkaline phosphatase (Shan et al., 2012; Xiao et al., 2012b; He et al., 2016). This explains the report that greater digestibility of AA and energy is observed with dietary Gln supplementation (Xiao et al., 2012a). Further, dietary Gln beneficially modulates intestinal immune responses by regulating intestinal redox status (Wang et al., 2008), inflammatory response, (Johnson et al., 2006; Zhong et al., 2012), and immune cell proliferation (Johnson et al., 2006; He et al., 2016). Collectively, it seems that dietary Gln supplementation beneficially modulates overall intestinal health by enhancing or maintaining intestinal barrier function, digestive and absorptive function, and immune response.

There have been several attempts to elucidate the synergistic effect of Gln and other AA, including Glu, Ala, Asp, and Gly (Xing et al., 2017; Yi et al., 2018; Qi et al., 2020; Yan et al., 2020; Xu et al., 2021). Although the supplementation of Gln in combination with other AA shows beneficial effects on intestinal health, there is limited evidence to conclude synergistic effects because the studies did not compare the independent effect of Gln in combination with other AA. Therefore, future studies need to expand the treatments to test the interactive effects of individual AA supplementation.

He et al. (2016) investigated the effects of dietary supplementation with different ratios of Glu and Gln (1:9 and 2:8) mixture and Glu (1%) or Gln (1%) independently. However, the addition of Glu at any proportion with Gln dissipated the beneficial effects of Gln supplementation on brush border enzyme activity, growth performance, and mTOR-encoding gene expression in the small intestine. By contrast, dietary Glu supplementation in the form of monosodium or α-ketoglutarate

shows intestinal and hepatic health benefits (Rezaei et al., 2013; Wang et al., 2015b). This suggests that pigs may have a preference for certain forms of Glu to generate benefits, which requires further study.

2.6.3. SULFUR-CONTAINING AMINO ACIDS – METHIONINE AND CYSTEINE

Unlike other AA, Met and Cys contain a sulfur atom in their molecules. This distinct difference from other AA grants vital biological function to Met and Cys. The SAA are metabolized via transmethylation, remethylation, and transsulfuration pathways (Stipanuk, 2004). Methionine is transmethylated intracellularly to homocysteine via *S*-adenosylmethionine (**SAM**) by a series of enzymes, including methionine adenosyltransferase (**MAT**), methyl transferases, and S-adenosylhomocysteine hydrolase (D'Mello, 2003). SAM is a biologically critical methyl donor involved in all methylation reactions, including nucleic acid methylation (Martínez-López et al., 2008). Furthermore, recent evidence shows that SAM mediates the immune system by regulating immune-related gene expression (Song et al., 2005; Ding et al., 2015). The transmethylation mainly occurs in the liver, but it also occurs in the small intestine to a lesser extent (Riedijk et al., 2007). Homocysteine undergoes either the remethylation pathway or the transsulfuration pathway (D'Mello, 2003). In a remethylation pathway, homocysteine is recycled back to Met, in which choline or folic acids provide a methyl group to homocysteine (Stipanuk, 2004). The homocysteine can also undergo an irreversible transsulfuration pathway, becoming cysteine via cystathionine, which is regarded as critical for health maintenance (Riedijk et al., 2007). Cysteine is distinguished from other AA in that it has a sulfhydryl (thiol) group in its structure (Circu and Aw, 2012). Cysteine serves as an extracellular reducing agent by being oxidized into cystine (**CySS**), in which two molecules of Cys form a disulfide bridge between two thiol groups (Circu and Aw, 2012). The extracellular redox potential of Cys/CySS plays critical roles in enzyme catalysis, gene expression,

and signaling pathways for cell proliferation and apoptosis (Zhu et al., 2012). Cys serves as a substrate for the syntheses of various proteins associated with the immune system (Cys-rich defensins, antimicrobial peptides, APP), GSH, coenzyme A, and Mo-coenzymes (Stipanuk, 2020). Glutathione, or γ-glutamyl-cysteinyl-glycine, is a tripeptide of Glu, Cys, and Gly and serves as a powerful intercellular antioxidant (Bhattacharyya et al., 2014a). Furthermore, Cys is a precursor of Tau that is associated with redox homeostasis, osmoregulation, cytoprotection, and neurotransmission (Stipanuk, 2020).

GSH regulates nutrient metabolism, gene expression, DNA and RNA synthesis, cell proliferation and apoptosis, signal transduction, cytokine production, and protein glutathionylation (Wu et al., 2004). Hydrogen sulfide, known as a gasotransmitter, is produced during the transsulfuration pathway and acts as a signaling and cytoprotective molecules (Kimura, 2015; Sbodio et al., 2019). There is growing evidence that hydrogen sulfide plays critical roles in modulating neurotransmission, vascular tone, and inflammation (Kimura, 2015).

Although Met is regenerated from homocysteine via the remethylation pathway, its net synthesis does not override the metabolic needs for Met, indicating that Met is a nutritional EAA in pigs (D'Mello, 2003). Thus, it is common for synthetic Met to be supplied in nursery pig diets to meet Met and Met + Cys requirements. The most common source of synthetic Met is $_{DL}$-Met (a racemic mixture) that is chemically produced. Before $_{D}$-Met is metabolized, it must be converted to an L isomer in two consecutive processes by amino acid oxidase and transaminase, mainly in the kidney and liver (D'Mello, 2003). Another common source of Met for pigs is a precursor of Met, $_{DL}$-2-hydroxy-(4-methylthio)butanoic acid ($_{DL}$-HMTBA). The $_{L}$- and $_{D}$-HMTBA is first metabolized into 2-keto-4 methylthiobutanoic acid by $_{L}$-2-hydroxy acid oxidase, and then to L-Met by L-2-hydroxy acid dehydrogenase, mainly in the liver (Zhang et al., 2018). Feed-grade $_{L}$-

Met that is microbially produced is now available (Yang et al., 2019). The biological efficacy of these Met sources is still controversial, and it is not discussed in the present review.

Traditionally, the Met+Cys requirement is suggested rather than the Cys requirement for pigs (NRC, 2012). Cys synthesis rate is delicately regulated by rate-limiting enzymes associated with the transsulfuration pathway (Sbodio et al., 2019). It is generally believed that systemic inflammation increases the Met transsulfuration rate to meet the increased needs for Cys (Malmezat et al., 2000b). This is mainly because of the increased synthesis and turnover of GSH (Rakhshandeh et al., 2019; Rakhshandeh et al., 2020). In this regard, the SAA requirement increases when pigs are immunologically suppressed (Rakhshandeh et al., 2014; Kahindi et al., 2017a).

Isotopic tracer research indicated that 20% and 25% of dietary Met and Cys, respectively, are metabolized by the intestine (Riedijk et al., 2007; Bauchart-Thevret et al., 2011). This reflects the high demands for methyl donors, GSH, and Cys-rich protein (e.g., mucins and defensins) by the intestinal epithelial cells in response to exogenous toxins and pathogens (Bauchart-Thevret et al., 2009a; Rakhshandeh et al., 2020). In this regard, the efficacy of dietary SAA supplementation seems apparent on intestinal integrity and redox status. Dietary Met supplementation, including SAA and Tau and GSH, can elevate the concentrations of AA in plasma, intestinal, and liver (Chen et al., 2014), reflecting the increased transsulfuration pathways. Dietary Met or Cys (via $_{L}$-Cys or N-acetylcysteine) supplementation improves intestinal health, with enhanced intestinal morphology, brush-border enzyme activity, tight junction proteins, and reduced apoptosis and expression of pro-inflammatory cytokines (Wang et al., 2013; Chen et al., 2014; Hou et al., 2015; Song et al., 2016; Yi et al., 2017; Su et al., 2018).

Table 2.3 Effects of dietary Met or Cys on growth performance, metabolism, and immune response in nursery pigs

Item	Reference	Basal	Supplementation level	Physiological status	Results[1]
Met	Tian et al. (2016)	Total Met 0.27% Total SAA[2] 0.60%	0.12% L-Met or 0.12% DL-Met	Nursery pigs (15.5 kg)	↑N retention rate ↑serum total protein ↓serum urea nitrogen
Met	Su et al. (2018)	Total Met 0.4%	0.12% L-Met	Intrauterine growth-retarded weanling pigs (4.9 kg)	↑intestinal Met concentration ↑intestinal morphology ↑intestinal maltase activity ↑intestinal GSH concentration ↓intestinal MDA and protein carbonyl concentrations ↓intestinal apoptotic indices ↑OCLN gene and protein expression in jejunum
Met	Chen et al. (2014)	SID[3] Met 0.24% SID SAA 0.47%	0.12%	weaned pigs (7.1 kg)	↑ADG and G:F ↑plasma Cys, Met, GSH, GSSG, and taurine concentrations ↑intestinal Met, Glu, Gln, Cys, GSH, polyamine concentrations ↑hepatic Met, Gln, Glu, Taurine, and polyamines concentrations ↑Cys/CySS redox potential in plasma, duodenum, and jejunum ↑TEER of the jejunum ↑intestinal morphology ↑jejunal OCLN protein expression ↓jejunal active caspase-3

Table 2.3 Effects of dietary Met or Cys on growth performance, metabolism, and immune response in nursery pigs (Continued)

Item	Reference	Basal	Supplementation level	Physiological status	Results[1]
Cys	Song et al. (2016)	Total Met 0.35% Total SAA 0.69%	0.25% or 0.5% L-Cys	Weaned pigs (6.7 kg)	↑Intestinal morphology ↑Jejunal TEER ↓Jejunal FD4 flux ↑OCLN and claudin-1 protein expression in SI ↓*caspase-3* gene expression in SI ↑*claudin-1* gene expression in SI ↓TNF-α, IL-6 and IL-8 gene expression in SI ↑CAT, SOD, GPx and GSH concentrations in SI ↓MDA concentration in SI ↓NF-κB protein expression in SI ↑Nrf2 protein expression in SI
Cys	Wang et al. (2013)	N/A[3]	0.05% (*N*-acetylcysteine)	Acetic acid-induced colitis in weaned pigs (6.4 kg)	↑goblet cells in colon ↓Intravillus lamina propria cell and lymphocytic density ↓MPO activity and MDA concentration in plasma ↓MPO activity and MDA concentration in mucosa ↓plasma TNF-α ↑plasma epidermal growth factor ↓Transforming growth factor-α in colonic mucosa ↓caspase-3 protein expression in colonic mucosa ↑claudin-1 protein expression in colonic mucosa ↑amphiregulin gene expression in colonic mucosa
Cys	(Hou et al., 2012)	Total Met 0.30% Total SAA 0.65%	0.05% (*N*-acetylcysteine)	LPS-challenged weaned pigs (11.6 kg)	↑Intestinal morphology ↑claudin-1 activity in SI ↓caspase-3 activity in SI ↑occludin protein expression in SI

Table 2.3 Effects of dietary Met or Cys on growth performance, metabolism, and immune response in nursery pigs (Continued)

Item	Reference	Basal	Supplementation level	Physiological status	Results[1]
Cys	Yi et al. (2017)	N/A	0.05% (*N*-acetylcysteine)	LPS-challenged weaned pigs (5.03 kg)	↓plasma cortisol concentration ↓DAO activity in plasma ↑maltase, sucrase, and lactase activities in SI ↑Cys GSH content in duodenum ↓caspase-3 protein expression ↑claudin-1, occludin expression ↑genes associated with PI3K/Akt/mTOR, EGFR, TLR4/NF-κB, AMPK, and type I interferons

[1]GSH, glutathione; MDA, malondialdehyde; OCLN, occludin; ADG, average daily gain; G:F, gain to feed ratio; GSSG, glutathione disulfide; CySS, cystine; TEER, transepithelial electrical resistance; FD4, FD4, fluorescein isothiocyanate-dextran (4kDa); TNF, tumor necrosis factor; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; SI, small intestine; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NRF2, nuclear factor erythroid 2-related factor 2; MPO, myeloperoxidase; EGFR, epidermal growth factor receptor; AMPK, AMP-activated protein kinase.
[2]Standardized ileal digestible.
[3]Information is not available.

Enhanced redox status was also observed by increasing Cys/CySS redox potential and the concentrations or activity of antioxidant enzymes, such as catalase, SOD, GSH peroxidase, and GSH, when nursery pigs were fed a SAA-fortified diet (Chen et al., 2014; Song et al., 2016; Yi et al., 2017; Su et al., 2018). However, there is no information on whether the benefits of Cys supplementation can be fully replaced by Met supplementation. Given that transmethylation and transsulfuration rates are tissue-specific, the conversion of Met to Cys may be insufficient for the needs of Cys under immune challenge (Le Floc'h et al., 2018). However, to date, feed-grade Cys is not available, and thus it is not practical to supplement nursery diets with $_{L}$-Cys. Thus, future studies should confirm that the modification of Met:Cys by dietary ingredients can generate similar effects with $_{L}$-Cys supplementation.

2.6.4. TRYPTOPHAN

Although Trp is quantitatively the least required among other EAA by pigs for optimal growth, it is the third or fourth limiting AA in typical nursery pig diets. Apart from being a substrate for protein, Trp is a precursor of serotonin and kynurenine (D'Mello, 2003). Indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase are two main rate-limiting enzymes that initiate Trp catabolism into kynurenine (Wu, 2018). While IDO is expressed throughout the body, tryptophan 2,3-dioxygenase is primarily expressed in the liver (Wu, 2018). Kynurenine acts as a ligand for the aryl hydrocarbon receptor, and the activated receptor stimulates antioxidant system-inducing transcription factor, nuclear factor erythroid 2-related factor 2 (**Nrf2**) and T cell differentiation into regulatory T-cells that produce anti-inflammatory cytokines, suppressing immune responses (O'Mahony et al., 2015). Regulatory T-cell-induced IL-10 and IL-22 are associated with intestinal mucosal wound repair, immunity, and integrity (Ding et al., 2020).

IDO activity can be modulated by pro-inflammatory cytokines, particularly INF-γ, and cellular kynurenine/Trp ratio (Moffett and Namboodiri, 2003). Given that IDO is a key regulator of kynurenine expression, it is generally considered that IDO has an immunosuppressing function (Moffett and Namboodiri, 2003). The kynurenine pathway accounts for 95% of Trp catabolism, leaving 5% of Trp catabolism that occurs via serotonin (Wu, 2018). A small subset of Trp undergoes the 5-hydroxytrpytamine (serotonin) pathway that depends on vitamin B_6-dependent decarboxylation. This mainly takes place in the gut, and serotonin plays a pivotal role in the brain-gut axis (O'Mahony et al., 2015). Serotonin mediates appetite by modulating satiety, insulin secretion and sensitivity, intestinal contraction, and expression of gut hormones, such as ghrelin (Le Floc'h and Seve, 2007). A recent study revealed that serotonin also interacts with immune cells, which mediate intestinal inflammation (Banskota et al., 2019). Gut-produced serotonin regulates glucose and lipids by promoting pancreatic β-cells, insulin secretion, hepatic gluconeogenesis, and lipolysis in adipocytes and suppressing hepatic glucose uptake (El-Merahbi et al., 2015). Growing evidence indicates that luminal serotonin that is produced by the intestinal microbiome is not only quantitatively significant but also influences host intestinal Trp metabolism and the brain-gut axis (Stasi et al., 2019). The serotonin can be further metabolized into N-acetyl-5methoxyserotonin or melatonin, an endogenous indolamine that possesses antioxidant, anti-inflammatory, and antiapoptotic properties (Le Floc'h et al., 2011).

Empirical studies have confirmed that dietary L-Trp supplementation promotes host as well as bacterial Trp metabolism (Mao et al., 2014b; Liang et al., 2018) and elevates serotonin levels in the hypothalamus in nursery pigs (Koopmans et al., 2006; Shen et al., 2012; Sterndale et al., 2020). This possibly enhances appetite, thereby increasing average daily feed intake (**ADFI**) and average daily gain (**ADG**) (Trevisi et al., 2009; Mao et al., 2014b; Shen et al., 2015; Liu et al.,

2019). Dietary Trp supplementation can dampen the hypothalamic-pituitary-adrenocortical axis in nursery pigs, thereby suppressing cortisol release (Koopmans et al., 2006; Shen et al., 2015). This is not only associated with pigs' performance but also with pigs' welfare, a concern in the modern swine industry.

Dietary Trp supplementation can ameliorate oxidative stress in immunologically challenged pigs by elevating antioxidant enzymes, including SOD, GSH peroxidase, thioredoxin reductase, and heme oxygenase (Mao et al., 2014b; Liu et al., 2019). The authors speculated that the beneficial effects on the antioxidant defense system are associated with the antioxidative properties of Trp and its metabolites. Furthermore, the benefit can be attributed to the fact that dietary Trp supplementation indirectly mediates *Nrf2,* a transcription factor that regulates the levels of endogenous antioxidant enzymes (Liu et al., 2019). Evidence shows that dietary Trp supplementation is beneficial on intestinal barrier function by enhancing tight junction protein (Liang et al., 2018; Liu et al., 2019). Furthermore, intestinal innate immunity is fortified by dietary Trp supplementation with greater expression of antimicrobial peptides and sIgA (Liang et al., 2019). Demands for dietary Trp for these biological functions observed above are relevant to our previous findings that optimal dietary Trp levels upshift when pigs are immunologically challenged compared with normal physiological condition (Jayaraman et al., 2017a; Jayaraman et al., 2017b). Recent studies have revealed that dietary Trp levels interact with intestinal microbial composition and microbial metabolites. Supplemental Trp beneficially modulated intestinal microbial composition by enriching Trp-metabolizing bacteria, including *Lactobacillus* spp. and *Clostridium* XI, and suppressing the abundance of opportunistic pathogens, including *Clostridium sensu stricto* and *Streptococcus* (Liang et al., 2018; Liang et al., 2019). By contrast, dietary Trp supplementation at 0.75% had negative effects on feed intake, tight junction protein,

Table 2.4 Effects of dietary Trp on growth performance, metabolism, and immune response in nursery pigs

Reference	Basal	Supplementation level	Physiological status	Results[1]
Mao et al. (2014b)	Total 0.18%	0.13 and 0.28%	diquat-challenged pigs	↑ ADG, ADFI, and G:F ↑ GPx activity in liver ↑ plasma Trp ↓ LNAA and urea nitrogen levels in plasma
Koopmans et al. (2006)	SID[2] Trp 0.20%	0.50%	weaned pigs	↑serotonin content in hypothalamus ↑intestinal morphology ↓salivary cortisol
Shen et al. (2015)	Total Trp 0.21	0.80%	nursery pigs (25.8 kg or 11.7 kg)	↑ADG, G:F ↓salivary cortisol concentration ↓plasma urea nitrogen
Shen et al. (2012)	SID Trp 0.20%	0.60%	nursery pigs (13.0 kg)	↑serotonin content in hypothalamus ↓plasma urea nitrogen concentration ↓MDA concentration in plasma and hypothalamus
Liang et al. (2018)	?	0.20% or 0.40%	Weaned pigs (7.6 kg)	↑ADFI, ADG ↑*AhR*, *CYP1A1*, *CYP1B1* gene expression in colon and cecum ↓*IL-8*, *TNF-α* gene expression in cecum and colon ↑ZO-1 and OCLN protein expression ↑propionate, isobutyrate, isovalerate concentrations in cecal or colon digesta ↑indole-3-acetic acid, indole, tryptamine concentration in cecal or clonic digesta

Table 2.4 Effects of dietary Trp on growth performance, metabolism, and immune response in nursery pigs (continued)

Reference	Basal	Supplementation level	Physiological status	Results[1]
Liang et al. (2019)	Total Trp 0.25%	0.2%, and 0.4%	Nursery pigs (7.6 kg)	↑Serum concentrations of Glu, Asp, His, Trp, Tau ↑ZO-1, ZO-3, claudin-1 and OCLN protein expression ↑*pBD-2*, *pBD-3*, and sIgA gene expression ↑*AKT*, *mTOR*, *4E-BP1*, and *P70* gene expression ↑jejunal microbial diversity ↑jejunal abundance of Trp-metabolizing bacteria (*Lactobacillus*, *Clostridium* XI) ↓jejunal abundance of opportunistic pathogens (*Clostridium sensu stricto*, *Streptococcus*)
Sterndale et al. (2020)	SID Trp 0.24%	0.11% Trp with reduced LNAA	ETEC-challenged pigs (6.3 kg)	↑plasma Trp concentration and haptoglobin ↑serum serotonin concentration ↑ADG and ADFI
Liu et al. (2019)	SID Trp 0.22%	0.15% or 0.31%	Diquat-challenged weaned pigs (6.6 kg)	↑ADG, ADFI ↑ Jejunal TEER ↓ Jejunal FD4 flux and DAO activity ↑ *ZO1*, *OCLN*, *claudin-1* gene expression in jejunum ↑ SOD, GPx, CAT activities in jejunum ↓MDA content in jejunum ↑*SOD1*, *HO1*, *GPx1*, *thioredoxin reductase* gene expression in jejunum ↑HO 1 expression in jejunum ↓reactive oxygen species production in jejunal mitochondrial ↑gene expression associated with mitochondrial biogenesis

Table 2.4 Effects of dietary Trp on growth performance, metabolism, and immune response in nursery pigs (continued)

Reference	Basal	Supplementation level	Physiological status	Results[1]
Trevisi et al. (2009)	total Trp 0.25%	0.10%	ETEC-challenged pigs (6.6 kg)	↑ADG, ADFI ↑Intestinal morphology

[1]ADG, average daily gain; ADFI, average daily feed intake; GPx, glutathione peroxidase; LNAA, large neutral amino acids; MDA, malondialdehyde; AhR, aryl hydrocarbon receptor; CYP1A1, cytochrome P450, family 1, subfamily A, polypeptide 1; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; IL, interleukin; TNF, tumor necrosis factor; OCLN, occludin; ZO, zonula occludens; ; pBD, porcine beta defensins; sIgA, secretory immunoglobulin A; AKT, protein kinase B; mammalian target of rapamycin; eukaryotic translation initiation factor 4E-binding protein 1; P70, ribosomal protein S6 kinase beta-1; TEER, transepithelial electric resistance; SOD, superoxide dismutase; DAO, diamine oxidase; CAT, catalase; HO, heme oxygenase.
[2]Standardized ileal digestible.

intestinal morphology, and intestinal barrier integrity. This indicates that dietary Trp supplementation above a certain level can generate toxic effects.

Dietary Trp has been researched in relation to other large neutral amino acids (**LNAA**), such as BCAA. This is because Trp competes with the same Leu-preferring transporter in the luminal membrane with other large neutral AA (D'Mello, 2003). Therefore, it is generally hypothesized that lowering dietary LNAA and thus plasma LNAA concentration can increase the synthesis of Trp metabolites, generating their benefits on immunity and the nervous system (Shen et al., 2012; Sterndale et al., 2020). Sterndale et al. (2020) reported that increasing the level of LNAA content can dissipate the benefit of Trp supplementation on serotonin synthesis and growth performance in ETEC-challenged pigs. Similarly, Shen et al. (2012) reported that 0.6% of L-Trp supplementation improved growth performance when dietary LNAA concentration was reduced from 4.5 to 3.9%. It was further found that reducing LNAA concentration can decrease salivary cortisol concentration regardless of dietary Trp level. Collectively, dietary LNAA levels should be considered when L-Trp is supplemented in a nursery diet for Trp metabolism and its efficacy.

2.6.5. BRANCHED CHAIN AMINO ACIDS (LEUCINE, ISOLEUCINE, AND VALINE)

Leu, Ile, and Val are collectively referred to as BCAA. As the name implies, BCAA have an aliphatic side-chain with a branched alkyl group and because of structural similarity BCAA's biological roles are similar and intricately interact with each other (D'Mello, 2003). BCAA are catabolized via two steps: transamination and decarboxylation. Unlike other AA, the initiation of BCAA catabolism occurs in extrahepatic tissues, such as intestine and skeletal muscle, but not in the liver because of the lack of BCAA aminotransferase or BCAA transaminase that catalyzes the conversion of BCAA and α-ketoglutarate to branched chain α-keto acids and Glu (Wu, 2018). The

released branched chain α-keto acids undergo irreversible oxidation in the liver, where branched-chain α-keto acid dehydrogenase complexes are highly expressed compared with other tissues. This enzyme oxidizes branched chain α-keto acids with coenzyme A into either 2-methylbutyryl-CoA, isobutyryl-CoA, or isovaleryl-CoA (Nie et al., 2018). These metabolites are further catabolized into acetyl-CoA and succinyl-CoA, which enter the Krebs cycle, and the carbon skeleton is recycled for glucose synthesis and Asp (Wu, 2018). The α-keto acid of Leu, α-ketoisocaproate unlike α-keto acids of Ile and Val can be converted to β-hydroxy-β-methylbutyrate by a-ketoisocaproate dioxygenase. β-hydroxy-β-methylbutyrate is known to stimulate myogenic differentiation and survival and mTOR pathway activity, thereby increasing muscle hypertrophy, minimizing muscle protein degradation, and protecting against skeletal muscle injury (Nie et al., 2018). Many studies have confirmed that dietary Leu enhances muscle protein anabolism via the activation of the mTOR pathway that promotes the initiation of translation by the phosphorylation of downstream effectors, such as 4EBP-1 and 70-kDa ribosomal protein S6 kinase (**P70**). Although dietary Leu can promote muscle protein synthesis, the negative effects of dietary Leu in a typical swine diet have been reported (Ren et al., 2015; Kwon et al., 2019). Typical swine diet contains excessive Leu, while Val and Ile are the fifth and sixth limiting AA, respectively. The balance of BCAA is nutritionally important because branched-chain amino transferases have high affinity for all BCAA, suggesting that one excessive BCAA can lead to other BCAA degradation and make them deficient (D'Mello, 2003; Wu, 2018).

Growing evidence exists regarding the interaction between immunity and BCAA metabolism. Immune cells express branched chain α-keto acid dehydrogenase and utilize BCAA as energy fuel, directly oxidizing BCAA or converting BCAA to other AA such as Glu (Calder, 2006). BCAA are essential nutrients for neutrophils and lymphocytes to differentiate and

synthesize effector and protective molecules (Calder, 2006; Zhang et al., 2017). In a study by Ren et al. (2015), feeding weaned pigs a protein-restricted diet that is deficient in Val and Ile led to suppressed concentration of sIgA, IgA, IgG, and IgM in the jejunum or ileum compared with feeding a control diet that meets the requirement. BCAA supplementation through L-Ile, L-Val, and L-Leu in a protein-restricted diet restored the levels of those Ig, reflecting the importance of BCAA for the synthesis of Ig. Evidence also shows that Ile stimulates antimicrobial peptide expression, particularly β-defensin, in the intestine by modulating c-myc, a multifunctional transcription factor, and the pathways of NF-κB and extracellular signal-regulated kinase (Gu et al., 2019). There are reports that Val also plays important roles in maintaining the immune system by promoting macrophage phagocytosis and regulating the maturation of monocyte-derived dendritic cells that link the innate and adaptive immune systems (Kakazu et al., 2007; Chen et al., 2017a).

There is consensus that BCAA regulates the expression and translocation of nutrient transporters, particularly glucose transporter, Na^+/glucose co-transporter, and AA transporters in muscle and intestine. Despite the poor understanding of their mode of action, it has been hypothesized that BCAA elevates insulin levels and stimulates protein kinase C, phosphoinositide 3-kinase, and extracellular signal-regulated kinase signaling pathways, thereby increasing glucose transporter translocation (Zhang et al., 2014; Zhang et al., 2017).

The abovementioned benefits have been clearly demonstrated in mammary tissues; thus, numerous studies have focused on sows to investigate the effects of dietary BCAA modification on lactating performance and suckling pig performance. However, this review discusses the

Table 2.5 Effects of dietary branched chain amino acids (BCAA) on growth performance, metabolism, and immune response in nursery pigs

BCAA	Reference	Basal	Supplementation level	Physiological status	Results[1]
Ile	Ren et al. (2019)	total Val 0.78% total Ile 0.64% total Leu 1.46%	0.40% (Ile)	ETEC-challenged pigs (5.5 kg)	↑ADG ↑total plasma protein ↑plasma IgA level ↓plasma endotoxin and IL-6 concentrations ↑SI *pBD-1*, *pBD-2*, *pBD-3* gene expression
Ile	Mao et al. (2018)	?	1.0% (Ile)	Rotavirus-challenged weaned pigs (7.0 kg)	↑ADFI, ADG, and G:F ↑serum Ile concentration ↑rotavirus antibody levels in serum and ileum ↓non-structural protein 4 content in ileum ↑CD4+, IgA, IgG, INF-β, IFN-γ, IL-1β, TNF-α, IL-10 concentration in serum ↑IFN-β, IFN-γ, IL-10, sIgA, and TNF-α concentration in ileum ↑*IFN-β*, *IFN-γ*, *IL-1β*, *IL-10*, and *TNF-α* gene expression in ileum and mesenteric lymph nodes ↑*NF-κB*, *TLR3*, *RIG-I*, pBD1-3 gene expression in ileum or mesenteric lymph nodes
BCAA	Zhang et al. (2013)	total Val 0.74% total Ile 0.60% total Leu 1.26%	0.34% (L-Val), 0.10% (L-Leu), 0.19% (L-Ile)	Weaned pigs (8.0 kg)	↑ADG, ADFI, G:F ↑serum Ile, Leu, and Val concentrations ↑Intestinal morphology ↑ASCT2, CAT-1, rBAT, 4F2hc gene expression in jejunum ↑rBAT protein expression in jejunum

Table 2.5. Effects of dietary branched chain amino acids (BCAA) on growth performance, metabolism, and immune response in nursery pigs (continued)

BCAA	Reference	Basal	Supplementation level	Physiological status	Results[1]
BCAA	Spring et al. (2020)	SID[2] Leu 1.08% SID Ile 0.40% SID Val 0.50%	0.57% (L-Val), 0.82% (L-Leu), 0.55% (L-Ile)	Weaned pigs (8.3 kg)	↑Ile, Val, Thr, Gly levels in plasma ↑2-ketoisocaproic acid, aminomalonate levels in plasma ↓urea, Met, 2,3-dihydroxybutanoic acid levels in plasma
BCAA	Zheng et al. (2016)	SID Leu 1.32% SID Ile 0.58% SID Val 0.68%	0.16% or 0.32% (L-Val), 0.24% or 0.48% (L-Leu), 0.17% or 0.34% (L-Ile)	Weaned pigs (8.4 kg and 9.2 kg)	↑ ADG, ADFI, and G:F ↑P70, mTOR protein expression in the hypothalamus and *longissimus dorsi* ↓*eIF2* expression in hypothalamus ↑*AgRP*, *NPY* gene expression ↓melanocortin-4 receptor gene and cocaine- and amphetamine-regulated transcript expression in hypothalamus ↑muscle mass
BCAA	Yin et al. (2020)	Total Val: 0.62 Total Leu: 1.21 Total Ile: 0.53	0.20% (L-Val), 0.25% (L-Leu), 0.18% (L-Ile)	Weaned pigs (6.39 kg, 7.68 kg, or 11.3 kg)	↑ADG, ADFI ↑serum BCAA concentrations ↑Spirochaetales, Gammaproteobacteria, Lactobacillales abundance in the gut

Table 2.5. Effects of dietary branched chain amino acids (BCAA) on growth performance, metabolism, and immune response in nursery pigs (continued)

BCAA	Reference	Basal	Supplementation level	Physiological status	Results[1]
BCAA	Habibi et al. (2021)	SID Leu 1.28% SID Val 0.63% SID Ile 0.57%	0.23% or 0.70% (L-Val), 0.09% or 0.82% (L-Leu), 0.13% or 0.51% (L-Ile)	Weaned pigs (9.5 or 10.5 kg)	↑ADG, ADFI ↑plasma BCAA concentrations ↓plasma urea concentration ↑intestinal morphology ↑PYY gene expression in intestine ↑CCK gene expression in jejunum and duodenum ↑GLP-1 concentration in plasma ↓T1R1 and T1R3 protein expression in duodenum and jejunum ↓POMC gene expression and eIF2α protein expression in hypothalamus ↑AgRP and NPY gene expression in hypothalamus

[1]ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed ratio; Ig, immunoglobulin; SI, small intestine; IFN, interferon; TNF, tumor necrosis factor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; pBD, porcine beta defensins; RIG-I, retinoic acid-inducible gene I; ASCT2, a sodium dependent neutral amino acid transporter; CAT, cationic transporter; rBAT, neutral and basic amino acid transporter; 4F2hc, 4F2 cell-surface antigen heavy chain; eIF2, eukaryotic translation initiation factor 2α; NPY, neuropeptide Y; PYY, peptide YY; CCK, cholecystokinin; GLP, glucagon-like peptide; T1R1, taste receptor type 1 member 1; T1R3, T1R1, taste receptor type 1 member 3; POMC, proopiomelanocortin; AgRP, agouti-related protein.
[2]Standardized ileal digestible.

effects of dietary BCAA in nursery pigs. Recently, growing interest in lowering dietary protein content has intrigued research on dietary BCAA levels and balance (Zhang et al., 2013; Zheng et al., 2016; Spring et al., 2020; Habibi et al., 2021). In nursery diet, lowering CP below 22% typically results in Val and Ile deficiency. Supplementing BCAA in a low protein diet reduces circulating urea N and elevates circulating free BCAA levels, which eventually improves muscle mass and growth performance (Zhang et al., 2013; Zheng et al., 2016; Habibi et al., 2021). As mentioned above, dietary BCAA levels are critical for intestinal development according to empirical studies in which low protein-induced villus atrophy was restored by BCAA supplementation. Furthermore, recent studies showed that dietary BCAA increases feed intake by upregulating the expression of orexigenic genes such as agouti-related peptide and neuropeptide Y while downregulating the expression of anorexigenic genes such asmelanocortin-4 receptor and cocaine- and amphetamine-regulated transcript in the hypothalamus (Zheng et al., 2016; Habibi et al., 2021). As commonly reported in growing pigs, Zheng et al. (2016) found that BCAA supplementation restored muscle mass deposition that was lagged by feeding a low protein diet by upregulating the mTOR signaling pathway in muscle and hypothalamus.

Duan et al. (2016b) conducted a series of studies with 9.9 kg of nursery pigs to test the optimal BCAA ratio in a low protein diet. Pigs fed a diet containing Leu:Ile:Val at the ratio of 1:0.51:0.63 or 1:0.75:0.75 had superior growth performance and levels of circulating BCAA compared with those fed a diet with 1:1:1 and 1:0.25:0.25 ratios. In terms of intestinal morphology and AA transporters, BCAA supplementation to a low protein diet at the ratio of 1:0.75:0.75 (Leu:Ile:Val) was optimal (Duan et al., 2018). This indicates that the overall BCAA ratio should be considered when individual BCAA is supplemented with a nursery diet. Surprisingly, however, very little information is available on whether single BCAA supplementation, such as L-Val, can

balance the diet and improve growth performance. This is a practically important area because feed-grade L-Leu is not commercially available, and the price of L-Ile is not reasonable at the moment.

2.6.6. THREONINE

Threonine is not synthesized in pigs *de novo;* thus, it is nutritionally regarded as an EAA. Thr is second- or third-limiting AA in nursery pig diets regardless of simple or complex diets. Feed grade of L-Thr is commonly supplemented in the diet to meet AA requirement. Unlike other FAA, Thr does not seem to participate in any critical metabolic pathway (D'Mello, 2003). Although Thr is mainly used for protein synthesis, it can go into catabolic pathways by the actions of threonine aldolase, threonine dehydrogenase, and threonine dehydratase, releasing Gly, acetyl-CoA, or propionyl-CoA (Tang et al., 2021).

In 1998, Stoll et al. found that 60% of dietary Thr was extracted by portal drained viscera during its first pass in piglets, suggesting high Thr metabolism in portal drained viscera. Floc'h and Sève (2005) further revealed that Thr does not undergo the threonine dehydrogenase catabolic pathway in the intestine, attributing the high first-pass extraction rate of dietary Thr by portal drained viscera to protein synthesis. The most well-known functional role of Thr is as a precursor of intestinal mucosal protein and mucins (Schaart et al., 2005). Mukkur et al. (1985) analyzed the chemical composition of mucins and reported that they are composed of 66% carbohydrate and 34% protein, with Thr and Val accounting for 44% of the AA. Intestinal mucin is continuously secreted from goblet cells into the gut lumen, forming an intestinal mucus layer. The mucus layer is a viscous fluid that coats the lining of the intestinal epithelium. This not only facilitates the movement of digesta as a lubricant but also participates in the innate immune system by providing

the physicochemical barrier and protecting the intestine from the adhesion of pathogen (Corfield et al., 2000). In the inner mucus layer, antimicrobial peptides, including lysozyme and defensins and secretary IgA (sIgA), which are secreted by intestinal cells (e.g., Paneth cell and M cell), serve as the innate immune system stabilizing the gut microbiota (Peterson and Artis, 2014). Recent evidence shows that the mucus layer interacts with the gut microbiota, mucus glycans being their energy source (Schroeder, 2019). This explains the findings that modification of dietary Thr content can alter the bacterial composition in the gut (Chen et al., 2017b; Dong et al., 2017). In addition to the contribution of Thr in mucin synthesis, it is known that Thr is a quantitatively major component AA of Ig, particularly sIgA (Bortoluzzi et al., 2018). Intestinal mucosal sIgA secretion accounts for two thirds of the entire Ig secretion in the body (Slack et al., 2012), reflecting the significance of Thr supply in the intestine. sIgA serves as a first line of defense in the innate immune system against pathogens by inhibiting pathogens or toxins that penetrate or adhere to the mucosal surface (Abbas et al., 2017).

Apart from the quantitative significance of Thr in protein syntheses, Thr seems to participate in signaling pathways. In a recent study by Zhao et al. (2020), dietary Thr levels were associated with muscle growth and muscle protein content. The authors found that an increase in dietary Thr can upregulate the expressions of muscle growth-related genes, including growth hormone, insulin-like growth factor 1, myoblast determination protein 1 (**MyoD1**), and myogenin (**MyoG**), and activate Akt/mTOR signaling pathway, resulting in improved muscle protein content in hybrid cat fish (Zhao et al., 2020). The authors further reported that dietary Thr supplementation can also regulate the Nrf2/Keap1 signaling pathway, enhancing muscle antioxidant capacity. Thus, future studies need to confirm that these benefits can be observed in pigs and investigate the mode of action of dietary Thr as a signaling molecule. There is a report that Thr can stimulate goblet cell

differentiation by modulating the expression of the Notch-Hes1-Math1 pathway, which eventually promotes the synthesis of mucin 2 (Zhang et al., 2019). Further, Thr is a vital nutrient in the differentiation of embryonic stem cells; however, this is not the scope of our review (Chen and Wang, 2014).

Surprisingly, studies on dietary Thr supplementation above the requirement have not been conducted actively in pigs compared to other species and other FAA. To the best of our knowledge, there are only two studies where the supplemental effects of dietary Thr in weaned pigs were investigated. In a study by Mao et al. (2014a), three dietary Thr levels (0.74%, 0.89%, and 1.11% of SID Thr) that were obtained with L-Thr supplementation were tested in pigs challenged with swine *Pseudorabies* live vaccine. Increasing dietary Thr levels decreased the serum IFN-γ concentrations and the expressions of mRNA encoding *TLR3*, *TLR7*, and *TLR9*, which were upregulated by the vaccination. The authors concluded that dietary Thr supplementation can be a beneficial nutritional strategy in immune-challenged weaned pigs by down-regulating TLR expressions and thus regulating the levels of T-helper cytokines. Similarly, in a study by Ren et al. (2014), intestinal integrity and immune responses were investigated in response to different dietary Thr levels in weaned pigs challenged with ETEC. L-Thr was supplemented in a basal diet that contained 0.37% (deficient diet) SID Thr to reach 0.75% SID Thr, close to NRC (2012) requirement and 1.1% supplemental diet. Increasing dietary SID Thr levels improved mucosal IgA concentration and duodenal villus height (**VH**) to crypt depth (**CD**) ratio. The two studies (Mao et al., 2014; Ren et al., 2014) commonly found an interaction between dietary Thr levels and immune challenge on immune response and intestinal integrity. This is supported by our previous study (Jayaraman et al., 2015) in which the optimal SID Thr:Lys for feed efficiency is upshifted in pigs

under unclean sanitary conditions that cause mild immune stimulation compared with those under clean sanitary conditions. According to an

Table 2.6 Effects of dietary Thr or amino acid (AA) blend on growth performance, metabolism, and immune response in nursery pigs

AA	Reference	Basal	Supplementation level	Physiological status	Results[1]
Thr	Mao et al. (2014a)	SID[2] 0.74%	0.15% or 0.37%	swine Pseudorabies live-vaccinated weaned pigs (6.9kg)	↓ serum IgM and IgG concentrations ↓ serum IFN-γ concentration ↓ serum IL-1β and TNF-α concentrations ↑ TLR3, TLR7, and TLR9 in lung, hilar lymph node, or cerebrum
Thr	Ren et al. (2014)	SID 0.35%	0.75% or 1.1%	ETEC-challenged weaned pigs (7.3 kg)	↑ ADG, G:F ↑ intestinal morphology
Asp-Glu-Gln	Qi et al. (2020)	?	0.1% Asp 0.5% Glu 1% Gln	Weaned pigs	↑*Acyl-CoA oxidase*, *malic enzyme 1*, and *sirtuin 1* gene expression in liver ↑serine/threonine protein kinase expression in liver ↑phosphor-ACC expression in liver
Gly-Gln	Yan et al. (2020)	?	0.25% (Gly-Gln)	weaned pigs (7.6kg)	↑ADG and ADFI ↑alpha diversity and abundance of anaerobes and phylum Fibrobacteres ↑SCFA concentrations in colon and ileum ↑ileal endocrine peptides (GLP-1 and 2 and EGF) ↑intestinal morphology ↓serum IL-1β, TNF-α, IL-6 ↑serum IL-10
Gly-Gln	Xu et al. (2021)	?	0.25% Gly-Gln	LPS-challenged weaned pigs (7.6 kg)	↑intestinal morphology ↑ ileal IL-10 content ↑ tight junction protein ↑ ileal SOD activity ↓ ileal MDA content and NOS activity ↑α-diversity, obligate anaerobes and SCFA-producing bacteria ↑ileal SCFA concentrations

Table 2.6 Effects of dietary Thr or amino acid (AA) blend on growth performance, metabolism, and immune response in nursery pigs (continued)

AA	Reference	Basal	Supplementation level	Physiological status	Results[1]
Ala-Gln	Xing et al. (2017)	?	0.5% Ala-Gln	LPS-challenged weaned pigs	↑intestinal morphology ↑EGF receptor and IGF-1R gene expression ↑Claudin-1 and ZO-1 protein and gene expression ↓Jejunal IL-1β and IL-8 gene expression
Trp + Met	Capozzalo et al. (2017a)	SID Trp 0.20% SID Met 0.35% SID SAA 0.69%	0.10% (L-Trp) 0.13% (DL-Met)	Weaned pigs (6.2 kg)	↑ADG and G:F ↓Fecal *Escherichia coli* score ↑C-reactive protein, haptoglobin, and apoliproprotein A1 concentrations in plasma ↓plasma urea nitrogen concentration ↑IL-1β
Blend	Yi et al. (2018)	?	1% AA blend (Glu:Gln:Gly:Arg:*N*-acetylcysteine at 5:2:2:1:0.5)	Weaned pigs (5.5 kg)	↓diarrhea incidence ↓DAO activity in plasma ↓H_2O_2, MDA, and HP70 levels in plasma ↓IL-1β and INF-γ gene expressions in SI ↓*Enterobacterium*, *Enterococcus*, *Clostridium coccoides* abundance in colon digesta ↑*Bifidobacterium*, *Lactobacillus* genus in colon digesta ↑CAT, SOD, and GPx activities in SI ↑AA concentrations in plasma ↑SI morphology ↑pBD-1, $b^{0,+}$AT, y^+LAT1, aquaporin 3, 8, and 10; NRF2 gene or protein expression in SI

[1]Ig, immunoglobulin; IFN, interferon; IL, interleukin; TLR, toll-like receptor; TNF, tumor necrosis factor; ADG, average daily gain; ADFI, average daily feed intake; SCFA, short chain fatty acids; EGF, epidermal growth factor; SOD, superoxide dismutase; MDA, malondialdehyde; NOS, nitric oxide synthetase; IGF-1R, insulin-like growth factor 1 receptor; ZO, zonula occludens; DAO, diamine oxidase; HP, heat-shock protein; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; SI, small intestine; pBD-1, porcine β-defensin; AT, amino acid transporter; LAT, L-type AA transporter; NRF2, nuclear factor erythroid 2-related factor 2.
[2]Standardized ileal digestible.

isotopic tracer study by Faure et al. (2007), Thr utilization for the syntheses of small intestinal and plasma proteins increased during sepsis in rats, suggesting the increased Thr requirement in immune-stimulated status.

It is worth noting that an increase in dietary fiber elevates the dietary Thr requirement for optimal protein deposition, possibly because of the increase in physical contact of dietary fiber and thus increase in protein renewal and mucin secretion in the GIT (Wellington et al., 2018). This may be relevant when a simple diet or a diet containing a high amount of alternative plant-based ingredients is fed, instead of a conventional complex diet, as a means to save feed cost.

2.7. PROSPECTIVE AND DIRECTION

Traditional requirements are estimated based on growth performance and protein deposition as response criteria. However, as discussed above, ISS alters the FAA metabolic pathway. Therefore, dietary FAA supplementation can be a nutritional strategy to enhance the immune system and minimize growth retardation in immune system-stimulated weaned pigs. Although the biologic function of AA has been well documented in different animals and physiologic states, further studies should focus on FAA in relation to specific weaning stressors, such as transportation, vaccination, pathogen infection, and thermal stress.

Due to the difficulty in quantifying the extent of immune stimulation, it would be impossible to determine the precise value of the requirement for each AA, unlike conventional requirements. It would be more practical to supplement FAA as an immunomodulatory feed additive for a certain period and in a certain environment in which the farmers believe that weaned pigs suffer from weaning stress. Surplus of dietary AA is a metabolic burden as well as economically wasteful. Therefore, we recommend providing AA requirements for two physiologic states of nursery pigs: normal physiological state and immune system stimulated-state.

3. MANUSCRIPT I

Diet Complexity and L-Threonine Supplementation: Effects on Nutrient Digestibility, Nitrogen and Energy Balance, and Body Composition in Nursery Pigs

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BK: conducted the animal trial, laboratory analyses, data collection, and wrote the paper; JL: participated in animal trial, laboratory analyses, and data collection; CMN had primary responsibility for the final content; and all authors: read and approved the final manuscript.

3.1. ABSTRACT

This study was conducted to investigate the effects of dietary complexity and L-Thr supplementation on energy and nutrient utilization in nursery pigs. Thirty-two nursery pigs (7.23 ± 0.48 kg) were randomly assigned to a 2 × 2 factorial treatment arrangement based on diet complexity (complex vs. simple) with different levels of L-Thr supplementation. The complex diet contained animal protein sources (e.g., fish meal, plasma) and a dairy product (e.g., dried whey) to mimic a conventional nursery diet. The simple diet was formulated with corn, wheat, and soybean meal. Both diets were supplemented with L-Thr to contain either 100% (STD Thr) or 115% (SUP Thr) of the estimated SID Thr requirement for 9 kg BW pigs (NRC, 2012). The pigs were individually housed in metabolism crates and fed an experimental diet *ad libitum* for a 7-day adaptation period and 5 days of total but separate urine and fecal collection. On day 14, all pigs were euthanized to determine body composition. The diet complexity, L-Thr supplementation, and their interactions were considered main effects. Pigs fed the complex diet tended to exhibit greater ($P < 0.10$) apparent total tract digestibility (ATTD) of ash and urinary energy output than those fed the simple diet. The complex diet had greater ($P < 0.05$) digestible energy and net energy contents than the simple diet. Furthermore, the complex diet-fed pigs had lower ($P < 0.05$) plasma urea nitrogen concentration on day 14 than simple diet-fed pigs. The SUP Thr decreased ($P < 0.05$) ATTD of acid detergent fiber but trended ($P < 0.10$) toward a decrease in urinary nitrogen (N) output and an increase in N retention and body N mass. In conclusion, the simple diet for nursery pigs had lower digestible and net energy contents than a complex diet. The SUP Thr can improve N utilization and body protein deposition, irrespective of diet complexity.

Key words: body composition, diet complexity, energy content, nursery pigs, nutrient digestibility, L-threonine

3.2. INTRODUCTION

Conventional nursery pig diets contain a variety of digestible and palatable ingredients, such as animal proteins and dairy products, resulting in high diet complexity and feed cost (Wolter et al., 2003). Many efforts (Dritz et al., 1996; Wolter et al., 2003; Mahan et al., 2004) have been made to simplify the conventional composition of complex nursery diets in order to reduce feed cost without compromising growth performance. However, simple diets generally contain high levels of soybean meal, which replaces animal proteins and dairy products used in complex diets, and, consequently, high levels of non-starch polysaccharides (**NSP**), which are known to have a negative effect on nutrient digestibility (Souffrant, 2001). Furthermore, simple diets generally contain more antinutritional factors such as antigenic compounds (e.g., conglycinin and β-conglycinin; Li et al. 1990), than conventional complex diets, which compromise intestinal integrity and immunity (Koo et al., 2017), possibly causing N imbalance and disrupting the nutrient and energy balance.

Threonine is usually the third-limiting AA in cereal-based swine diets and a major substrate for the synthesis of intestinal mucins and Ig in pigs (Le Floc'h et al., 2018). Previous studies reported that dietary $_{L}$-Thr supplementation over the requirement improved intestinal integrity and mucin production in nursery pigs (Ren et al., 2014; Zhang et al., 2019). However, despite the close relationship between gut integrity and nutrient digestibility in young pigs (Pluske et al., 2018b), no studies have focused on the impact of dietary Thr supplementation above requirement on nutrient and energy utilization in pigs.

PUN has been widely used as a simple biomarker to test dietary AA balance in animal nutrition. The urea is the major product of N in excessive or oxidized AA in the body and is excreted through urine (Barrett, 2014). In light of this, attempts have been made to extrapolate

urinary N excretion and N retention from the PUN concentration in growing pigs (Zervas and Zijlstra, 2002a, b). However, no information is available on the correlation between PUN and urinary N excretion or N retention and deposition in young pigs.

We hypothesized nursery pigs fed a simple diet would show poorer nutrient and energy utilization than those fed a conventional complex diet. Dietary L-Thr supplementation may ameliorate the negative effects of a simple diet, thereby maintaining body composition comparable with that of pigs fed a complex diet. The objective of this study was to investigate the effects of diet complexity and L-Thr supplementation on nutrient and energy balance and body composition in nursery pigs, as well as, to determine the correlation between PUN concentration and N balance or body N mass at slaughter.

3.3. MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Committee (AC11414), and the pigs were cared for according to the guidelines of the Canadian Council on Animal Care (2009).

3.3.1. ANIMALS, HOUSING, DIETS, AND EXPERIMENTAL DESIGN

Thirty-two male piglets at 28 days of age (TN70 × TN Tempo; Topigs Norsvin, Winnipeg, MB, Canada) with an initial BW of 7.23 ± 0.48 kg were obtained from the Glenlea Research Station at the University of Manitoba. During the lactation period, the piglets were surgically castrated on d 3 of age and exposed to creep feed from day 10 of age until weaning at 21 day of age. For a week post-weaning, all pigs were fed a commercial starter diet. The pigs were individually housed for 14 days in adjustable metabolism crates (1.80 × 0.60 m) with smooth, transparent plastic sides and plastic-covered expanded metal sheet flooring. Each crate was equipped with a stainless-steel

feeder and a nipple drinker, which allowed the pigs *ad libitum* access to feed and water. The pigs were randomly assigned to a 2 × 2 factorial design based on diet complexity (complex vs. simple) and L-Thr supplementation. The complex diet contained animal protein sources (e.g., fish meal, spray-dried animal plasma) and a dairy product (e.g., dried whey) to mimic a conventional nursery diet (Table 3.1). The simple diet was formulated with corn, wheat, and soybean meal. L-Thr was supplemented to each diet to supply 100% (**STD Thr**) and 115% (**SUP Thr**) of estimated NRC (2012) requirement for 9 kg of BW, which correspond to 0.790% and 0.909% of SID Thr, respectively. All the experimental diets were formulated to meet or exceed the NRC (2012) requirement for 9 kg of BW. Room temperature was maintained at 29 ± 1ºC during week 1 and 28 ± 1ºC during week 2.

3.3.2. EXPERIMENTAL PROCEDURE AND SAMPLE PREPARATION

The pigs were allowed a 7-day period of adaptation to experimental diets and environmental conditions. On days 8 and 12, 3 g of ferric oxide was fed as an indigestible marker (Product number 310050; Sigma-Aldrich, St. Louis, MO) to mark the beginning and the end of fecal collection. From days 9 to 14, total feces were collected twice daily, in the morning and in the evening, and stored at −20°C. Urine was collected every morning in jugs containing 10 mL of 6 *N* hydrochloric acid to minimize N losses. The collected urine was weighed for 5 days, from days 9 to 14, and the subsample of the urine was obtained, strained through glass wool, and stored at −20°C. On days 7 and, blood samples (10 mL) from the jugular veins of all the pigs were collected in vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). For body compositions analysis, all pigs were euthanized on day 14 by captive bolt following stresnil-xylazine (2:4 mg/kg) sedation. The abdominal cavity was opened from the sternum to the pubis to expose the entire GIT, which was then emptied and weighed to calculate empty BW.

Table 3.1 Diet composition and nutrients contents of experimental diets, % (as-fed basis)[1]

	Complex		Simple	
Item	STD Thr	SUP Thr	STD Thr	SUP Thr
Ingredient				
Corn	33.123	33.002	33.175	33.055
Wheat	30.00	30.00	30.00	30.00
Soybean meal	13.00	13.00	30.30	30.30
Spray dried animal plasma	5.00	5.00	-	-
Fish meal	5.00	5.00	-	-
Dried whey	10.00	10.00	-	-
Vegetable oil	0.80	0.80	1.80	1.80
Limestone	1.09	1.09	1.30	1.30
Monocalcium phosphate	0.20	0.20	1.05	1.05
Salt	0.35	0.35	0.35	0.35
Vitamin-mineral premix[1]	1.00	1.00	1.00	1.00
$_{L}$-Lys·HCl	0.322	0.322	0.552	0.552
$_{DL}$-Met	0.097	0.098	0.185	0.185
$_{L}$-Thr	0.018	0.138	0.162	0.282
$_{L}$-Trp	-	-	0.012	0.012
$_{L}$-Val	-	-	0.114	0.114
Calculated nutrients				
Metabolizable energy, Mcal/kg	3.30	3.30	3.30	3.30
Total calcium, %	0.80	0.80	0.80	0.80
STTD[3] phosphorus, %	0.40	0.40	0.40	0.40
SID[4] Lys, %	1.35	1.35	1.35	1.35
SID[4] Thr, %	0.79	0.91	0.79	0.91

[1]STD Thr, the standard NRC (2012) level of standardized ileal digestible Thr (0.79%); SUP Thr, 15% over the standardized ileal digestible Thr requirement.
[2]Supplied per kilogram of diet: vitamins A, 2,200 IU; vitamin D_3, 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; thiamine, 1.5 mg; riboflavin, 4 mg; niacin, 30 mg; pantothenic acid, 12 mg; vitamin B_{12}, 0.02 mg; folic acid, 0.3 mg; 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; biotin 0.2 mg.
[3]Standardized total tract digestible.
[4]Standadized ileal digestible.

The carcass and internal organs were frozen separately at –20°C. Care was taken to ensure all blood was collected.

The blood samples were centrifuged at 2,000 × g for 10 min at 4°C to obtain plasma, which was immediately stored at –80°C until required for PUN analysis. Fecal samples were dried in a forced-air oven (CDO-28, Cascade Sciences, Hillsboro, OR) at 60°C for 3 days, weighed, pooled on a pig basis, and finely ground before chemical analysis. Frozen urine samples were thawed and pooled independently for each pig, sieved through cotton gauze, and filtered with glass wool. Frozen carcasses, organs, and blood were ground as described by Ayoade et al. (2012) with minor modifications. In brief, carcasses and organs were cut into smaller pieces using an electric band saw and then ground in a meat grinder (model H600, Hobart Manufacturing Co. Ltd., Toronto, ON, Canada) to pass through a 12-mm die. The ground pieces were mixed in a mixer for 10 min to ensure even distribution and facilitate uniform sampling. Samples of approximately 500 g were taken from the mixtures and were later freeze-dried. The samples were weighed before and after freeze-drying to calculate the body water content.

3.3.3. CHEMICAL ANALYSES

Diet, fecal, and mixed carcass samples were finely ground with a grain miller (50−200 μm of fineness; HC-700, Boshi Electronic Instrument, Guangzhou, China). Diet and fecal samples were analyzed for DM, N, neutral detergent fiber (**NDF**), acid detergent fiber (**ADF**), and ash content. Diet samples were further analyzed for ether extract (**EE**), starch, NSP, and AA. Carcass samples were analyzed for DM, N, EE, and ash. Urine samples were analyzed for gross energy (**GE**) and N.

The DM (method 934.01), EE (method 920.39A), and ash (942.05) were determined according to the methods of the Association of Official Analytical Chemists (AOAC, 2006). The

N content was determined using a combustion analyzer (model CNC-2000; Leco Corporation, St. Joseph, MI; method 984.13A-D) and was used to calculate the crude protein concentration (N × 6.25). The GE was determined using an isoperibol bomb calorimeter (Parr Instrument Co., Moline, IL), which had been calibrated using benzoic acid as a standard. The ash content was determined according to AOAC method 942.05 (2006). The organic matter was calculated as DM minus ash. The starch content was measured using an assay kit (Megazyme Total Starch assay kit; Megazyme International Ltd., Wicklow, Ireland). The NSP content was analyzed using gas-liquid chromatography (neutral sugar components; Varian CP-3380 Gas Chromatograph, Varian Medical Systems Inc., Palo Alto, CA), colorimetry (uronic acids; Biochrom Ultrospec 50, Biochrom Ltd., Cambridge, UK), and the procedure described by Englyst and Cummings (1988) with some modifications (Slominski and Campbell, 1990). The ADF and NDF contents were analyzed according to the method of Goering and van Soest (1970) using α-amylase (product number, A3306; Sigma-Aldrich, St. Louis, MO). The hemicellulose content was calculated as NDF minus ADF content. The AA composition in the diets was determined after acid hydrolysis according to method 994.12 of the AOAC (2006). The total sulfur AA content was determined after performic acid oxidation followed by acid hydrolysis (AOAC method 985.18; 2006). The gross energy of urine was determined as described by Koo et al. (2018). In brief, 0.5 g of cellulose was dried at 100°C for 24 h, and 2 mL of urine sample was sprayed over it. The urine-cellulose mixture, along with a sample of pure cellulose, was again dried at 50°C for 24 h and then weighed to estimate the urine DM. The GE of the dried urine-cellulose mixture and the pure cellulose was determined using a bomb calorimeter, with which the GE of the urine samples was calculated by the difference method. Plasma samples were analyzed for PUN contents at the Veterinary Diagnostic Services of

Manitoba Agriculture a VITROS 250 Chemistry System (Ortho-Clinical Diagnostics Inc., Raritan, Rochester, NY).

3.3.4. CALCULATION AND STATISTICAL ANALYSES

The apparent total tract digestibility (**ATTD**) of nutrients and energy and nutrient retention (%) were determined by the total collection method using the equations described by Kong and Adeola (2014). The digestible energy (**DE**) and ME contents of the experimental diets were determined using the following equations:

DE (kcal/kg) = [(ATTD of GE, %) × (GE content in diets, kcal/kg)]/100 and

ME (kcal/kg) = [(GE retention, %) × (GE content in diets, kcal/kg)]/100.

The DE and ME contents were used to calculate the net energy (**NE**) according to the equations established by Noblet et al. (1994):

NE = 0.700 × DE + 1.61 × EE + 0.48 × starch – 0.91 × crude protein – 0.87 × ADF and

NE = 0.726 × ME + 1.33 × EE + 0.39 × starch – 0.62 × crude protein – 0.83 × ADF,

where NE, DE, and ME are expressed in kilocalories per kilogram, and EE, starch, crude protein, and ADF are expressed in grams per kilogram. All data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) with each animal used as an experimental unit. The model included diet complexity, the level of SID Thr, and their interaction. The LSMEANS statement with the Tukey-adjusted PDIFF option was used to calculate and separate the mean values for each treatment. The correlation between PUN and N balance was evaluated by a simple linear correlation analysis (Pearson correlation coefficient) using the CORR procedure of SAS. Results were considered significant at $P < 0.05$, and tendencies were observed at $0.05 < P \leq 0.10$.

Table 3.2 Analyzed nutrient components of experimental diets, % (as-fed basis)

	Complex		Simple	
Item	STD Thr	SUP Thr	STD Thr	SUP Thr
Dry matter	91.7	91.6	90.5	90.4
Organic matter	86.7	86.4	86.1	85.8
Crude protein	20.0	21.1	20.1	20.1
Gross energy, Mcal/kg	4.04	4.04	3.99	3.97
Ether extract	2.81	2.86	3.26	3.50
Starch	64.1	62.5	65.5	64.5
NSP, mg/g[1]				
Arabinose	10.12	11.24	13.38	13.41
Xylose	15.77	16.48	17.03	16.86
Galactose	8.99	9.76	12.70	12.53
Glucose	25.76	26.68	30.31	31.31
Uronic acid	8.32	8.29	14.50	13.94
Total	68.96	72.45	87.92	88.05
Neutral detergent fiber	7.7	8.7	8.6	8.0
Acid detergent fiber	3.0	2.4	3.4	3.0
Indispensable amino acids				
Arg	1.08	1.10	1.16	1.20
His	0.64	0.67	0.54	0.58
Ile	0.66	0.62	0.62	0.71
Leu	1.61	1.61	1.48	1.54
Lys	1.50	1.54	1.53	1.57
Met	0.43	0.44	0.50	0.50
Phe	0.87	0.87	0.91	0.93
Thr	0.89	1.02	0.82	0.97
Val	0.89	0.85	0.82	0.91
Dispensable amino acids				
Ala	1.11	1.14	1.04	1.01
Asp	1.83	1.89	1.99	2.02
Cys	0.43	0.43	0.35	0.36
Glu	1.24	1.27	1.27	1.27
Gly	3.69	3.76	4.06	4.13
Pro	0.85	0.86	0.79	0.80
Ser	1.16	1.20	1.12	1.11
Tyr	0.60	0.59	0.54	0.54

[1]Non-starch polysaccharides; rhamnose and mannose were not detected.

3.4. RESULTS AND DISCUSSION

The complex diet in this study was formulated to include animal protein sources such as fish meal, plasma meal, and dried whey to mimic a conventional nursery pig diet. In contrast, the simple diet contained a high proportion of soybean meal (30.3%) to replace the animal protein sources. Both basal diets contained L-Thr to supply 0.79% of SID Thr (**STD Thr**), which is recommended for pigs with 9 kg of BW when fed 3.3 Mcal/kg of diet (NRC, 2012). An additional 0.12% of L-Thr was supplemented to each diet at the expense of corn to supply 0.91% SID Thr (SUP Thr). The supplementation level (115% of the requirement) was based on our previous study (Jayaraman et al., 2015), where the optimum dietary SID Thr was obtained for nursery pigs whose immune system is compromised. The SID Lys contents in all diets remained at 1.35% to precisely meet the estimated requirement of NRC (2012) and to maintain SID Lys:SID Thr within STD Thr-diets and SUP Thr-diets. The SID contents of all other essential AA exceeded the requirement. Analyzed diet composition showed that the additional Thr supplementation clearly increased the total Thr contents (Table 3.2) in both the complex and simple diets. The pigs were fed ad libitum to consider whether feed intake that may be affected by different diet composition could be a factor that might contribute to nutrient and energy digestibility. However, neither diet complexity nor SID Thr contents affected the ADG and ADFI throughout the experimental period (data not shown).

Complex diets are generally considered more digestible compared to simple diets. This is because animal protein sources and dairy products are processed and highly digestible. Furthermore, soybean meal and other plant-based protein sources are relatively rich in antinutritional factors such as NSP as well as in antigenic compounds, which could possibly interfere with digestion and absorption in the gut (Li et al., 1990; Montagne et al., 2003). Indeed,

Table 3.3 Effect of dietary complexity and threonine supplementation on apparent total tract digestibility (ATTD), nitrogen balance, and plasma urea nitrogen (PUN) concentration[1]

	Complex		Simple			*P*-values[2]		
Item	STD Thr	SUP Thr	STD Thr	SUP Thr	SEM	Diet	Thr	Diet × Thr
No. of replicates	8	8	9	7				
ATTD, %								
Dry matter	90.2	88.8	88.4	89.4	0.66	0.303	0.797	0.057
Organic matter	91.1	89.7	89.4	90.4	0.61	0.450	0.775	0.047
Nitrogen	86.1	86.0	84.2	86.0	1.16	0.390	0.446	0.426
NDF[3]	71.8	72.7	68.8	69.1	2.00	0.090	0.769	0.857
ADF[4]	68.2	54.5	63.3	58.7	3.03	0.893	0.003	0.124
Hemicellulose	74.1	79.8	72.5	75.5	2.74	0.259	0.100	0.600
Ash	76.0	74.9	67.9	70.5	1.85	0.001	0.675	0.282
Nitrogen balance								
Intake, g/d	19.7	20.2	16.9	18.1	1.69	0.133	0.599	0.824
Fecal output, g/d	2.6	2.7	2.7	2.5	0.22	0.719	0.763	0.517
Urinary output, g/d	2.0	1.2	1.4	1.0	0.34	0.218	0.058	0.472
Retained, % of intake	74.1	79.9	76.0	79.7	2.79	0.760	0.081	0.686
Retained, g/d	15.1	16.3	12.8	14.6	1.74	0.237	0.356	0.874
PUN, mmol/L								
day 7	3.21	2.36	3.65	3.37	0.501	0.129	0.235	0.546
day 14	2.68	2.30	3.62	3.32	0.449	0.025	0.422	0.935
Average	2.94	2.33	3.64	3.34	0.409	0.032	0.244	0.678

[1]STD Thr, the standard NRC (2012) level of standardized ileal digestible Thr (0.79%); SUP Thr, 15% over the standardized ileal digestible Thr requirement.

[2]Diet, main effect of diet complexity; Thr, main effect of the levels of dietary Thr supplementation; Diet × Thr, interactive effect of diet complexity and Thr supplementation.

[3]Neutral detergent fiber.

[4]Acid detergent fiber.

analyzed NSP contents confirmed that the simple diets contained approximately 8.8% NSP, which was 24% higher than the content in the complex diets. Our previous study (Koo et al., 2017) showed that pigs fed a complex diet had greater ATTD of DM, OM, and NDF than those fed a simple animal protein–free diet. However, in this study, only a higher ATTD of ash was observed in the complex diet compared to the simple diet group ($P < 0.01$), whereas no differences were observed in the ATTD of DM, OM, CP, and GE (Table 3.3). This discrepancy may be attributed to the effect of the interaction between diet complexity and L-Thr supplementation on the ATTD of DM ($P = 0.06$) and OM ($P < 0.05$) in this study, which offsets the effects of diet complexity. Indeed, when the ATTD of the complex (STD Thr) and simple (STD Thr) groups were separately compared using Student's t-test, a greater ($P < 0.05$) ATTD of DM and OM was observed in the complex (STD Thr) group. Because both diets were formulated to contain identical digestible phosphorus contents, the greater ATTD of ash with the complex diet may reflect the fact that nursery pigs digest other minerals more readily from animal proteins than from soybean meal. In fact, plasma meal and fish meal are relatively rich in chloride, sodium, sulfur, iron, and zinc, which are reserved in an inorganic form (Delaney, 1975; NRC, 2012), whereas minerals such as manganese, magnesium, and zinc in plant-based ingredients are commonly present in organic forms such as phytate-bound minerals, which are poorly available to pigs (Adeola, 1995; Woyengo and Nyachoti, 2013). This is in accordance with our previous study (Koo et al., 2017), where a significantly greater ATTD of ash was found in pigs fed a complex diet than in those fed a simple animal protein–free diet, whereas the difference was not observed when the simple diet contained a certain amount of animal protein.

The PUN concentration has been regarded as a reliable biomarker for determining the balance of dietary AA (Brown and Cline, 1974). In this study, simple diet-fed pigs had an increased

($P < 0.05$) PUN concentration on day 14 than the complex diet-fed pigs (Table 3.3). Similarly, pigs fed a simple diet showed a greater ($P < 0.05$) average of PUN concentrations on days 7 and 14 than those fed a complex diet. An increase in PUN indicates an excess or deficiency of certain AA (Brown and Cline, 1974). Given that both diets were formulated to contain comparable total N contents, it is likely that the simple diet had certain limiting AA. The SID essential AA in both diets exceeded the estimated NRC (2012) requirement for 9 kg of BW, while the SID Lys and Thr in the basal diet precisely met the requirement. Thus, it is probable that a relatively high NSP content or antigenic compounds (e.g., glycinin and β-conglycinin) in the simple diet inhibited dietary AA digestion or absorption and increased endogenous AA loss (Schulze et al., 1994; Qin et al., 1996; Souffrant, 2001), rendering Lys or Thr to be limiting AA.

The complex diet contained greater ($P < 0.05$) DE and NE than the simple diet, whereas only a numerical difference ($P = 0.107$) was observed in ME (Table 3.4). Given that there were no differences in the energy-partitioning efficiencies (e.g., ME:DE or NE:ME), the observed difference appears to be mainly a result of the higher GE content and energy digestibility of the complex diet. However, only a numerical difference in the ATTD of GE was observed between the two diets, suggesting that the numerically greater GE content in the complex diet may have been the main contributor to the greater DE and NE content compared with the simple diet. It should also be noted that, contrary to our expectation, the calculated ME content (3.3 Mcal/kg) was lower than the actual measurement (3.4 Mcal/kg), which probably reflects an underestimation of the amount of feces or urine excreted. This underestimation is likely greater with nursery pigs, which produce smaller quantities of feces and urine than growing pigs. Furthermore, there may be an overestimation of tabulated values of ingredients used in the present study.

Table 3.4 Effect of dietary complexity and threonine supplementation on apparent total tract digestibility (ATTD), nitrogen balance, and plasma urea nitrogen (PUN) concentration[1]

	Complex		Simple			*P*-values[2]		
Item	STD Thr	SUP Thr	STD Thr	SUP Thr	SEM	Diet	Thr	Diet × Thr
No. of replicates	8	8	9	7				
ATTD, %								
Dry matter	90.2	88.8	88.4	89.4	0.66	0.303	0.797	0.057
Organic matter	91.1	89.7	89.4	90.4	0.61	0.450	0.775	0.047
Nitrogen	86.1	86.0	84.2	86.0	1.16	0.390	0.446	0.426
NDF[3]	71.8	72.7	68.8	69.1	2.00	0.090	0.769	0.857
ADF[4]	68.2	54.5	63.3	58.7	3.03	0.893	0.003	0.124
Hemicellulose	74.1	79.8	72.5	75.5	2.74	0.259	0.100	0.600
Ash	76.0	74.9	67.9	70.5	1.85	0.001	0.675	0.282
Nitrogen balance								
Intake, g/d	19.7	20.2	16.9	18.1	1.69	0.133	0.599	0.824
Fecal output, g/d	2.6	2.7	2.7	2.5	0.22	0.719	0.763	0.517
Urinary output, g/d	2.0	1.2	1.4	1.0	0.34	0.218	0.058	0.472
Retained, % of intake	74.1	79.9	76.0	79.7	2.79	0.760	0.081	0.686
Retained, g/d	15.1	16.3	12.8	14.6	1.74	0.237	0.356	0.874
PUN, mmol/L								
day 7	3.21	2.36	3.65	3.37	0.501	0.129	0.235	0.546
day 14	2.68	2.30	3.62	3.32	0.449	0.025	0.422	0.935
Average	2.94	2.33	3.64	3.34	0.409	0.032	0.244	0.678

[1]STD Thr, the standard NRC (2012) level of standardized ileal digestible Thr (0.79%); SUP Thr, 15% over the standardized ileal digestible Thr requirement.

[2]Diet, main effect of diet complexity; Thr, main effect of the levels of dietary Thr supplementation; Diet × Thr, interactive effect of diet complexity and Thr supplementation.

[3]Neutral detergent fiber.

[4]Acid detergent fiber.

In this study, SUP Thr exhibited lower ($P < 0.01$) ATTD of ADF. No clear explanation for this can be offered based on our results. However, given that pig's digestive enzymes cannot hydrolyze ADF (Souffrant, 2001), the difference may be attributed to a re-equilibrium of the gut microbiota through dietary L-Thr supplementation. This is partially supported by a previous study (Dong et al., 2017), in which supplementing a low-protein diet with L-Thr modified the cecal microbiota composition by promoting saccharolytic bacteria (e.g., Faecalibacterium and Parabacteroides). Similarly, Faure et al. (2007) suggested that dietary supplementation of AA mixture containing L-Thr altered the overall commensal microbiota growth, favoring *Bacteroides* and *Lactobacillus*. However, dietary L-Thr is known to be rapidly absorbed through B system transporters such as B^0AT1 at the brush border in the small intestine (Maenz and Patience, 1992), and thus it is postulated that it indirectly alters the gut microbiota composition by mediating intestinal integrity and secretion, thereby altering the microbial niche environment (Li et al., 2015). Dietary L-Thr supplementation may have altered the absorption kinetics of dietary AA and specific endogenous losses such as proteins and phospholipids (Have et al., 2007; Dong et al., 2017), which modifies the amount and profile of nutrients that reach the hindgut. Furthermore, our results indicating an increase trend in the ATTD of hemicellulose in SUP Thr-group ($P = 0.100$) suggest that L-Thr supplementation selectively altered the microbiota composition by promoting hemicellulolytic and suppressing cellulolytic bacteria. Also, the observed effect of the interaction between diet complexity and L-Thr supplementation on the ATTD of DM ($P = 0.06$) and OM ($P < 0.05$) suggests that the modification may be mediated by contents such as that of NSP in the diet. However, microbiological measurements are required to elucidate these findings.

Interestingly, SUP Thr tended to decrease the daily N excretion ($P = 0.058$), thereby increasing the N retention rate ($P = 0.081$), irrespective of diet complexity (Table 3.3). This

suggests that the absorbed Thr increased protein synthesis and reduced oxidation of dietary AA thus reduced excretion through urine. Previous studies using the stable isotope methodology revealed that the major metabolic fate of Thr is its incorporation into various proteins, particularly mucosal (e.g., mucins and sIgA) in pigs (Floc'h and Sève, 2005; Schaart et al., 2005). As reported in a companion paper using the same pigs, SUP Thr significantly improved goblet cell density and VH in the jejunum. Therefore, it is likely that NRC (2012) requirement for SID Thr does not supply sufficient Thr for the gut mucosa to achieve the maximum mucosal protein synthesis, thereby mobilizing Thr in body protein and increasing urinary excretion of N that was not involved in the mucosal protein synthesis (Obled, 2003). This is in line with our study, where SUP Thr tended to increase body protein mass ($P = 0.061$) as shown in Table 3.5. However, our speculation was based on the assumption that the Thr requirement suggested by NRC (2012) is sufficient for optimal whole-body protein synthesis.

Dietary AA undergo oxidative catabolism, producing NH_3 in the body as a result of protein turnover and oxidation of the excessive AA after protein synthesis through deamination and transamination (Wu, 2009). The produced NH_3 is converted into urea, which is transported through the blood to the kidneys for excretion through urine. In light of this, attempts have been made to predict the urinary N excretion and eventually estimate N retention or protein deposition in growing pigs (Zervas and Zijlstra, 2002a, b) because urine collection is a tedious task requiring specially designed metabolism crates. In this study, as shown in Table 3.6, the average PUN concentration of days 7 and 14 was negatively correlated with the N retention rate ($P < 0.01$; $R^2 = 0.27$), the daily N retention amount ($P < 0.01$; $R^2 = 0.28$), and the body N mass ($P < 0.05$; $R^2 = 0.13$), whereas there was a trend for a positive correlation with the urinary N output ($P = 0.059$; $R^2 = 0.12$). These correlations clearly demonstrate the aforementioned fate of N in the body.

Table 3.5 Effect of dietary complexity and threonine supplementation on body weight (BW) and body composition[1]

Item	Complex		Simple		SEM	*P*-values[2]		
	STD Thr	SUP Thr	STD Thr	SUP Thr		Diet	Thr	Diet × Thr
No. of replicates	8	8	9	7				
Live BW	12.7	12.8	11.8	13.0	0.65	0.507	0.286	0.373
Empty BW, kg	12.1	12.3	11.2	12.2	0.64	0.419	0.331	0.518
Body composition, % of empty BW								
Water mass	73.7	73.8	73.5	73.4	0.37	0.356	0.968	0.764
Protein mass	14.5	15.5	15.1	15.3	0.50	0.686	0.191	0.444
Lipid mass	7.3	7.2	7.5	7.7	0.34	0.306	0.955	0.650
Ash mass	2.5	2.6	2.6	2.5	0.06	0.846	0.516	0.252
Body composition, kg								
Water mass	8.93	9.10	8.26	8.98	0.464	0.373	0.320	0.530
Protein mass	1.76	1.99	1.66	1.89	0.119	0.402	0.061	0.972
Lipid mass	0.90	0.90	0.85	0.94	0.071	0.956	0.480	0.498
Ash mass	0.31	0.31	0.29	0.30	0.013	0.262	0.431	0.888

[1]STD Thr, the standard NRC (2012) level of standardized ileal digestible Thr (0.79%); SUP Thr, 15% over the standardized ileal digestible Thr requirement.

[2]Diet, main effect of diet complexity; Thr, main effect of the levels of dietary Thr supplementation; Diet × Thr, interactive effect of diet complexity and Thr supplementation.

Table 3.6 Pearson correlation coefficients (R) between plasma urea nitrogen (PUN) concentration and nitrogen (N) balance

	N urinary output, g/day	N retention, % of intake	N retention, g/d	Body protein, %	Body protein mass, kg
PUN (day 7)					
R	0.33	-0.46	-0.46	0.02	-0.37
P-value	0.069	0.008	0.008	0.889	0.036
PUN (day 14)					
R	0.26	-0.45	-0.47	0.04	-0.26
P-value	0.144	0.010	0.006	0.813	0.150
PUN (Average)					
R	0.34	-0.52	-0.53	0.04	-0.36
P-value	0.059	0.003	0.002	0.832	0.041

However, the obtained R^2 of each correlation suggests that the PUN concentration is not a sound variable for predicting either urinary N output and N retention or body protein mass. In fact, the significant difference in PUN between the simple diet- and the complex diet-fed pigs did not coincide with N retention, suggesting that PUN concentration does not always correspond to N retention. Similarly, Zervas and Zijlstra (2002a) found that the PUN concentration was highly correlated with urinary N excretion (R^2 = 0.71), but the 95% prediction intervals of their models demonstrated the inaccuracy of using predictions of N status. This may be attributed to individual variations in physiological condition, particularly regarding postprandial periods and urination times. Furthermore, it should be noted that a paucity of AA and peptides can contribute to the total urinary N, and that approximately 25% of systemic urea is transported into the intestinal lumen thereby excreted through feces as ammonium ion (Barrett, 2014). This may account for the closer correlations of PUN with N retention and body protein mass with higher R^2 compared with urinary N output.

3.5. CONCLUSION

The simple diet had lower DE and NE contents than the complex diet. Although the PUN concentrations were higher with the simple than with the complex diet, no difference in N retention was observed between the two groups. Therefore, the lower energy content of the simple diet, rather than N utilization, may be the decisive factor leading to the consistently reported poor growth performance of simple diet-fed pigs compared to complex diet-fed pigs. Addition of dietary $_{L}$-Thr to supply 115% of the NRC (2012) requirement did not increase the nutrient and energy digestibility but did increase the dietary N retention rate by reducing urinary N excretion, irrespective of diet complexity. This benefit appears to extend to a body protein mass increase. Thus, this requires additional study with a large number of animals to confirm that the SID Thr

requirement suggested by the NRC (2012) is underestimated for optimal body protein accretion. In terms of DM and OM digestibility, SUP Thr interacts with diet complexity. This mechanism requires further research, particularly in relation with the gut microbiota. Finally, although PUN is highly correlated with the body's N status, it is not a sound biomarker for extrapolating urinary N excretion and N retention. Therefore, it seems necessary to collect urine and feces in order to evaluate dietary N balance accurately in nursery pigs.

4. MANUSCRIPT II

Diet Complexity and L-Threonine Supplementation: Effects on Growth Performance, Immune Response, Intestinal Barrier Function, and Microbial Metabolites in Nursery Pigs

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BK: conducted the animal trial, laboratory analyses, data collection, and wrote the paper; JC: participated in animal trial, laboratory analyses, and data collection; CY provided essential materials for analyses, provided laboratory equipment, and reviewed the paper; CMN had primary responsibility for the final content; and all authors: read and approved the final manuscript.

TRANSITION STATEMENT

Manuscript I and II were written based on data obtained from Experiment 1. Manuscript I focused on the effect of dietary Thr supplementation in relation with diet complexity on nutrient utilization and body composition in nursery pigs. Samples were further analyzed for gut health indicators to explorer the effects of dietary Thr supplementation in association with diet complexity on immunity, microbial metabolites, and intestinal barrier function. Based on the data, Manuscript II was written.

4.1. ABSTRACT

The aim of this study was to investigate the effects of diet complexity and L-Thr supplementation level on the growth performance, immune response, intestinal barrier function, and microbial metabolites in nursery pigs. Thirty-two weaned pigs (BW 7.23 ± 0.48 kg) were randomly assigned to dietary treatments in a 2 × 2 factorial arrangement based on diet complexity (complex or simple) and dietary Thr content. The complex diet contained fish meal, plasma protein and dried whey to mimic a conventional nursery diet. The simple diet was formulated with corn, wheat, and soybean meal and did not contain any animal products. L-Thr was supplemented to each diet to supply either 100% (STD Thr) or 115% (SUP Thr) of the NRC (2012) requirement for SID Thr. Pigs were individually housed and fed experimental diets ad libitum for 14 d. Diet complexity, dietary Thr content, and their interactions were considered main effects. Pigs fed the simple diet had greater ($P < 0.05$) plasma interleukin (IL)-10 and IL-6 concentrations compared to those fed the complex diet on days 7 and 14, respectively. Simple diet-fed pigs tended to show greater ($P < 0.10$) expression of genes encoding for tumor necrosis factor-α, claudin-1, and ZO-1 in the jejunum compared to complex diet-fed pigs. The simple diet–fed pigs had greater ($P < 0.05$) concentrations of NH_3-N in the jejunum digesta than did complex diet–fed pigs. The SUP Thr increased ($P < 0.05$) VH and goblet cell density in villi and crypts in the jejunum and deepened ($P < 0.05$) crypts in the

proximal colon. The SUP Thr resulted in the upregulation ($P < 0.05$) of occludin gene expression and a tendency toward downregulation ($P = 0.10$) of IL-6 gene expression in the jejunum. Interactions ($P < 0.05$) between diet complexity and L-Thr supplementation level were observed in goblet cell density in the crypt, NH_3-N concentration in the jejunum, and the contents of acetate, propionate, and total volatile fatty acids in the colon. In conclusion, feeding a simple diet to nursery pigs resulted in systemic and intestinal inflammation. The SUP Thr diet did not normalize the simple diet-induced inflammation but improved gut integrity. SUP Thr seems to have greater benefits with a simple diet than with a complex diet. Therefore, SUP Thr in a simple diet could be a beneficial nutritional strategy for enhancing gut health.

Key words: diet complexity, immune response, intestinal barrier function, nursery pigs, threonine.

4.2. INTRODUCTION

Nursery pig diets have been conventionally formulated with digestible and palatable ingredients that include animal protein sources and dairy products (Mahan et al., 2004). However, this has resulted in a highly complex diet composition and, consequently, high feed costs. Many attempts have therefore been made to simplify the conventional complex diet by increasing the proportion of soybean meal as a way to save on feed costs in nursery pig production. Previous studies have confirmed that simple diets do not compromise the growth performance at the end of the nursery phase (Skinner et al., 2014; Koo et al., 2017). However, concerns remain that the large amounts of non-starch polysaccharides and antigenic compounds in soybean meal may stimulate the pig's immune system and impair intestinal integrity (Koo et al., 2017), thereby leading to poor growth performance in pigs raised in a commercial swine barn environment (Pastorelli et al., 2012). These concerns have restricted the actual introduction of simple diets in nursery production and have stressed the need to find an economic nutritional strategy that can fortify the gut barrier function of pigs to allow a practical introduction of a simple diet in nursery production. This might be possible by focusing on dietary supplementation with Thr, a major component of mucins and γ-globulins, including Ig (Wang et al., 2009). Dietary Thr is mostly metabolized in the intestine for incorporation into the functional proteins (Floc'h and Sève, 2005; Schaart et al., 2005). When pigs are immunologically challenged, body proteins are mobilized to supply the intestine with additional Thr, which suggests that supplementation with dietary Thr above the requirement is necessary for optimal growth and immune system function in animals (Faure et al., 2007; Jayaraman et al., 2015). In this regard, previous studies (Mao et al., 2014a; Ren et al., 2014) have reported that dietary L-Thr supplementation over the NRC (2012) recommended levels improved intestinal morphology and immune status by regulating Ig and cytokines in pigs. However,

although diet composition is one of the major factors affecting gut integrity, immune system, and gut microbiota during the post-weaning period (Pluske et al., 2018a), most pig studies (Mao et al., 2014a; Ren et al., 2014) on Thr supplementation have focused on the effects of dietary Thr content in response to pathogen infection. In the present study, we hypothesized that pigs fed a simple diet would elicit systemic and gut inflammation and show impairment in their gut integrity and barrier function when compared with pigs fed a complex diet and that L-Thr supplementation would ameliorate the inflammation and the gut impairment. The objective of this study was to investigate the effects of diet complexity and L-Thr supplementation levels on the growth performance, immune response, intestinal barrier function, and microbial metabolites in nursery pigs.

4.3. MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Committee (AC11414), and the pigs were cared for according to the guidelines of the Canadian Council on Animal Care (2009).

4.3.1. ANIMALS, HOUSINGS, DIETS, AND EXPERIMENTAL DESIGN

Thirty-two male piglets (TN 70 × TN Tempo; Topigs Norsvin, Winnipeg, MB, Canada) with an initial BW of 7.23 ± 0.48 kg were obtained from the Glenlea Research Station at the University of Manitoba. The pigs were weaned at 21 d of age and fed a commercial diet for one week before the experiment commenced. The pigs were randomly assigned to a 2 × 2 factorial arrangement based on diet complexity and the levels of dietary L-Thr supplementation to give 8 replicates per treatment. Pigs were individually housed for 14 d in adjustable metabolism crates (1.80 × 0.60 m) with smooth, transparent plastic sides and plastic covered expanded sheet-metal flooring. Each crate was equipped with a stainless-steel feeder and a nipple drinker, allowing the pigs ad libitum access to feed and water. The complex diet contained animal protein sources (e.g., fish meal, spray-

dried animal plasma) and a dairy product (e.g., dried whey) to mimic a conventional nursery diet (Table 3.1). These animal protein sources and dairy product were replaced with soybean meal to make the simple diet. The two respective diets were supplemented with L-Thr to supply the standard NRC (2012) level of Thr (STD Thr) or 15% over the SID Thr requirement (SUP Thr) for pigs weighing 9 kg. All the experimental diets were formulated to meet or exceed the requirements for EAA, Ca, and standardized total tract digestible P. Room temperature was maintained at 29 ± 1ºC during week 1 and 28 ± 1ºC during week 2.

4.3.2. SAMPLING AND MEASUREMENTS

Freshly voided feces were grab-sampled, weighed, and immediately frozen at –20°C for DM determination as an indicator of fecal consistency. This procedure was performed every morning and evening from d 2 to d 14. On days 7 and 14, the BWs of the pigs and the feed disappearance were recorded to determine the ADG, ADFI, and gain to feed ratios (**G:F**). On the same days, blood samples (10 mL) from all the pigs were collected from the jugular vein into vacutainer tubes (Becton Dickson, Rutherford, NJ).

On day 14, all pigs were euthanized using captive bolt following sedation with stresnil/xylazine (2:4 mg/kg) sedation in order to collect tissue sample collection. The abdominal cavity was opened from the sternum to the pubis to expose the entire GIT. A sample of jejunum and colon were taken 2 m away from the ileocecal junction and 20 cm away from cecum, respectively. The jejunum sample was rinsed with Krebs Ringer buffer, immersed in Krebs Ringer buffer, and then immediately transported to the laboratory for *ex vivo* Ussing chamber analysis. Samples for mRNA gene expression were washed with 1 × phosphate buffered saline, immediately snap-frozen in liquid nitrogen, and then stored in a –80 °C freezer. Samples for histomorphology were immediately stored in 10% buffered formalin to fix the villi, crypts, and goblet cells (GC). The

contents of the jejunum and proximal colon were collected for analysis of short chain fatty acids (SCFA) and immediately snap-frozen and transferred into a –80 C° freezer. The pH of the jejunum and colon contents was measured by direct submergence of the pH probe (AB15 plus, Fisher Scientific, Toronto, ON, Canada).

4.3.3. TOTAL RNA EXTRACTION, COMPLEMENTARY DNA SYNTHESIS AND REAL-TIME PCR

Jejunum samples for mRNA gene expression were immersed in liquid nitrogen in a mortar and ground with a pestle. Total RNA was extracted from 80 mg ground jejunum samples using a TRIzol^{TM} Plus RNA Purification Kit (Invitrogen Canada Inc., Burlington, ON, Canada) according to the manufacturer's protocol. The quantity and quality of the isolated RNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The integrity of the total RNA was confirmed by agarose gel electrophoresis. From the extracted RNA, first-strand complementary DNA was synthesized using a high-capacity complementary DNA synthesis kit (Applied Biosystems, Burlington, ON, Canada) following the supplier's protocol. Quantitative real-time PCR was performed in duplicate reactions, using a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON, Canada), as described by Waititu et al. (2017). For the qPCR amplification, negative controls were prepared by replacing the complementary DNA with nuclease-free water. A melt curve analysis was performed with a temperature gradient of 0.1 °C/s from 70°C to 95°C to confirm that only specific products were amplified. Pairs of primers for each gene were designed with Primer-Blast based on the published cDNA sequences in the gene bank of the National Center for Biotechnology Information (Table 4.1). The specificity of all primers was confirmed by a melting curve analysis, and all assays had efficiency between 90% and 110%.

4.3.4. HISTOMORPHOLOGY MEASUREMENT

After fixation in 10% buffered formalin, the specimens were embedded in paraffin and cut into 5 µm sections. Each section was dewaxed and immersed sequentially in xylene, 95% ethanol, and 100% ethanol for 5 min; this sequential immersion was repeated twice. After rinsing with water, the sections were stained with 0.5% periodic acid solution for 5 min, followed by Schiff reagent staining for 10 min. The sections were counterstained with hematoxylin for 10 sec and then dehydrated in alcohol, cleared, and mounted on slides for viewing with an Axio Scope A1 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) coupled with an Infinity 2 digital camera (Lemenera Corporation, Ottawa, ON, Canada). Images from all the circular basolateral membranes of the specimens were captured for evaluation of villi and crypts. VH and CD were measured for all the distinguishable villi and crypts in the captured images using ImageJ software (National Institutes of Health, MD). The numbers of GC on the corresponding villi and crypts were counted manually.

4.3.5. EX VIVO USSING CHAMBER ANALYSES

A modified Ussing chamber (VCC-MC6; Physiologic Instruments Inc., San Diego, CA) was used to study gut permeability across the jejunal epithelial tissues. The serosal and muscle layers of the jejunum were removed using microforceps, and the epithelial tissues were placed in the tissue holder with an aperture of 1 cm^2. Each holder was mounted in a two-coupled chamber containing pairs of current (Ag wire) and voltage (Ag/AgCl pellet) electrodes housed in 3% agar bridges and filled with 3 M KCl. The samples for Ussing chamber analysis were processed

Table 4.1 Primers used for qPCR analyses of immune cytokines, tight junction protein, and mucin 2

Target[1]	Amplicon size, bp	Primer sequence (5' to 3')	Reference[2]
GAPDH	142	F: GTGAACGGATTTGGCCGC	NM_001206359.1
		R: AAGGGGTCATTGATGGCGAC	
IL-1β	91	F: TGGCTAACTACGGTGACAACA	NM_214055
		R: CCAAGGTCCAGGTTTTGGGT	
IL-4	243	F: TCCACGGACACAAGTGCGAC	NM_214123
		R: TGTTTGCCATGCTGCTCAGG	
IL-6	151	F: AAGGTGATGCCACCTCAGAC	M86722
		R: TCTGCCAGTACCTCCTTGCT	
IL-8	126	F: AGAGGTCTGCCTGGACCCCA	NM_213867
		R: GGGAGCCACGGAGAATGGGT	
IL-10	220	F: CATCCACTTCCCAACCAGCC	NM_214041
		R: CTCCCCATCACTCTCTGCCTTC	
TNF-α	151	F: ATGGATGGGTGGATGAGAAA	X54001
		R: TGGAAACTGTTGGGGAGAAG	
CLDN1	93	F: CTGTGGATGTCCTGCGTGT	NM_001244539.1
		R: GGTTGCTTGCAAAGTGGTGTT	
OCLN	163	F: GAGAGAGTGGACAGCCCCAT	NM_001163647
		R: TGCTGCTGTAATGAGGCTGC	
ZO-1	200	F: GATCCTGACCCGGTGTCTGA	XM_021098856
		R: TTGGTGGGTTTGGTGGGTTG	
MUC2	90	F: CCAGGTCGAGTACATCCTGC	XM_021082584.1
		R: GTGCTGACCATGGCCCC	

[1]GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; TNF-α, tumor necrosis factor alpha; CLDN1, claudin-1; OCLN, occludin; ZO-1, zonula occludens 1; MUC2, mucin 2.
[2]GenBank accession reference number.

within 15 min post-mortem to ensure the retention of the electrophysiological properties of the jejunal samples. Both mucosal and serosal chambers were bathed in 5 mL of Kreb Ringer buffer containing 115 mM NaCl, 2.4 mM K_2HPO_4, 0.4 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1μm indomethacin, and 10 mM D-glucose. The bathing medium in the chambers was continuously aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37 °C in a water bath. The electrode potential and the solution resistance were corrected before the tissues were mounted in the chamber. The clamps were connected to the software (Physiological Instruments) for automatic data collection and calculation. After a 10 mins period to allow establishment of electrophysiological equilibrium, the transepithelial electrical resistance (**TEER**) was recorded at 5 s intervals over a 1 h period. In addition, the flux of 4kDa fluorescein isothiocyanate dextran (**FD4**) was measured by adding 0.1 mg/mL of FD4 (Sigma-Aldrich, St. Louis, MO) to the mucosal chamber at the end of the equilibrium period, and 2 mL of the serosal buffer was sampled at 60 min and transferred to a light protection tube. The optical density of the samples was read at 450 nm with the emission wavelength set at 540 nm.

4.3.6. SAMPLE PREPARATION AND CHEMICAL ANALYSES

Diet samples were finely ground with a grain miller (50–200 μm fineness; HC-700, Boshi Electronic Instrument, Guangzhou, China) and analyzed for AA. Fecal samples were oven-dried at 60 °C for 3 d and weighed. Blood samples were centrifuged at 2,000 × g for 10 min at 4 °C to recover plasma, which was immediately stored at –80 ˚C until required for cytokine analyses. Plasma samples were used to measure the concentration of IL-6 and IL-10 with a quantitative sandwich enzyme-linked immunosorbent assay technique using porcine IL-6 and IL-10 immunoassay kits (Porcine IL-6 ELISA Kit and Porcine IL-10 ELISA Kit; Sigma-Aldrich) according to the manufacturer’s instructions. The optical densities were read on a

spectrophotometer (SoftMax Pro; Molecular Devices, Abingdon, Oxfordshire, UK) at 450 nm with the emission wavelength set at 540 nm. The SCFA concentrations were determined by gas chromatography (Varian Chromatography System, model Star 3400; Varian Medical Systems, Palo Alto, CA) with a capillary column (30 × 0.5 mm; Restek Corp., Belefonte, PA), according to the method described by Erwin et al. (1961). Briefly, 1 mL of 25% metaphosphoric acid was mixed with 5 mL of digesta fluid in a 15-mL centrifuge tube, and the mixture was frozen overnight. The acidified samples were then thawed, neutralized with 0.4 mL of 25% NaOH, and vortexed. A 0.65 mL volume of 0.3 M oxalic acid was then added and the samples were vortexed again. 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 NH_3-N concentration in the jejunum and colon digesta samples were determined using the method described by Novozamsky et al. (1974). Briefly, 1.5 mL of a reagent containing 200 mL of 0.05% sodium nitroprusside and 10 mL of 4% ethylenediaminetetraacetic acid was added to 50 µL of sample in a 10-mL test tube. A solution containing 10% sodium hypochlorite (2.5 mL) was then added to the mixture. Test tubes containing the resulting mixture were placed in a test tube rack and incubated in complete darkness for 30 min, and then the optical density of the mixture was immediately read at 630 nm using a spectrophotometer (SoftMax Pro).

4.3.7. CALCULATIONS AND STATISTICAL ANALYSES

The glyceraldehyde 3-phophate dehydrogenase (**GAPDH**) gene was a control gene and used to normalize the transcriptional levels for immune cytokines, tight junction protein, and mucin protein. The relative expression was expressed as a ratio of the target gene to the simple diet (STD Thr)-fed group gene, using the formula $2^{-\Delta\Delta Ct}$ according to Livak and Schmittgen (2001), where $\Delta\Delta Ct = (Ct_{target} - Ct_{GAPDH})_{treatment} - (Ct_{target} - Ct_{GAPDH})_{simple\ diet\ (STU\ Thr)}$.

The concentrations of IL-6, IL-10, FD4, and NH_3-N were determined by calculating the concentration from a regression equation of the standard curves with R^2 values of 0.963, 0.998, 0.999, and 0.998, respectively.

All data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Inst. Inc., Cary, NC) with each animal used as the experimental unit. The model included diet complexity, the level of L-Thr supplementation, and their interaction. The LSMEANS statement with the Tukey-adjusted PDIFF option was used to calculate and separate the mean values for each treatment. Results were considered significant at $P < 0.05$ and tendencies were observed at $0.05 < P \leq 0.10$.

4.4. RESULTS

4.4.1. GROWTH PERFORMANCE AND FECAL DRY MATTER

Dietary treatment did not affect the ADG, ADFI, or G:F throughout the experimental period (Table 4.2). Pigs fed a complex diet had lower ($P < 0.05$) fecal DM than those fed the simple diet for the first week of the experimental period, whereas no difference was observed during the second week.

4.4.2. PLASMA CYTOKINE CONTENTS AND INTESTINAL HISTOMORPHOLOGY

Although no difference in IL-6 was observed across dietary treatments on day 7, the plasma IL-6 contents were higher ($P < 0.05$) in the simple diet–fed pigs than in the complex diet–fed pigs on day 14, as shown in Table 4.3. Conversely, plasma IL-10 contents were higher in the pigs fed simple diets than those fed complex diets ($P < 0.05$), but no difference was observed on day 14. The SUP Thr did not change plasma cytokine contents on either days 7 or 14. However, SUP Thr

Table 4.2 Effect of diet complexity and threonine supplementation on growth performance and fecal dry matter[1]

	Complex		Simple			*P*-value[2]		
Item	STD Thr	SUP Thr	STD Thr	SUP Thr	SEM	Diet	Thr	Diet × Thr
No. of animals	8	8	9	7				
Body weight, kg								
d 0	7.2	7.2	7.2	7.3				
d 7	8.9	8.7	8.4	9.0	0.35	0.863	0.536	0.235
d 14	12.7	12.8	11.8	13.0	0.65	0.507	0.286	0.373
Average daily gain, g/d								
d 0 – 7	241	213	172	239	32.2	0.469	0.529	0.120
d 7 – 14	548	591	477	563	49.7	0.288	0.172	0.644
d 0 – 14	395	402	324	401	38.1	0.318	0.246	0.334
Average daily feed intake, g/d								
d 0 – 7	320	294	247	307	32.4	0.316	0.579	0.164
d 7 – 14	630	651	542	604	52.3	0.172	0.404	0.677
d 0 – 14	475	473	395	455	40.3	0.198	0.445	0.404
Gain to feed ratio, g/g								
d 0 – 7	0.74	0.71	0.69	0.77	0.053	0.988	0.628	0.278
d 7 – 14	0.87	0.90	0.88	0.94	0.032	0.461	0.171	0.721
d 0 – 14	0.83	0.84	0.82	0.88	0.029	0.539	0.186	0.500
Fecal dry matter, %								
d 0 – 7	31.8	31.6	33.2	37.6	1.55	0.017	0.161	0.137
d 7 – 14	44.3	42.0	44.1	45.4	2.90	0.551	0.861	0.513
d 0 – 14	37.9	36.5	38.7	41.4	1.90	0.125	0.718	0.253

[1]STD Thr, the standard NRC (2012) level of standardized ileal digestible Thr (0.79%); SUP Thr, 15% over the standardized ileal digestible Thr requirement.

[2]Diet, main effect of diet complexity; Thr, main effect of the levels of dietary Thr supplementation; Diet × Thr, interactive effect of diet complexity and Thr supplementation.

Table 4.3 Effect of diet complexity and threonine supplementation on plasma cytokine contents and histomorphology[1]

	Complex		Simple			*P*-value[2]		
Item[3]	STD Thr	SUP Thr	STD Thr	SUP Thr	SEM	Diet	Thr	Diet × Thr
IL-6, pg/mL								
day 7	1,309	1,556	1,697	2,316	549.1	0.274	0.408	0.722
day 14	555	945	2,297	2,211	688.6	0.040	0.826	0.730
IL-10, pg/mL								
day 7	112	45	239	245	74.7	0.027	0.663	0.602
day 14	70	34	144	106	53.3	0.181	0.488	0.987
Jejunum								
Villus height, μm	345	375	347	393	17.9	0.547	0.024	0.591
Crypt depth, μm	229	245	247	227	14.6	0.985	0.885	0.172
Villus height : crypt depth	1.5^{xy}	1.6^{xy}	1.4^{y}	1.8^{x}	0.09	0.520	0.031	0.074
Goblet cells/100 μm villi	2.5^{y}	2.8^{xy}	2.2^{y}	3.8^{x}	0.35	0.312	0.005	0.056
Goblet cells/villi	8.7^{y}	10.5^{xy}	7.7^{y}	15.0^{x}	1.46	0.191	0.002	0.041
Goblet cells/100 μm crypt	6.7^{y}	6.8^{xy}	6.1^{y}	8.0^{x}	0.35	0.323	0.004	0.011
Goblet cells/crypt	15.1	16.4	15.3	18.2	1.34	0.434	0.086	0.511
Colon								
Crypt depth, μm	167^{xy}	191^{x}	150^{y}	197^{x}	9.9	0.578	0.001	0.243
Goblet cells/100 μm crypt	9.2	16.0	5.9	8.8	4.36	0.210	0.246	0.641
Goblet cells/crypt	15.5	28.7	8.8	17.9	7.55	0.227	0.127	0.779

[1]STD Thr, the standard NRC (2012) level of standardized ileal digestible Thr (0.79%); SUP Thr, 15% over the standardized ileal digestible Thr requirement. Data represented as least square means of 7 – 9 pigs.

[2]Diet, main effect of diet complexity; Thr, main effect of the levels of dietary Thr supplementation; Diet × Thr, interactive effect of diet complexity and Thr supplementation.

[3]IL = interleukin.

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

increased ($P < 0.05$) the VH, VH:CD, and GC density of the villi (*n*/100 µm of villi and *n*/villi) and crypts (*n*/100 µm of crypts) in the jejunum. However, SUP Thr did not alter the GC density in the colon, although it deepened ($P < 0.01$) the CD. Diet complexity did not affect the histomorphology in either the jejunum or the colon. However, interactive effects ($P < 0.05$) were observed for diet complexity and the level of dietary $_{L}$-Thr supplementation on GC density (*n*/villi and *n*/100 µm of villi). In addition, trends toward an interaction were observed in VH:CD ($P = 0.07$) and GC density (*n*/100 µm villi; $P = 0.06$).

4.4.3. RELATIVE mRNA GENE EXPRESSION

The expression of genes encoding TNF-α ($P = 0.06$), claudin 1 ($P = 0.08$), and ZO-1 tended to be higher ($P = 0.07$) in pigs fed the simple diet than in those fed the complex diet (Table 4.4). SUP Thr upregulated ($P < 0.05$) occludin gene expression in the jejunum. Furthermore, a tendency ($P = 0.07$) was observed for an increase in IL-6 gene expression in the jejunum of pigs fed the SUP Thr diet than those fed the STD Thr. An interactive effect ($P = 0.10$) was observed between diet complexity and Thr supplementation level on the expression of the IL-6 gene in the jejunum.

4.4.4. CONCENTRATIONS OF MICROBIAL METABOLITES AND PH IN THE JEJUNUM AND COLON CONTENTS

Diet complexity did not affect the SCFA contents or the pH value of the jejunum (Table 4.5). However, the NH_3-N concentration in the jejunum tended to be higher ($P = 0.07$) in the simple diet–fed pigs than in the complex diet–fed pigs. The butyrate concentration in the colon was higher ($P < 0.05$) in the simple diet–fed pigs than in those fed the complex diet. Interactive effects ($P < 0.05$) were observed for diet complexity and level of $_{L}$-Thr supplementation for the NH_3-N and pH levels in the jejunum and the acetate, propionate, total VFA, and total SCFA levels in the colon.

A trend for the interaction was also found for valerate content ($P = 0.08$) and pH ($P = 0.09$) in the colon.

4.4.5. ORGAN WEIGHTS AND GUT PERMEABILITY

The kidney weights (% of empty BW) were greater in the pigs fed the simple diet than in those fed the complex diet (Table 4.6). Although SUP Thr did not change organ weights, a trend ($P = 0.062$) was observed for an interaction between diet complexity and the level of L-Thr supplementation for the liver weight. Dietary treatment did not affect TEER or FD4 flux in the jejunum (Figure 4. 1).

4.5. DISCUSSION

The conventional complex diet for pigs was mimicked in the present study by including spray-dried animal plasma, fish meal, and dried whey. These ingredients were replaced in the simple diet with 17.3% soybean meal, bringing its dietary content to 30.3%. Each diet was supplemented with 0.12% L-Thr to supply 115% of the NRC (2012) SID Thr requirement. Our analysis showed SUP Thr diets contained 0.13% greater total Thr content when compared with the STD Thr diets, confirming the successful mixing of the added L-Thr in the diets. The SID Lys contents remained constant across the diets, at 1.35%, to maintain the SID Lys:SID Thr ratio in the STD Thr and SUP Thr diets. The level of SUP Thr was based on our previous study (Jayaraman et al., 2015) which showed 15% more dietary Thr is required for nursery pigs when their immune system is stimulated. In general, the major limitation regarding the use of soybean meal in a weaner pig diet is the presence of antigenic compounds, such as glycinin and β-conglycinin (Li et al., 1990). These antigens cause a hypersensitivity that elicits overreaction of the immune system and commonly results in inflammation and proliferation of immune cells, particularly in nursery pigs, as their immune systems are still immature (Hao et al., 2009). The process of hypersensitivity appears to

be modulated by cytokines, which play roles in cell signaling among several immune cell types, including macrophages, lymphocytes, and mast cells. In fact, previous studies (Guo et al., 2007; Hao et al., 2009) have reported that oral gavage with β-conglycinin, one of antigenic compounds in soybean meal, significantly increased plasma IL-4, IL-5, TNF-α, and IFN-γ in both piglets and rats. Therefore, our findings of greater IL-6 and TNF-α gene expression in response to the simple diet may be a consequence of hypersensitivity to antigenic compounds provided by the simple diet. However, one point to note is that the inflammation state appears to be maintained by IL-10, a typical anti-inflammatory cytokine, which functions by suppressing the action of pro-inflammatory cytokines like IL-6 during the first week of the experimental period. This may be associated with the quantitative threshold of antigens that allows nursery pigs to maintain homeostasis. In fact, the pigs fed simple diets consumed approximately 2.5 times more feed in week 2 than in week 1, indicating more consumption of antigens from soybean meal.

Intestinal permeability is associated with the functioning of the intramembrane multiprotein complexes known as tight junctions (Vereecke et al., 2011). Two transmembrane proteins of tight junctions, occludin and claudin, create the link between two adjacent epithelial cells, whereas ZO-1, a cytoplasmic adaptor protein, is associated with cytoskeletal tethering and binding of the transmembrane proteins (Cummins, 2012). Intestinal tight junctions rely on cytokines for modulation of their function (Capaldo and Nusrat, 2009), with TNF-α serving as a key mediator of intestinal permeability and inflammation by activating the myosin light chain kinase gene thereby promoting acto-myosin contractility (Capaldo and Nusrat, 2009). Furthermore, Yang et al. (2003) reported a modulation of ZO-1 stability in the tight junction complex in mice in response to IL-6. In the present study, the intestinal tight junction

Table 4.4 Effect of diet complexity and threonine supplementation on relative mRNA gene expression ($2^{-\Delta\Delta Ct}$) in jejunum[1,2]

	Complex		Simple			*P*-value[3]		
Item[4]	STD Thr	SUP Thr	STD Thr	SUP Thr	SEM	Diet	Thr	Diet × Thr
IL-1β	1.33	1.06	1.00	2.14	0.435	0.401	0.326	0.120
IL-4	1.22	0.84	1.00	0.59	0.394	0.530	0.297	0.959
IL-6	0.46	0.44	1.00	0.40	0.182	0.143	0.069	0.097
IL-8	0.47	0.86	1.00	0.76	0.299	0.450	0.796	0.278
IL-10	0.66	0.66	1.00	0.96	0.252	0.170	0.930	0.943
TNF-α	0.62	0.43	1.00	1.15	0.306	0.060	0.943	0.546
CLDN1	0.61	0.55	1.00	0.90	0.222	0.076	0.709	0.924
OCLN	0.70	1.10	1.00	1.11	0.133	0.211	0.042	0.233
ZO-1	0.79	0.81	1.00	0.95	0.094	0.070	0.867	0.706
MUC2	0.94	0.95	1.00	0.77	0.169	0.692	0.489	0.450

[1]STD Thr, the standard NRC (2012) level of standardized ileal digestible Thr (0.79%); SUP Thr, 15% over the standardized ileal digestible Thr requirement. Data represented as least square means of 7 – 9 pigs.

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

[3]Diet, main effect of diet complexity; Thr, main effect of the levels of dietary Thr supplementation; Diet × Thr, interactive effect of diet complexity and Thr supplementation.

[4]IL = interleukin; TNF-α = tumor necrosis factor alpha; CLDN1 = claudin 1; OCLN = occludin; ZO-1 = zonula occludens-1; MUC2 = Mucin 2.

Table 4.5 Effect of diet complexity and threonine supplementation on microbial metabolites and pH in jejunum and colon digesta[1]

	Complex		Simple			*P*-value[2]		
Item	STD Thr	SUP Thr	STD Thr	SUP Thr	SEM	Diet	Thr	Diet × Thr
Jejunum[3]								
Acetate	15.8	18.8	16.0	11.9	4.09	0.389	0.897	0.362
Propionate	0.6	0.3	0.7	1.1	0.38	0.156	0.874	0.323
Butyrate	0.5	0.5	0.4	0.3	0.13	0.155	0.738	0.598
Isovalerate	1.3	1.2	1.4	0.8	0.28	0.600	0.177	0.309
Valerate	0.1	0.1	0.1	0.1	0.05	0.643	0.739	0.255
Lactate	11.1	8.2	10.9	11.2	2.79	0.598	0.628	0.564
BCFA[4]	1.4	1.4	1.6	0.9	0.32	0.567	0.208	0.286
Total VFA	18.2	20.9	18.6	14.2	4.43	0.450	0.836	0.395
Total SCFA	29.3	29.2	29.5	25.3	4.20	0.549	0.589	0.607
Ammonia N	73.6^{y}	93.8^{xy}	158.5^{x}	76.3^{y}	18.82	0.067	0.091	0.007
Colon								
Acetate	113.6^{x}	87.1^{y}	106.8^{xy}	110.2^{xy}	7.17	0.238	0.097	0.036
Propionate	72.6	52.9	64.7	74.7	6.38	0.254	0.422	0.020
Isobutyrate	1.5	2.0	1.9	1.2	0.51	0.651	0.842	0.242
Butyrate	35.9	32.9	37.8	46.7	3.94	0.043	0.430	0.120
Isovalerate	2.6	3.4	3.0	2.1	0.77	0.515	0.921	0.224
Valerate	12.8	10.0	10.1	14.4	2.06	0.670	0.692	0.082
Lactate	21.5	28.3	9.7	20.4	11.71	0.376	0.434	0.859
BCFA[4]	16.9	15.5	15.0	17.6	2.27	0.947	0.778	0.348
Total VFA	239.0^{xy}	188.4^{y}	224.3^{xy}	249.2^{x}	14.69	0.106	0.359	0.011
Total SCFA	260.5	216.7	234.0	269.5	20.22	0.493	0.829	0.046
Ammonia N	254.2	320.3	271.0	301.4	41.89	0.979	0.231	0.653

[1]STD Thr, the standard NRC (2012) level of standardized ileal digestible Thr (0.79%); SUP Thr, 15% over the standardized ileal digestible Thr requirement. Data represented as least square means of 7 – 9 pigs.

[2]Diet, main effect of diet complexity; Thr, main effect of the levels of dietary Thr supplementation; Diet × Thr, interactive effect of diet complexity and Thr supplementation.

[3]Isobutyrate was not detected.

[4]Branched fatty acids = isobutyrate + isovalerate + valeric acid.

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

Table 4.6 Effect of diet complexity and threonine supplementation on organ weights[1]

	Complex		Simple			*P*-value[2]		
Item	STD Thr	SUP Thr	STD Thr	SUP Thr	SEM	Diet	Thr	Diet × Thr
Empty BW	12.13	12.33	11.24	12.23	0.641	0.419	0.331	0.518
% of empty BW								
Liver	4.15	3.58	3.40	3.65	0.223	0.116	0.454	0.062
Spleen	0.27	0.29	0.29	0.26	0.036	0.796	0.819	0.539
Kidneys	0.74	0.76	0.66	0.72	0.029	0.027	0.155	0.388
Stomach	0.88	0.80	0.81	0.85	0.051	0.893	0.644	0.199
Small intestine	4.26	4.42	4.33	4.59	0.203	0.562	0.281	0.790
Large intestine	1.66	1.73	1.96	1.91	0.168	0.140	0.945	0.714
Gastrointestinal tract[3]	6.81	6.95	7.09	7.35	0.283	0.205	0.462	0.834

[1]STD Thr, the standard NRC (2012) level of standardized ileal digestible Thr (0.79%); SUP Thr, 15% over the standardized ileal digestible Thr requirement. Data represented as least square means of 7 – 9 pigs.

[2]Diet, main effect of diet complexity; Thr, main effect of the levels of dietary Thr supplementation; Diet × Thr, interactive effect of diet complexity and Thr supplementation.

[3]Stomach + small and large intestine.

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

permeability was assessed by measuring TEER and FD4 flux in the jejunum. The pigs fed the simple diet showed a greater expression of IL-6 and TNF-α than those fed the complex diet, but they maintained their TEER and FD4 fluxes in the jejunum at levels comparable to those of the pigs fed the complex diet. This finding contrasts with those of previous studies (Zhao et al., 2014; Wu et al., 2016), in which the permeability of epithelial cells was dose-dependently compromised by sensitization to soy protein antigens. Our findings indicated that the jejunal permeability may have been maintained by transcriptional regulation of tight junction proteins. In fact, trends for upregulated genes encoding claudin-1 and ZO-1 gene were observed upregulated in pigs fed the simple diet when compared with pigs fed the complex diet.

Soy antigens are well known to cause villus atrophy and crypt proliferation (Dréau et al., 1994; Hao et al., 2009). In this regard, we previously showed that simple diet–fed pigs had deeper CD and shorter VH in duodenum and ileum than complex diet–fed pigs (Koo et al., 2017). However, no differences in VH and CD were noted in the jejunum between complex-fed and simple-fed pigs in the present study. Various factors such as different ages, feed intakes, diet compositions, and feeding regimens may have resulted in the discrepancy (Pluske et al., 1997). The effects of the simple diet on intestinal morphology may also occur in a segment-dependent manner, as no difference was observed in jejunal morphology in our previous study (Koo et al., 2017).

A disruption in gut health could be caused by NH_3 produced in the gut lumen by microbial fermentation of nitrogenous compounds. The produced NH_3, as a toxic compound, can irritate intestinal mucosa and be absorbed into the bloodstream via nonionic diffusion (Williams et al., 2001). Our assessment of NH_3-N content in digesta was therefore as an indicator of protein fermentation as well as gut health. Because the diets were formulated to contain identical CP

content, the NH_3-N content in the jejunum indicated a higher content of indigestible protein and greater protein fermentation with the simple diet than in the complex diet. Notably, urea is utilized and converted into NH_3 by the urease of ureolytic bacteria (Vince et al., 1973). Considering that approximately 25% of the circulating urea is excreted into the gut lumen (Barrett, 2014), the N balance in the body may partially contribute to the NH_3 concentration found in the gut. In fact, a 39% greater plasma urea nitrogen content was observed in the pigs fed the simple diet when compared with those fed the complex diet.

In contrast to NH_3 production in the jejunum, the simple diet appeared to have a beneficial effect on microbial fermentation in the proximal colon by promoting butyrate production. Butyrate is a well-documented microbial metabolite that plays a trophic role in colonocytes as a major source of energy for their metabolic activities and as a stimulant of epithelial proliferation in pigs (Montagne et al., 2003). This may be associated with the higher NSP content in the simple diet than in the complex diet. The simple diet had a 24% greater NSP content (8.8%), which originated from the xylogalacturonan, hemicellulose, and pectin known to occur in soybean meal (Knudsen, 2014). This potential association is partially supported by previous studies (Levrat et al., 1991; Jiménez-Escrig et al., 2008) in which inclusion of soy fiber in a rat diet significantly promoted butyrate production in the gut. The presence of butyrate in the large intestine is reported to promote water and sodium absorption; thus, butyrate has the ability to limit osmotic diarrhea (Montagne et al., 2003). A butyrate effect may therefore partially explain the greater fecal DM in the pigs fed the simple diet than those fed the complex diet. In addition, the kidney is a major osmoregulatory organ in the body (Strange, 1992), so a modification of electrolyte absorption could explain the heavier kidneys in the pigs fed the complex diet than those fed the simple diet.

Threonine is of great importance in maintaining the immune system because it is a major AA component of mucins and Ig (Li et al., 2007). Studies using isotope tracers revealed that dietary Thr is mainly metabolized in the intestine by incorporation into newly synthesized proteins (Floc'h and Sève, 2005; Schaart et al., 2005). In the present study, SUP Thr improved the VH and GC density in the jejunum. This is in agreement with previous findings (Ren et al., 2014; Min et al., 2017; Chen et al., 2018) where dietary supplementation with L-Thr above the requirement enhanced intestinal morphology or GC density in pigs and broiler chickens. The interactive effects of diet complexity and L-Thr supplementation level on gut integrity showed the patterns that were similar to those seen for microbial metabolites. This suggests that SUP Thr may have enhanced intestinal morphology and GC density through an interplay with microbial metabolites. This is because intestinal NH_3 and VFA contents are closely associated with enterocyte atrophy and proliferation, respectively (Williams et al., 2001).

The GC are propagated from stem cells at the base of the crypt, and as they mature, they migrate up to the villus tip to condense with mucin granules (Specian and Oliver, 1991). Therefore, the increased GC density in the crypts could reflect a promotion of GC proliferation by SUP Thr. However, SUP Thr did not alter mucin 2 (MUC2) gene expression, indicating that the STD Thr level may have not been sufficient for optimum GC proliferation and the synthesis of MUC2. The improved GC density in response to SUP Thr seems to parallel the improved VH, because GC secrete MUC2 as well as trefoil factor 2 and resistin-like molecule β, which are responsible for maintaining intestinal integrity (McGuckin et al., 2009).

Evidence is growing that dietary Thr supplementation beneficially modifies the gut microbiota composition in poultry (Chen et al., 2017b; Dong et al., 2017) and in rats (Faure et al., 2006). The authors of these previous studies speculated that dietary Thr supplementation

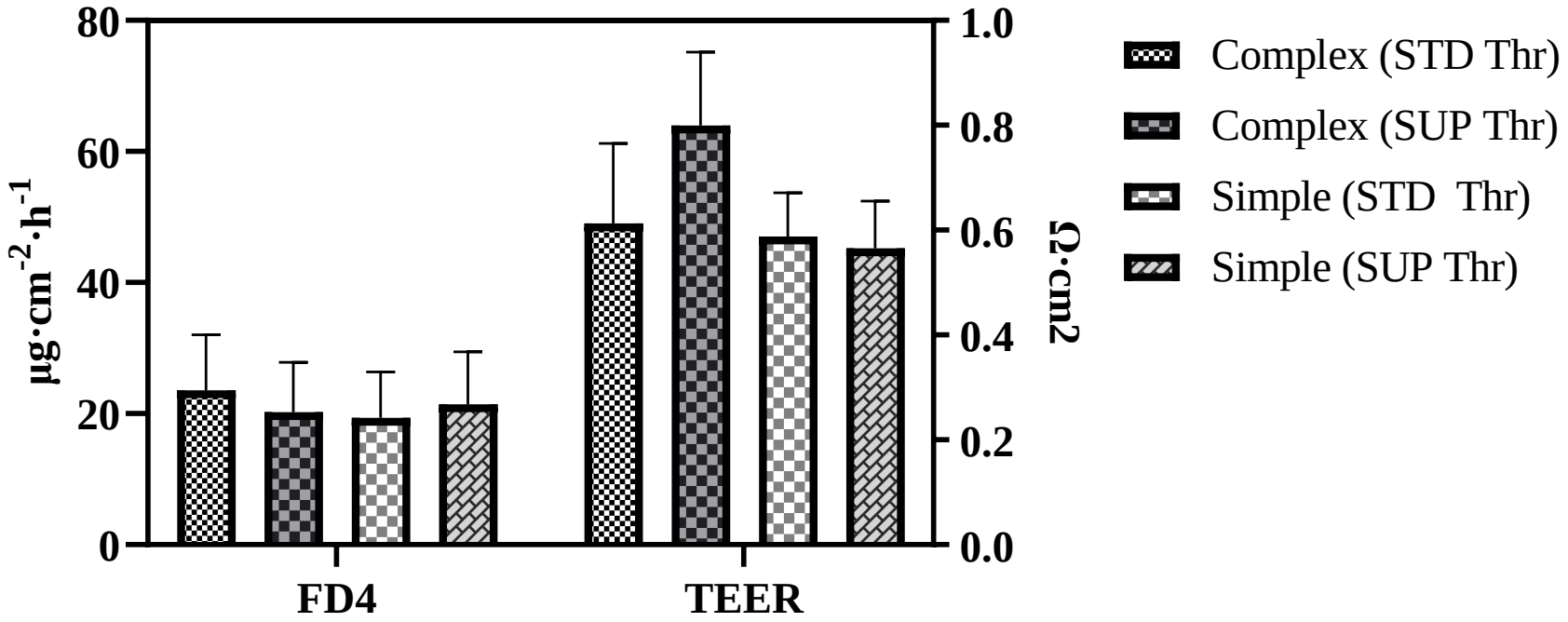

Figure 4.1 Effect of diet complexity and threonine supplementation on fluorescein isothiocyanate dextran 4kDa (FD4) flux and transepithelial electrical resistance (TEER) in jejunum. Complex = animal protein sources containing complex diet, Simple = corn-wheat-soybean meal based simple diet, STD Thr = the standard NRC (2012) level of standardized ileal digestible Thr (0.79%), SUP Thr = 15% over the standardized ileal digestible Thr requirement. No significant effects ($P > 0.10$) of dietary complexity, Thr supplementation, and their interaction were observed.

promoted saccharolytic bacteria by stimulating the secretion of mucin (glycoconjugates) that serve as substrates for these microorganisms. However, the present results indicate that SUP Thr seems to modify intestinal microbial fermentation in an interactive manner with diet composition. Interestingly, SUP Thr in simple diet decreased NH_3 content to the level observed with the complex diet**.** Conversely, SUP Thr in the simple diet increased VFA production compared to that in the complex diet. Therefore, the promotion of saccharolytic bacteria by SUP Thr may have led to a preferential fermentation of carbohydrates over protein, thereby increasing the VFA production and decreasing NH_3 production. However, we found no benefits of SUP Thr with the complex diet in terms of microbial metabolite production, suggesting a requirement for adequate dietary carbohydrates to generate benefits with respect to microbial metabolites.

The microbial production of VFA in the gut lumen is known to have gut anti-inflammatory activity through regulation of T cell differentiation (Kim et al., 2014), whereas microbial production of NH_3 elicits inflammation in the host (Williams et al., 2001). In this regard, our findings on IL-6 gene expression paralleled the changes in gut microbial metabolites (e.g., VFA and NH_3-N). IL-6 gene expression may also have been associated with enhanced GC density, as the GC secrete MUC2, a major component of the mucus that coats intestinal epithelium and protects the gut from immune stimulants, such as microorganisms, toxins, and antigens (McGuckin et al., 2009).

As mentioned earlier, the production of pro-inflammatory molecules is a key modulator affecting the function of tight junction proteins in the intestine. The reduction in IL-6 gene expression by SUP Thr may be a contributing factor to the upregulation of occludin gene expression. This is partially supported by work of Chen et al. (2018) which showed that SUP Thr downregulated IL-1β and upregulated ZO-1 gene expression in the ileum of broiler chickens

challenged with *Escherichia coli* LPS. However, as was seen in the present study regarding the effects of diet complexity on gut permeability, the upregulation of the occludin gene expression by SUP Thr did not extend to improvement in TEER and FD4 flux. This indicated that the expression of tight junction protein-encoding genes does not translate into modification of the intestinal barrier function. Intestinal permeability is maintained in a systematic way (Wijtten et al., 2011); therefore, the pigs in the present study may have maintained an optimal gut permeability without a requirement for additional tight junction proteins. However, as Wang et al. (2015a) pointed out, the increase in intestinal permeability post-weaning is associated with a significant reduction in the expression of genes encoding occludin, claudin-1, ZO-2, and ZO-3. Therefore, the upregulation of occludin expression in the jejunum by SUP Thr may be beneficial for maintaining intestinal permeability when intestinal homeostasis is compromised.

The present understanding of the cellular function of the colonic crypt remains incomplete. To date, the existing evidence supports a role for the colonocyte in vectorial transport, mostly of electrolytes, and in innate immunity by serving as a barrier against various pathogens and their toxins (Kiela and Ghishan, 2016; Litvak et al., 2018). Therefore, crypt development seems beneficial for maintaining immunity and ion transport by increasing cell renewal and maturation. In the present study, SUP Thr deepened the crypts in the colon. Supplementary L-Thr is mostly absorbed in the small intestine, so it may be indirectly involved in alterations of CD, but additional studies are required to confirm this possibility.

4.6. CONCLUSIONS

Feeding a simple diet to nursery pigs resulted in systemic and intestinal inflammation, possibly due to a higher consumption of the antigenic compounds present in soybean meal and a greater NH_3 production in the jejunum when compared with feeding a complex diet. The SUP Thr diet,

which included 115% of the NRC (2012) requirement for SID Thr, did not normalize the simple diet-induced inflammation. However, SUP Thr did normalize NH_3 production in the jejunum and resulted in downregulation of IL-6 gene expression in the jejunum. SUP Thr also improved gut integrity and architecture, as indicated by greater VH, GC density, and occludin gene expression in the jejunum. SUP Thr seems to have greater benefits with a simple diet than with a complex diet in terms of production of gut microbial metabolites (e.g., NH_3, VFA), intestinal morphology, and inflammatory status in the jejunum. Therefore, SUP Thr in a simple diet could be a beneficial nutritional strategy for enhancing gut health. However, additional strategies should be combined with SUP Thr to maintain the systemic inflammatory state of pigs fed a simple diet. In addition, further study with large numbers of animals is required to investigate whether the benefits of SUP Thr in the simple diet can lead to superior growth performance in nursery pig production. Further research on gut microbiota is also warranted to understand the mode of action of SUP Thr on microbial metabolites.

5. MANUSCRIPT III

Comparative Effects of Dietary Methionine and Cysteine Supplementation on Redox Status and Intestinal Integrity in Immunologically Challenged-Weaned Pigs

TRANSITION STATEMENT

Experiment 1 (Manuscript I and II) found that the stress that can be triggered by a simplified nursery diet is partially ameliorated by supplementing the diet with $_{L}$-Thr. Experiment 2 was designed to investigate the efficacy of FAA against systemic inflammation and oxidative stress that are commonly followed by weaning. Sulfur amino acids were chosen based on their biological function and tested against the weaning stressors. In contrast to Experiment 1, immune challenge model was applied in Experiment 2 to focus on the systemic effects of FAA.

5.1. ABSTRCAT

Sulfur amino acids play key roles in immune responses and redox status in animals. Under inflammatory status, sulfur amino acid metabolism increases mainly because of high demands for methylation and the glutathione (GSH) synthesis. The study aimed to investigate the effects of dietary Met and Cys supplementation in immunologically challenged weaned pigs. Forty weaned piglets (6.5 ± 0.3 kg) were randomly allocated to five treatment groups. The treatment included: (1) sham-challenged control (SCC), (2) challenged control (CC), (3) MET (CC + 0.1% $_{DL}$-Met), (4) CYS (CC + 0.1% $_{L}$-Cys), and (5) MET+CYS (CC + 0.1% $_{DL}$-Met + 0.1% $_{L}$-Cys). On day 7, all pigs were intramuscularly injected with either *Escherichia coli* O55:B5 LPS or phosphate-buffered saline. Blood, liver, and jejunum samples were analyzed for immune response and redox status. The CC group had lower ($P < 0.05$) villus surface area and higher ($P < 0.05$) FD4 than the SCC

group. A lower ($P < 0.05$) GSH concentration was observed in the jejunum of pigs in the CC group than those in the SCC group. Dietary Cys supplementation increased ($P < 0.05$) villus surface area, GSH levels, and reduced ($P < 0.05$) the flux of FD4 in the jejunum. Dietary Met supplementation enhanced ($P < 0.05$) hepatic GSH content. Pigs in the MET group had lower serum IL-8 concentration than those in the CC group. There was a Met × Cys interaction ($P < 0.05$) in serum IL-4 and IL-8 concentrations, and Trolox equivalent antioxidant capacity. Dietary $_{L}$-Cys supplementation restored intestinal integrity and GSH levels that were damaged by lipopolysaccharides administration. Dietary $_{DL}$-Met supplementation improved hepatic GSH and reduced systemic inflammatory response, but antagonistic interaction with dietary $_{L}$-Cys supplementation was observed in the inflammatory response and redox status.

Key words: cysteine, intestinal integrity, methionine, oxidative stress, pigs

5.2. INTRODUCTION

Methionine is a nutritionally EAA in animals (Elango, 2020). Apart from being a building block of proteins, Met possesses biological significance with the presence of methyl groups and sulfur atoms (Stipanuk, 2004). Methionine undergoes the transmethylation pathway, becoming SAM that serves as a methyl donor for numerous metabolic processes including the synthesis of DNA, RNA, and histones (Stipanuk, 2004). The SAM is also a substrate for polyamines, which are associated with the innate immune response and proliferation of intestinal epithelial cells (Martínez-López et al., 2008; Ding et al., 2015). Methionine serves as a precursor of Cys by transferring its sulfur to serine via the transsulfuration pathway (Stipanuk, 2004). Cysteine is a rate-limiting substrate for the syntheses of GSH and taurine, which play pivotal roles in cellular redox status and osmoregulation (Yin et al., 2016). The catabolism of Cys can release H_2S, a gasotransmitter that modulates the immune system (Magierowski et al., 2015).

The liver is the major organ for SAA metabolism in the body, with high activities of enzymes involved in transmethylation and transsulfuration pathways (Stipanuk, 2004). The small intestine also expresses the enzymes to a lesser extent but plays a significant role in whole body SAA metabolism (Riedijk et al., 2007). Isotopic tracer research indicated that 20% and 25% of dietary Met and Cys were metabolized by the intestine, respectively (Riedijk et al., 2007; Bauchart-Thevret et al., 2011). This reflects the high demands for methyl donors, GSH, and Cys-rich protein (e.g., mucins and defensins) by the intestinal epithelial cells in response to exogenous toxins and pathogens (Bauchart-Thevret et al., 2009a; Rakhshandeh et al., 2020). During the acute phase of inflammation, SAA metabolism increases mainly because of the increased demand for APP and GSH (Rakhshandeh et al., 2019; Rakhshandeh et al., 2020). Dietary SAA for protein deposition (growth) is redirected toward mounting an immune response, suggesting greater dietary SAA

requirement for optimal growth (Litvak et al., 2013; Rakhshandeh et al., 2014). In this regard, dietary SAA supplementation could be a nutritional strategy to maintain the redox status and immune system (Shen et al., 2014; Song et al., 2016; Su et al., 2018), while minimizing body protein mobilization.

Dietary SAA content can be simply elevated by supplementing synthetic Met or Cys. However, a body of evidence shows that metabolic aspects of supplemented Met and Cys may differ in the body. The Cys for intestinal metabolism is mainly sourced from lumen absorption, showing no extraction from the arterial blood (Rémond et al., 2011). By contrast, Riedijk et al. (2007) reported that the intestine did not utilize first-pass Met, but did rely on arterial Met supply. Furthermore, Met appears to be prioritized for protein synthesis in the liver by suppressing the transsulfuration pathway under SAA-deficient conditions in pigs (Bauchart-Thevret et al., 2009b). In this context, it is postulated that the effects of supplemented Met and Cys on redox status and inflammation may differ in a tissue- and status-dependent manner. However, no studies have investigated comparative effects with respect to dietary Met and Cys supplementation. Thus, the objective of our study was to investigate the effects of dietary Met and Cys supplementation on redox status and intestinal integrity in pigs challenged with LPS.

5.3. MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Committee (AC11414), and pigs were cared for according to the guidelines of the Canadian Council on Animal Care (2009).

5.3.1. ANIMALS, EXPERIMENTAL DESIGN, AND DIETS

Forty female piglets (TN 70 × TN Tempo; Topigs Norsvin) with an initial BW of 6.5 ± 0.3 kg were obtained from Glenlea Research Station at the University of Manitoba. Pigs were individually

housed in pens and randomly allocated based on their BW to five treatment groups. The treatments were: (1) sham-challenged control (**SCC**), (2) challenged control (**CC**), (3) CC + 0.1% $_{DL}$-Met (**MET**), (4) CC + 0.1% $_{L}$-Cys (**CYS**), and (5) CC + 0.1% $_{DL}$-Met + 0.1% $_{L}$-Cys (**MET+CYS**). One batch of basal diet was formulated to meet the NRC requirement for SID Met but contained 0.1% less than the requirement for SAA (Table 5.1). The levels of all other nutrients in the basal diet were equal to or over the NRC requirement for 8 kg of BW with 14.3 MJ/kg of ME. Either 0.1% of $_{DL}$-Met (MetAMINO, Evonik Nutrition & Care GmbH) or $_{L}$-Cys (Sigma-Aldrich) was supplemented to the basal diet to make the MET and CYS diets, respectively, which supplied 100% of the NRC requirement for SID SAA. The MET+CYS diet was prepared by supplementing the basal diet with 0.1% of $_{DL}$-Met and 0.1% of $_{L}$-Cys simultaneously.

5.3.2. EXPERIMENTAL PROCEDURE, SAMPLING, AND MEASUREMENTS

Piglets were allowed a 7-day adaptation period to their respective diets; thereafter, a single dose of *Escherichia coli* O55:B5 LPS (300 μg/mL; Sigma-Aldrich) was administrated intramuscularly at 0.1 mL/kg BW to all pigs in the CC, Met, Cys, and Met+Cys groups, whereas pigs in the SCC group were intramuscularly injected with an equal volume of sterilized phosphate buffered saline (**PBS**). All pigs and feeders were weighed on days 7 and 9 to calculate BW gain and feed intake. Blood samples (5 mL) from the jugular veins of all pigs were collected into two vacutainer tubes to obtain whole blood (BD Vacutainer® spray-coated K_2DETA Tubes) and serum (BD Vacutainer® Plus Plastic Serum Tubes) on days 6 (24 h pre-inoculation), 7 (5 h post-inoculation), and 8 (24 h post-inoculation). Rectal temperature was determined before each time of blood collection. On day 9, all pigs were euthanized by captive bolt following stresnil-xylazine (2:4 mg/kg) sedation to allow for tissue collection. A sample of jejunum was taken 2 m away from

Table 5.1 Composition of experimental diets, as-fed basis, %

	Experimental diets[1]			
	Basal	Met	Cys	Met+Cys
Ingredient				
Corn	45.80	45.80	45.80	45.80
Wheat	17.00	17.00	17.00	17.00
Soybean meal	22.00	22.00	22.00	22.00
Fish meal	6.00	6.00	6.00	6.00
Dried whey	5.00	5.00	5.00	5.00
Vegetable oil	0.50	0.50	0.50	0.50
Cornstarch	0.20	0.20	0.20	0.20
Limestone	1.04	1.04	1.04	1.04
Monocalcium phosphate	0.30	0.30	0.30	0.30
Salt	0.30	0.30	0.30	0.30
Vitamin-mineral premix[2]	1.00	1.00	1.00	1.00
$_{L}$-Lysine-HCl	0.53	0.53	0.53	0.53
$_{DL}$-Methionine	0.09	0.19	0.09	0.19
$_{L}$-Cysteine	-	-	0.10	0.10
$_{L}$-Threonine	0.16	0.16	0.16	0.16
$_{L}$-Tryptophan	0.01	0.01	0.01	0.01
$_{L}$-Valine	0.07	0.07	0.07	0.07
Calculated Nutrient				
SID[3] lysine	1.42	1.42	1.42	1.42
SID[3] methionine	0.41	0.51	0.41	0.51
SID[3] cysteine	0.27	0.27	0.37	0.37
SID[3] methionine + cysteine	0.68	0.78	0.78	0.88
Metabolizable energy, Mcal/kg	3.41	3.41	3.41	3.41

[1]Basal diet was supplemented with either 0.1% $_{DL}$-Met or $_{L}$-Cys at the expense of cornstarch for Met and Cys diets, respectively. 0.1% of each $_{DL}$-Met and $_{L}$-Cys were supplemented to basal diet for Met+Cys diet.

[2]Supplied per kilogram of diet: vitamins A, 2,200 IU; vitamin $D_{3,}$ 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; thiamine, 1.5 mg; riboflavin, 4 mg; niacin, 30 mg; pantothenic acid, 12 mg; vitamin B_{12}, 0.02 mg; folic acid, 0.3 mg; 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; biotin 0.2 mg.

[3]Standardized ileal digestible.

and protein analysis was immediately snap-frozen in liquid nitrogen, and then stored in a −80°C freezer. Another subsample of jejunum was immediately immersed in Krebs Ringer buffer and then immediately transported to the laboratory for *ex vivo* Ussing chamber analyses. Jejunum samples for histomorphology were immediately stored in 10% buffered formalin to fix the villi, crypts, and goblet cells. Samples for hepatic gene expression and protein analysis were the ileocecal junction. The jejunum sample was rinsed with cold PBS. The sample for gene expression collected from the middle of the right lobe of the liver. After sampling, the content of GIT was removed and weight to calculate the empty BW. Jejunum and liver samples stored at -80°C were immersed in liquid nitrogen in a mortar and ground with a pestle for subsequent analyses for mRNA abundance and protein assay.

5.3.3. WHITE BLOOD CELL PROFILES AND SERUM CYTOKINE

The whole blood samples were analyzed for total white blood cell (**WBC**) counts using an automatic blood analyzer (Advia 2120i, SIEMENS Healthineers, Erlangen, Germany). The proportions of WBC profiles were determined from the stained blood smears based on WBC morphology using light microscopy at 100× magnification. The concentrations of each WBC were calculated based on the proportion and total WBC concentrations.

Serum samples were analyzed for the concentrations of cytokines, including TNF-α, IL-1α, IL-1β, IL-1 receptor antagonist (**IL-1ra**), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, and IL-18, using a commercial multiplex assay using laser bead technology at Eve Technologies Corporation.

5.3.4. RNA ISOLATION, COMPLEMENTARY DNA SYNTHESIS, AND QUANTITATIVE REAL-TIME PCR

A TRIzol Plus RNA purification kit (Invitrogen Canada Inc.) was used to isolate RNA from 80 mg of the ground jejunum. The quantity and quality of the isolated RNA were evaluated using a

Nanodrop 2000 spectrophotometer (ThermoFisher Scientific). The integrity of the total RNA was confirmed by agarose gel electrophoresis. A total of 2 μg of total RNA was used to synthesize cDNA using a high-capacity cDNA synthesis kit (Applied Biosystems) following the supplier's protocol. Quantitative real-time PCR was performed in duplicate reactions, using a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories), as described by Waititu et al. (Waititu et al.). For qPCR amplification, negative controls were prepared by replacing the cDNA with nuclease-free water. A melt curve analysis was performed with a temperature gradient of 0.1°C/s from 70°C to 95°C to confirm that only specific products were amplified. Pairs of primers for each gene were designed and checked for target identity using the National Center for Biotechnology Information database. Each set of primers used for qPCR amplification confirmed that their efficiencies were between 90% and 110%.

5.3.5. HISTOMORPHOLOGY MEASUREMENT

After fixation in 10% buffered formalin, the specimens were embedded in paraffin and cut into 5 μm sections. Each section was dewaxed and immersed sequentially in xylene, 95% ethanol, and 100% ethanol for 5 min; this sequential immersion was repeated twice. After rinsing with water, the sections were stained with 0.5% periodic acid solution for 5 min, followed by Schiff reagent staining for 10 min. The sections were counterstained with hematoxylin for 10 sec and then dehydrated in alcohol, cleared, and mounted on slides for viewing with an Axio Scope A1 microscope coupled with an Infinity 2 digital camera (Lumenera Corporation). Images from all the circular basolateral membranes of the specimens were captured for evaluation of villi and crypts. VH, villus width, and CD were measured for all the distinguishable villi and crypts in the captured images using ImageJ software (National Institutes of Health) and averaged for each pig. Villus absorptive surface area was calculated using the formula: Villus absorptive surface area =

$2\pi \times$ (average villus width/2) × VH. The numbers of goblet cells on the corresponding villi were counted manually.

5.3.6. REDOX STATUS INDICATORS

The Trolox equivalent antioxidant capacity (**TEAC**) of the liver sample was measured using a colorimetric Antioxidant Assay kit (Sigma-Aldrich) according to the supplier's manual with Trolox as a standard. Total GSH and oxidized GSH (**GSSG**) of jejunum and liver samples were measured using a Glutathione Colorimetric Detection kit (Thermo Fisher Scientific). The reduced GSH content was calculated by subtracting GSSG from the total GSH. The SOD activities of the jejunum and liver samples were measured using a SOD Determination kit (Thermo Fisher Scientific), and then SOD concentrations were calculated using a SOD standard curve. Aliquots of each isolated protein were taken for the analysis of their protein content using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific); then, the measured TEAC and concentrations of GSH and SOD were corrected for the unit of protein content.

5.3.7. EX VIVO USSING CHAMBER ANALYSES

A modified Ussing chamber (VCC-MC6; Physiologic Instruments Inc.) was used to study gut permeability across the jejunal epithelial tissues. The serosal and muscle layers of the jejunum were removed using microforceps, and the epithelial tissues were placed in the tissue holder with an aperture of 1 cm^2. Each holder was mounted in a two-coupled chamber containing pairs of current (Ag wire) and voltage (Ag/AgCl pellet) electrodes housed in 3% agar bridges and filled with 3 M KCl. The samples for Ussing chamber analysis were processed quickly, within 15 min postmortem, to ensure the retention of the electrophysiological properties of the jejunal samples. Both mucosal and serosal chambers were bathed in 5 mL of Krebs-Ringer buffer containing 115 mM NaCl, 2.4 mM K_2HPO_4, 0.4 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$,

1μm indomethacin, and 10 mM D-glucose. The bathing medium in the chambers was continuously aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37 °C in a water bath. The electrode potential and the solution resistance were corrected before the tissues were mounted in the chamber. The clamps were connected to the software (Physiological Instruments) for automatic data collection and calculation. After a 10 min period to allow the establishment of electrophysiological equilibrium, the TEER was recorded at 15 min intervals over a 1 h period. The flux of FD4 was measured by adding 0.1 mg/mL of FD4 (Sigma-Aldrich) to the mucosal chamber at the end of the equilibrium period, and 2 mL of the serosal buffer was sampled at 60 min and transferred to a light protection tube. The optical density of the sample was read at 450 nm with the emission wavelength set at 540 nm.

5.3.8. CALCULATIONS AND STATISTICAL ANALYSES

The geometric means of glyceraldehyde 3-phosphate dehydrogenase and hypoxanthine-guanine phosphoribosyltransferase expression were used to normalize the transcriptional levels for immune cytokines and enzymes associated with SAA metabolism. The relative expression was expressed as a ratio of the target gene to the SCC group gene, using the formula $2^{-\Delta\Delta Ct}$ according to Livak and Schmittgen (Livak and Schmittgen, 2001), where $\Delta\Delta Ct = (Ct_{target} - Ct_{geometric\ mean})_{treatment} - (Ct_{target} - Ct_{geometric\ mean})_{SCC}$.

All data were analyzed using single degree of freedom contrasts in the mixed procedure of SAS (SAS Inst.) to test the effects of LPS administration (SCC vs. CC), Met supplementation, Cys supplementation, and interaction between Met and Cys supplementation. The LSMEANS statement with the Tukey-adjusted PDIFF option was used to calculate and separate the mean values for each treatment. Results were considered significant at $P < 0.05$ and tendencies were observed at $0.05 \leq P < 0.10$.

5.4. RESULTS

5.4.1. GROWTH PERFORMANCE AND RECTAL TEMPERATURE

Dietary Met or Cys supplementation did not affect growth performance and rectal temperature during the pre-challenge and post-challenged periods (Table 5.2). However, during the postchallenge period, pigs in the CC group showed lower ($P < 0.01$) BW gain (g/day), feed intake (g/day), and gain:feed than those in the SCC group. Pigs in the CC group had a higher ($P < 0.05$) change (%) in rectal temperature at 5 h post-challenge but not at 24 h post-challenge than pigs in the SCC group (Table 5.2).

5.4.2. ORGAN WEIGHT, JEJUNUM MORPHOLOGY, AND *EX VIVO* USSING CHAMBER ANALYSES

There were no differences in organ weights between pigs in the SCC and CC groups (Table 5.3). Dietary Met supplementation decreased ($P < 0.05$) the weight (% of empty BW) of the liver, GIT, and portal-drained viscera. However, there were Met × Cys interaction ($P < 0.05$) effects on the weight of the GIT and portal-drained viscera. LPS-challenged control pigs had lower ($P < 0.05$) VH, villus width, villus absorptive surface area, and CD than sham-challenged control pigs. Dietary Cys supplementation increased ($P < 0.05$) villus width and villus absorptive surface area (Table 5.3). Additionally, a trend towards an increase ($P < 0.10$) in VH to CD ratio in pigs fed a diet supplemented with Cys was observed. There were trends ($P < 0.10$) toward interactive effects on the number of goblet cells, VH to CD ratio, and villus absorptive surface area. No

Table 5.2 Growth performance and rectal temperature of weaned pigs fed diets supplemented with either DL-Met or L-Cys in response to LPS administration[1, 2]

		LPS-challenged[3]				
	SCC	CC	Met	Cys	Met+Cys	Pooled SEM
Average daily gain, g/day						
Pre-challenge	239	235	222	205	208	28
Post-challenge[a]	258	118	111	147	79	31
Overall	245	200	184	185	169	26
Average daily feed intake, g/day						
Pre-challenge	316	304	315	304	325	19
Post-challenge[a]	391	302	294	322	307	22
Overall	339	304	307	298	319	17
Gain:feed, *g/g*						
Pre-challenge	0.77	0.74	0.69	0.68	0.65	0.07
Post-challenge[a]	0.67	0.40	0.37	0.45	0.32	0.08
Overall	0.74	0.64	0.59	0.59	0.54	0.06
Rectal temperature						
Pre-challenge, ˚C	39.4	39.4	39.3	39.4	39.1	0.26
Pre-challenge/5h post challenge, %[a]	-0.55	2.12	1.95	2.61	2.53	0.95
Pre-challenge/24h post challenge, %	1.32	1.24	1.40	1.50	2.17	0.81

[1]On days 7, all pigs were injected with *E. coli* lipopolysaccharides or phosphate-buffered saline.
[2]SCC, sham-challenged control; CC, challenged control; Met, Met-supplemented diet; Cys, Cys-supplemented diet supplemented diet; Met+Cys, Met and Cys-supplemented diet; Values are least-squares means, $n = 8$ per treatment.
[3]No significant effects of Met, Cys, and the interaction were observed ($P > 0.10$).
[a]SCC vs. CC ($P < 0.05$)

Table 5.3 Organ weights and intestinal morphology in pigs fed diets supplemented with either $_{DL}$-Met or $_{L}$-Cys in response to LPS administration[1]

		LPS-challenged					*P* values[4]		
	SCC	CC	Met	Cys	Met+Cys	Pooled SEM	Met	Cys	M×C
Empty BW, kg	8.02	7.69	7.59	7.54	7.53	0.25			
% of empty BW									
Spleen	0.34	0.38	0.39	0.38	0.36	0.03	0.76	0.61	0.66
Liver	3.65	3.97	3.35	3.62	3.47	0.16	0.013	0.46	0.12
Pancreas	0.13	0.15	0.11	0.14	0.28	0.08	0.491	0.29	0.23
Gastrointestinal tract	8.98	8.86	7.28	8.72	8.01	0.23	< 0.01	0.18	0.048
PDV[2]	9.46	9.39	7.77	9.23	8.65	0.24	< 0.01	0.12	0.030
Jejunum morphology									
Villus height, μm^{a}	416	335	366	380	358	23	0.83	0.41	0.23
Crypt depth, μm^{a}	238	211	209	192	207	10	0.48	0.25	0.34
Villus width, μm^{a}	166	135	142	159	150	6	0.95	0.009	0.17
GC[3]	1.81	2.27	1.76	1.83	2.66	0.36	0.64	0.51	0.056
Villus height: crypt depth	1.76	1.59	1.76	2.04	1.76	0.14	0.68	0.093	0.091
Villus surface area, mm^{2a}	0.21	0.14	0.16	0.19	0.17	0.01	0.96	0.029	0.091

[1]SCC, sham-challenged control; CC, challenged control; Met, Met-supplemented diet; Cys, Cys-supplemented diet supplemented diet; Met+Cys, Met and Cys-supplemented diet; BW, body weight; Values are least-squares means, $n = 8$ per treatment.
[2]Portal-drained viscera = pancreas + stomach + small intestine + large intestine + spleen.
[3]GC, the number of goblet cells per 100 *μm villus height*.
[4]Met, effect of Met supplementation; Cys, effect of Cys supplementation; M×C, effect of interaction between Met and Cys supplementation.
[a]SCC vs. CC ($P < 0.05$)

difference was observed in TEER among the treatments. A tendency ($P < 0.10$) toward the higher FD4 flux in the CC group compared with the SCC group was observed (Figure 5.1). Dietary Cys supplementation tended to decrease ($P < 0.10$) FD4 flux.

5.4.3. WHITE BLOOD CELLS PROFILE AND MRNA GENE EXPRESSION IN JEJUNUM AND LIVER

There were no differences in WBC concentrations between the SCC and CC groups at 24 h pre-challenge (Table 5.4). However, pigs in the CC group had lower ($P < 0.05$) concentrations of total WBC, neutrophils, lymphocytes, and monocyte, neutrophils but higher ($P < 0.05$) band cell concentration than those in the SCC group at 5 h postchallenge. Dietary Met supplementation increased ($P < 0.05$) lymphocyte content but decreased ($P < 0.05$) monocyte content at 24 h postchallenge.

The CC group tended to have a greater ($P < 0.10$) mRNA abundance of *TNF-α* and lower ($P < 0.10$) mRNA abundance of cystathionine gamma-lyase (***CSE***) in the jejunum (Table 5.5). Dietary Met supplementation tended to upregulate ($P < 0.10$) the gene encoding cystathionine β-synthase (***CBS***) in the jejunum. Dietary Cys supplementation increased ($P < 0.05$) the mRNA abundance of glutathione synthase (***GSS***) in the jejunum. Additionally, there was a trend towards a higher ($P < 0.10$) glutathione-disulfide reductase (***GSR***) mRNA abundance in the jejunum of pigs fed Cys-supplemented diets. An interactive effect ($P < 0.05$) of Met × Cys was observed for *IL-1β* gene expression in the jejunum. Pigs fed Met-supplemented diets showed higher ($P < 0.05$) mRNA abundance of *CBS* in the liver. Furthermore, dietary Met supplementation tended to increase ($P < 0.10$) the abundance of mRNA encoding *MAT1A* in the liver. Pigs in the Met group had greater ($P < 0.05$) mRNA abundance of *IL-6*, *IL-10*, and *TNF-α* in the liver. Met × Cys

Table 5.4 White blood cell (WBC) concentrations (× 10^6/mL) in nursery pigs fed diets supplemented with either $_{DL}$-Met or $_{L}$-Cys in response to LPS administration[1]

		LPS-challenged[2]				
	SCC	CC	Met	Cys	Met+Cys	Pooled SEM
Pre-challenge						
WBC	15.29	15.06	15.21	13.80	14.26	1.60
Neutrophils	9.41	8.52	8.47	8.69	7.94	1.29
Band cells	0.11	0.13	0.42	0.13	0.10	0.11
Lymphocytes[c]	5.45	5.96	5.86	4.23	5.61	0.51
Monocytes[c]	0.25	0.34	0.36	0.59	0.50	0.10
5 h post-challenge						
WBC[a]	13.95	9.33	8.49	10.12	7.88	1.38
Neutrophils[a]	8.22	4.31	3.67	4.86	4.06	1.03
Band cells[a]	0.37	2.71	3.00	3.42	2.50	0.49
Lymphocytes[a]	4.87	2.12	1.67	1.69	1.26	0.38
Monocytes[a]	0.42	0.15	0.13	0.15	0.03	0.12
24 h post-challenge						
WBC	16.58	18.77	18.10	16.32	18.21	1.57
Neutrophils	9.54	10.00	8.54	8.64	7.33	1.23
Band cells	0.39	1.62	2.39	1.39	2.86	0.91
Lymphocytes[b]	5.72	6.33	6.55	5.28	7.28	0.53
Monocytes[b]	0.61	0.61	0.21	0.68	0.42	0.13

[1]SCC, sham-challenged control; CC, challenged control; Met, Met-supplemented diet; Cys, Cys-supplemented diet supplemented diet; Met+Cys, Met and Cys-supplemented diet; Values are least-squares means, $n = 8$ per treatment.
[2]No effects of Met, Cys, and the interaction were observed ($P > 0.10$).
[a]SCC vs. CC ($P < 0.05$)
[b]Effect of Met supplementation ($P < 0.05$).
[c]Effect of Cys supplementation ($P < 0.10$).

interaction effect ($P < 0.05$) was observed in the mRNA abundance of *IL-6* and *TNF-α* in the liver. There was a trend ($P < 0.10$) for the interaction on *IL-10* mRNA abundance in the liver.

5.5.4. SERUM CYTOKINE CONCENTRATIONS AND OXIDATIVE STRESS INDICATORS IN JEJUNUM AND LIVER

Pigs in the CC group had higher ($P < 0.05$) serum TNF-α and IL-8 concentrations than those in the SCC group (Table 5.6). The CC group tended to have higher ($P < 0.10$) serum IL-1ra concentration than the SCC group. Pigs in the Met group had lower ($P < 0.05$) serum IL-8 concentration than those in the CC group. However, there were interactions ($P < 0.05$) of Met × Cys interaction effects on serum IL-4 and IL-8 concentrations. Similarly, trends ($P < 0.10$) were found for the interaction on serum IL-1α, IL-2, and IL-10.

The CC group showed lower ($P < 0.05$) GSH level in the jejunum than the SCC group (Figure 5.2). Similarly, a tendency ($P < 0.10$) was observed for a lower GSH level in the liver of pigs in CC than those in SCC. Dietary Met supplementation increased ($P < 0.05$) levels of total GSH and reduced GSH in the liver (Figure 5.3). By contrast, dietary Cys supplementation elevated ($P < 0.05$) levels of GSH and reduced GSH in the jejunum. A tendency ($P < 0.10$) for the increased GSH:GSSG was observed in pigs fed Cys supplemented diets. There were trends ($P < 0.10$) for a Met × Cys interactive effects on TEAC and SOD content in the liver.

5.5. DISCUSSION

After weaning, animals are vulnerable to enteric disease due to their immature digestive and immune systems and commonly undergo inflammation and oxidative stress (Moeser et al., 2017; Pluske et al., 2018a). LPS or endotoxin is the structural component of bacteria and activates the innate immune system (Wassenaar and Zimmermann, 2018). Purified LPS has been widely used

Table 5.5 Relative mRNA abundance ($2^{-\Delta\Delta Ct}$) in jejunum and livers of nursery pigs fed diets supplemented with either DL-Met or L-Cys in response to LPS administration[1, 2]

		LPS-challenged					*P* values[3]		
	SCC	CC	Met	Cys	Met+Cys	Pooled SEM	Met	Cys	M×C
Jejunum									
IL-1β	1.15	1.27	0.81	0.72	1.52	0.32	0.59	0.79	0.048
IL-6	1.12	1.23	1.17	0.84	1.07	0.19	0.65	0.20	0.43
IL-10	1.17	1.20	1.03	0.95	1.49	0.38	0.68	0.78	0.33
TNF-α[a]	1.04	1.49	1.10	1.02	1.13	0.21	0.49	0.27	0.21
MAT2A	1.14	0.76	1.09	1.02	1.65	0.31	0.11	0.17	0.62
CBS	1.05	0.77	1.19	0.82	1.24	0.25	0.088	0.84	0.98
CES[a]	1.51	0.63	0.56	0.86	0.84	0.36	0.89	0.44	0.93
GPx1	1.07	1.02	0.97	1.02	0.96	0.12	0.64	0.96	0.97
GSR[a]	1.03	0.82	0.84	0.97	1.02	0.09	0.68	0.070	0.86
GSS	1.02	0.95	1.04	1.23	1.18	0.09	0.83	0.023	0.45
Liver									
IL-1β	1.04	1.18	1.27	0.95	0.84	0.27	0.97	0.20	0.69
IL-6	1.12	1.08	1.74	1.33	1.11	0.18	0.21	0.29	0.015
IL-10	1.51	1.04	1.95	1.11	0.81	0.36	0.30	0.071	0.041
TNF-α	1.11	1.15	1.23	1.29	1.09	0.17	0.71	0.99	0.39
MAT1A	1.03	1.14	1.40	1.18	1.42	0.14	0.071	0.84	0.92
CBS	1.01	1.16	1.45	1.30	1.43	0.11	0.046	0.56	0.41
CES	1.02	0.97	1.14	1.14	1.26	0.12	0.23	0.24	0.84
GPx1	1.04	1.24	1.09	1.03	1.05	0.13	0.63	0.30	0.48
GSR	1.03	1.29	1.16	1.12	1.05	0.12	0.39	0.28	0.81
GSS	1.16	1.24	1.21	0.95	0.97	0.21	0.98	0.19	0.90

[1]SCC, sham-challenged control; CC, challenged control; Met, Met-supplemented diet; Cys, Cys-supplemented diet supplemented diet; Met+Cys, Met and Cys-supplemented diet; Values are least-squares means, $n = 8$ per treatment.

[2]IL, interleukin; TNF-α, tumor necrosis factor-alpha; MAT, methionine adenosyltransferase; CBS, cystathionine beta synthase; CES, cystathionine gamma-lyase; GPx1, glutathione peroxidase 1; GSR, glutathione-disulfide reductase; GSS, glutathione synthetase.

[3]Met, effect of Met supplementation; Cys, effect of Cys supplementation; M×C, effect of interaction between Met and Cys supplementation.

[a]SCC vs. CC ($P < 0.10$).

in different nutritional studies to elucidate the roles of nutrients and test nutritional strategies against inflammation and oxidative stress (Hou et al., 2012; Waititu et al., 2016). Thus, in the present study, LPS was administered to pigs to mimic the postweaning immune status and to investigate the effects of dietary Met or Cys supplementation under the status.

The pathogenesis of LPS has been well documented. LPS serves as a ligand for TLR-4/myeloid differentiation factor 2 on immune cells, which initiates the cascade of innate immune systems (Wang et al., 2016). The augment of TNF-α and IL-1 release has been observed immediately after LPS recognition by immune cells, and these cytokines increase the synthesis of prostaglandins, which act on the hypothalamus, elevating the thermoregulatory set point (Abbas et al., 2017). This corresponds with our results on febrile response, upregulation of the gene encoding TNF-α in the jejunum, and serum TNF-α concentration in LPS-administered pigs. Immune stimulation dramatically redistributes WBC profile (Abbas et al., 2017). LPS administration induces IL-8 and IL-1ra to recruit neutrophils to the infection site and to regulate the inflammatory response by IL-1, respectively (Yoshimura et al., 1997; Arend et al., 1998; Hoffmann et al., 2002). It has been well documented that TNF-α and IL-6 elevation that results from LPS activates the differentiation of myeloid progenitor cells into neutrophils (Soler-Rodriguez et al., 2000; Ai and Udalova, 2020). This is consistent with our results showing increased band neutrophils in CC group pigs compared to the SCC group pigs immediately after LPS administration. Furthermore, inflammation increases vascular permeability and facilitates the infiltration of leukocytes (e.g., neutrophils and monocytes) into tissues (Abbas et al., 2017). This may explain the lower concentration of circulating WBC in CC compared with SCC. A previous study also found leukopenia in pigs following LPS administration (Rakhshandeh et al., 2012; Huntley et al., 2018). Changes in leukocyte profile and cytokine levels together with lower

Table 5.6 Serum cytokine concentrations (ng/mL) in pigs fed diets supplemented with supplemented with either DL-Met or L-Cys at 24 h after LPS administration[1]

		LPS-challenged					*P* values[2]		
	SCC	CC	Met	Cys	Met+Cys	Pooled SEM	Met	Cys	M×C
INFγ	13.70	7.42	7.60	5.10	20.44	5.26	0.13	0.30	0.14
IL-1α	0.09	0.11	0.07	0.07	0.16	0.04	0.49	0.49	0.081
IL-1β	0.78	1.14	0.50	0.72	1.36	0.42	1.00	0.59	0.11
IL-1ra[b]	1.84	3.87	3.80	3.14	4.67	0.82	0.35	0.93	0.31
IL-2	0.98	1.09	0.72	0.70	1.77	0.39	0.34	0.38	0.057
IL-4	3.05	4.19	2.15	1.74	6.31	1.12	0.24	0.43	0.003
IL-6	0.30	0.40	0.22	0.25	0.63	0.20	0.59	0.49	0.15
IL-8[a]	0.18	0.39	0.10	0.15	0.25	0.06	0.12	0.48	0.003
IL-10	1.76	2.29	1.27	1.40	3.52	0.88	0.47	0.50	0.074
IL-12	1.13	0.86	0.59	0.66	1.09	0.26	0.73	0.53	0.15
IL-18	3.21	3.88	2.09	2.50	5.22	1.70	0.77	0.59	0.17
TNF-α[a]	0.10	0.25	0.24	0.17	0.34	0.06	0.18	0.87	0.11

[1]SCC, sham-challenged control; CC, challenged control; Met, Met-supplemented diet; Cys, Cys-supplemented diet supplemented diet; Met+Cys, Met and Cys-supplemented diet; Values are least-squares means, $n = 8$ per treatment.

[2]Met, effect of Met supplementation; Cys, effect of Cys supplementation; M×C, effect of interaction between Met and Cys supplementation.

[a,b]SCC vs. CC ($P < 0.05$ and $P < 0.10$, respectively).

BW gain indicated that LPS administration in our study successfully stimulated the immune system.

Under inflammatory conditions, multiple enzymes in immune cells, including lipoxygenase, myeloperoxidase, nitric oxide synthase, and cyclooxygenase, and the respiratory burst of phagocytes are stimulated, which results in greater release of reactive oxygen species (Bhattacharyya et al., 2014b) than under normal physiologic conditions. In this regard, LPS administration commonly increases the production of oxidants above the level that systemic antioxidants can sequester (Hou et al., 2012; Abdel-Salam et al., 2014). This aligns with our findings demonstrating lower TEAC in the liver of the CC group than in SCC.

The liver is the major site where LPS is detoxified by Kupffer cells; it has a high rate of oxidant production (Yao et al., 2016). Glutathione is an endogenous nonenzymatic antioxidant and plays a key role in maintaining cellular redox status (Bhattacharyya et al., 2014b). The GSH has a short lifespan, and its turnover and synthesis is accelerated after immune stimulation (Rakhshandeh et al., 2019). Unlike other studies in which GSH levels in tissue are maintained or elevated following LPS injection (Malmezat et al., 2000a; Rakhshandeh et al., 2019), the present study showed a lower GSH concentration in the liver and intestine in the CC group. This may be associated with the lower intake of SAA after LPS administration. There may have been a lack of SAA for a higher rate of GSH turnover. This is partially supported by a study by Castellano et al. (2015) in which a Met-deficient diet lowered the level of GSH in the muscle in growing pigs. Cys is the rate-limiting substrate for GSH synthesis, and the plasma Cys flux increases mainly for the higher GSH turnover when the immune system is stimulated in pigs (Rakhshandeh et al., 2020). This reflects that dietary Cys supplementation may help replenish the depleted GSH under oxidative stress conditions. Previous studies found that Cys supplementation via $_{L}$-Cys or *N*-

acetylcysteine enhanced the GSH levels in the intestines of immune-stimulated pigs (Hou et al., 2012; Song et al., 2016). This is consistent with our finding of the elevated GSH level in the jejunum of pigs fed a Cys-supplemented diet. The GSS is a homodimeric enzyme that catalyzes the condensation of γ-glutamylcysteine and glycine, forming a molecule of GSH (Yin et al., 2016). Luo et al. (1998) postulated that the depletion of GSH pool in skeletal muscle was associated with decreased activity of GSS in humans after surgical trauma. This suggests that the elevated GSH level in the jejunum may be attributed to the upregulation of the *GSS*-encoding gene in pigs fed the Cys-supplemented diet.

GSR is the key antioxidant enzyme that maintains the GSH:GSSG ratio. A higher ratio is desirable, suggesting a higher reducing potential (Bhattacharyya et al., 2014b). Our study showed that Cys supplementation also upregulated *GSR* gene expression in the jejunum, which may have led to higher GSH:GSSG. This is consistent with previous studies that found dietary Cys supplementation increased GSR activity in rats and pigs (Lee et al., 2013; Song et al., 2016). The benefit of Cys supplementation on GSH levels was not shown in the liver, but greater TEAC was observed. The enhanced GSH levels in the jejunum by dietary Cys supplementation were likely to sequester the intestinal oxidants and save the hepatic antioxidant pool, leading to greater TEAC in the liver. However, there is disagreement with the effect of Cys supplementation on hepatic GSH. Lin and Yin (2008) reported that Cys supplementation to water via *N*-acetyl cysteine, S-ethyl cysteine, *S*-propyl cysteine, or Cys restored the lowered hepatic GSH due to a high-fat diet in mice. Similarly, sucrose-induced hepatic GSH depletion was restored by consuming a Cys-rich protein diet in rats (Blouet et al., 2007). By contrast, the hepatic GSH content was not affected by 1% or 2% of dietary Cys supplementation in rats (Lee et al., 2013). Thus, it seems that the effect of Cys

supplementation on hepatic GSH content appears to vary depending on the source of Cys and physiologic state.

It has been revealed that inflammation and redox signaling systemically govern the fate and permeability of intestinal epithelial cells, which are relevant to intestinal integrity (Vereecke et al., 2011; Circu and Aw, 2012). Pro-inflammatory cytokines trigger the cascade of mitogen-activated protein kinase signaling and lead to apoptosis of enterocytes on the villus, mainly at the tip (Parker et al., 2019). Furthermore, the expression of myosin light-chain kinase that causes cytoskeletal contraction is activated under inflammatory conditions, thereby increasing intestinal permeability (Al-Sadi et al., 2009). A lower reducing potential in GSH–GSSG couple is associated with the apoptosis of intestinal epithelial cells and disruption of tight junction protein (Rao, 2008; Circu and Aw, 2012). In this regard, impaired intestinal integrity has been consistently shown after LPS administration (Hou et al., 2012; Song et al., 2016). This is consistent with our results in which pigs in the CC group showed shorter and narrower villi and increased FD4 flux than those in the SCC group. Higher FD4 flux indicates greater intestinal paracellular permeability, suggesting the disruption of tight junction proteins (Wijtten et al., 2011). In the present study, Cys supplementation seemed to restore the villus absorptive surface area of the jejunum and paracellular permeability. The supplemented Cys may have sequestered the oxidants via GSH preservation in the intestine, preventing their attacks on intestinal integrity. This is in line with previous studies in which dietary Cys supplementation through L-Cys or N-acetylcysteine maintained the VH (Hou et al., 2012) and FD4 flux (Song et al., 2016) against LPS administration.

Cysteine is nutritionally regarded as a dispensable AA because it can be synthesized *de novo* from Met via the transsulfuration pathway (Stipanuk, 2004). Transsulfuration of Met occurs mainly in the liver, but in the small intestine to a lesser extent (Riedijk et al., 2007). Thus, it was

generally hypothesized that dietary Met can fulfill the SAA requirement for protein deposition and GSH or taurine pool in the liver as well as intestine. CBS catalyzes the conversion of homocysteine into cystathionine as the first irreversible step of the transsulfuration pathway (Stipanuk, 2004). In the present study, Met supplementation upregulated CBS-encoding genes in the jejunum and liver. This may be associated with the increase in transmethylation of Met surplus and the accumulation of SAM (Chen et al., 2014). Indeed, the expression of CBS is positively regulated by the content of SAM (Sbodio et al., 2019). However, contrary to the effect of dietary Cys supplementation, dietary Met supplementation with upregulated *CBS*-gene expression did not restore the GSH levels and intestinal integrity in the jejunum. This suggests that the conversion of supplemental Met into Cys via the transsulfuration pathway was not as efficient as supplemental Cys in the intestine. Our study showed that LPS administration suppressed the expression of the *CSE* gene in the jejunum. Considering CSE is an enzyme that catalyzes cystathionine to Cys, this suppression may be an attributing factor for the lack of effects of Met supplementation on the GSH content in the jejunum. However, the Met supplementation enhanced the hepatic GSH levels. Given that transsulfuration is the sole pathway of Met catabolism (Stipanuk, 2004), supplemental Met that bypassed the intestine appeared to undergo transsulfuration in the liver, where the transsulfuration enzymes are more active than in the intestine (Riedijk et al., 2007). This postulation is further supported by an isotope tracer study, in which no first-pass metabolism of dietary Met was observed in the gut, although 20% of dietary Met was extracted from the artery (Riedijk et al., 2007). The authors suggested that dietary Met was prioritized for its metabolism in the liver. In this context, LPS administration in the present study may have increased the synthesis of proteins including GSH and APP in the liver, reducing the Met efflux into the bloodstream from where the intestine mainly sources dietary Met.

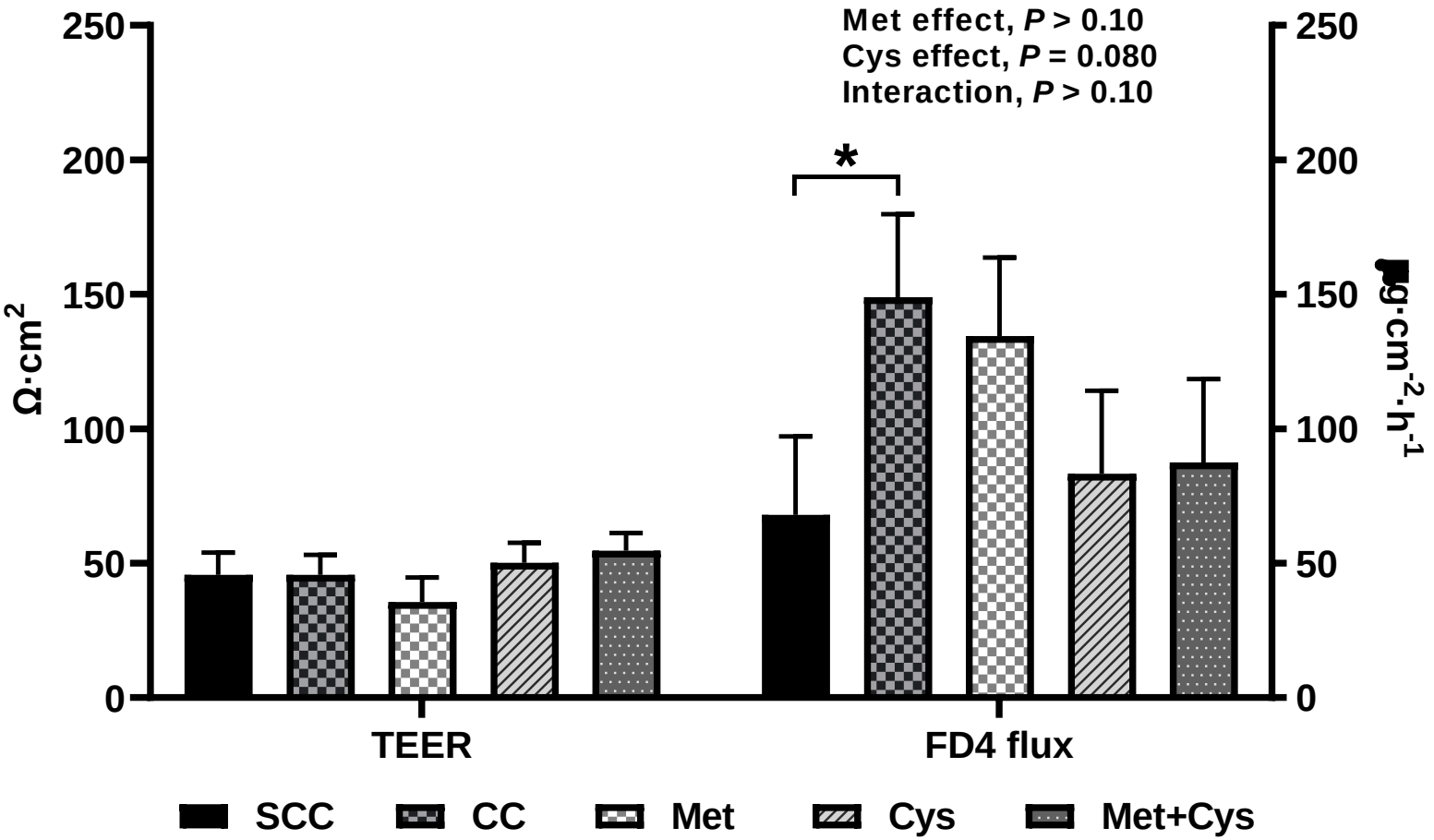

Figure 5.1 Transepithelial electrical resistance (TEER) and flux of fluorescein isothiocyanate dextran-4 kDa (FD4) in the jejunum of pigs fed diets supplemented with either $_{DL}$-Met or $_{L}$-Cys in response to LPS administration. SCC, sham-challenged control; CC, challenged control; Met, Met-supplemented diet; Cys, Cys-supplemented diet supplemented diet; Met+Cys, Met, and Cys-supplemented diet; *indicates the significant difference between SCC and CC (P = 0.065). No significant differences (P > 0.10) among treatments were observed in TEER. Values are mean ± SEM, n = 8 per treatment.

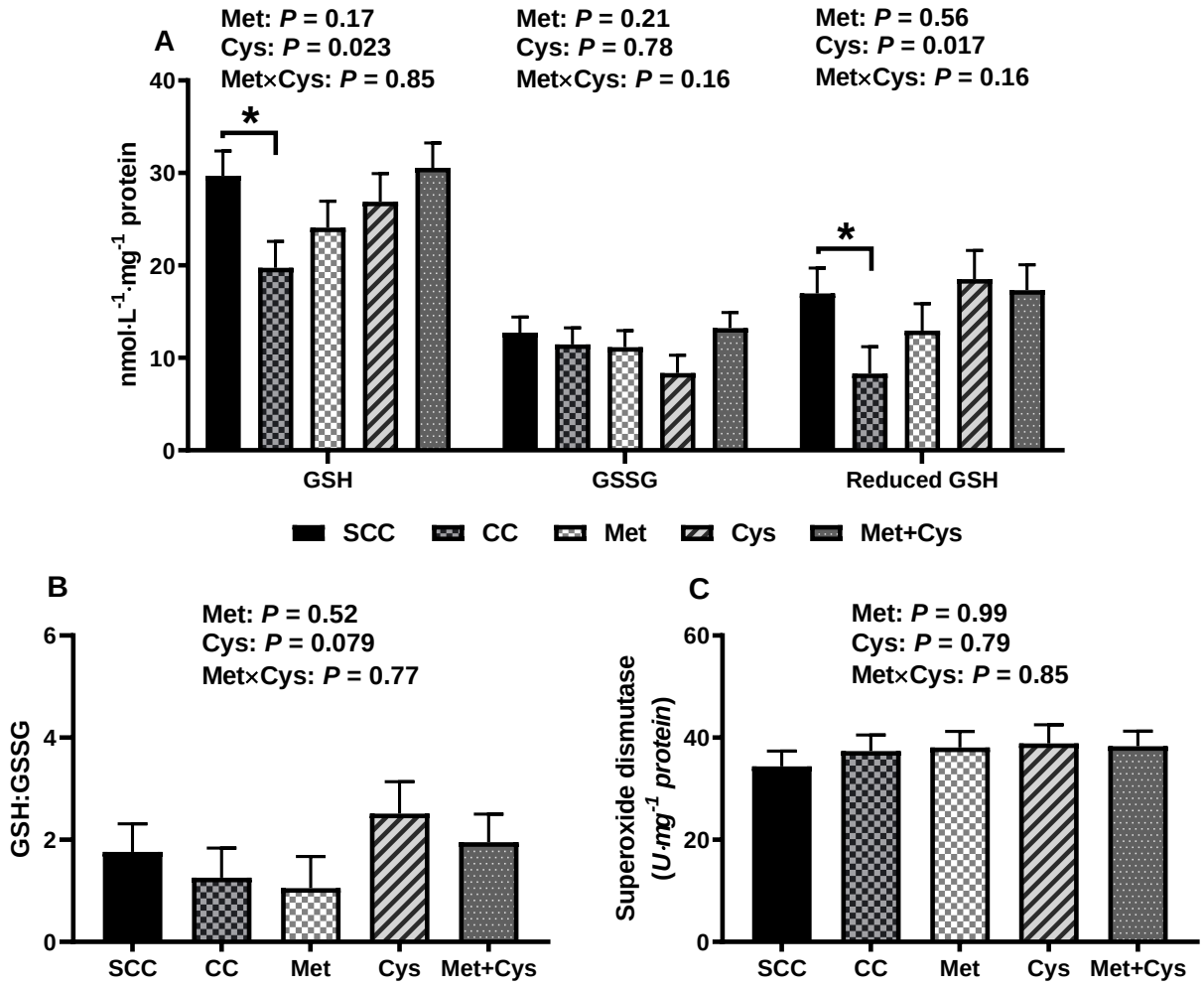

Figure 5.2 Effects of dietary DL-Met and L-Cys supplementation on total glutathione (GSH), oxidized GSH (GSSG), and reduced GSH concentrations (A), GSH:GSSG (B), and superoxide dismutase concentrations (C) in the jejunum of pigs challenged with *Escherichia coli* lipopolysaccharides. SCC, sham-challenged control; CC, challenged control; Met, Met-supplemented diet; Cys, Cys-supplemented diet supplemented diet; Met+Cys, Met, and Cys-supplemented diet; *indicates the difference between SCC and CC ($P < 0.05$). Values are mean ± SEM, $n = 8$ per treatment.

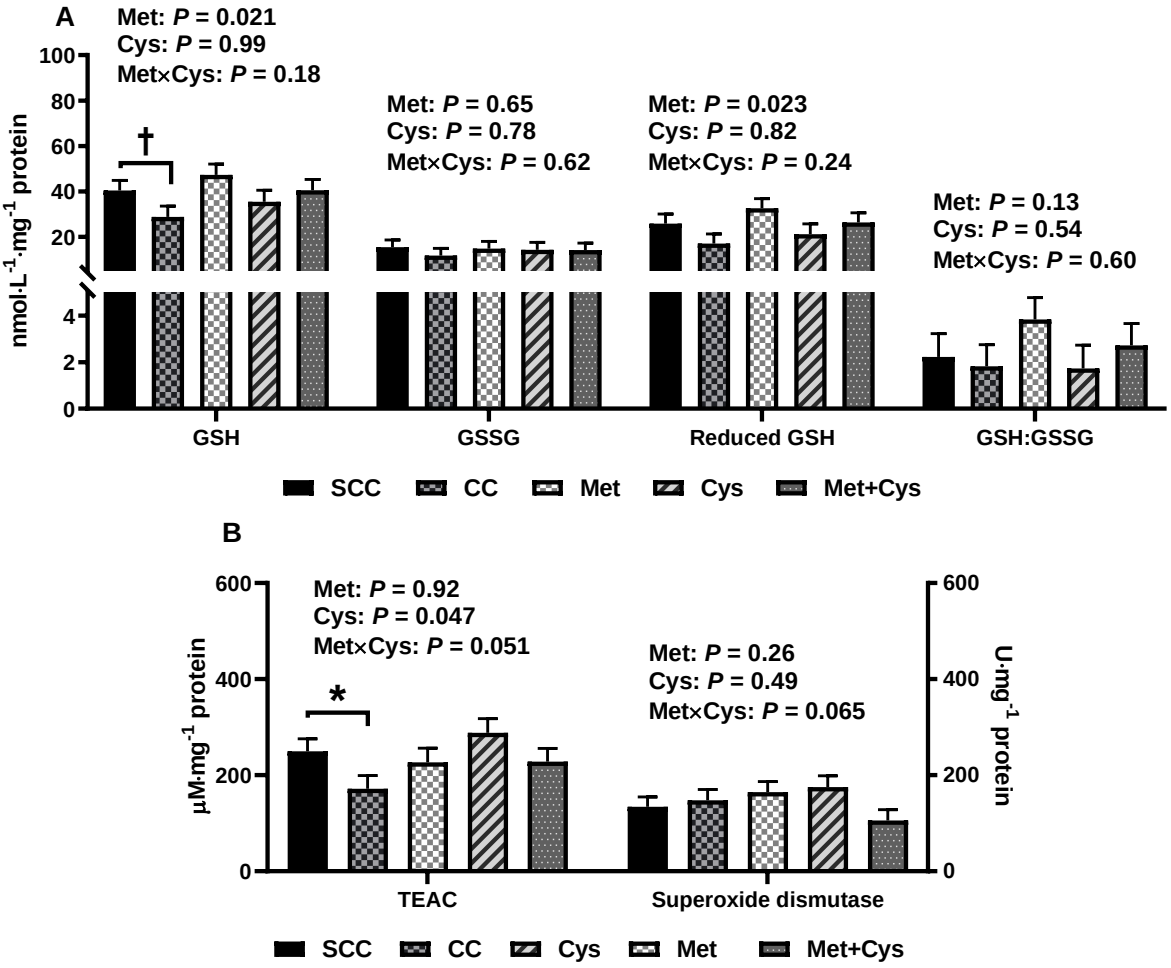

Figure 5.3 Effects of dietary $_{DL}$-Met and $_{L}$-Cys supplementation on hepatic GSH/GSSH couples (A) and Trolox equivalent antioxidant capacity (TEAC) and superoxide dismutase content (B) in the liver of pigs challenged with *Escherichia coli* lipopolysaccharides. SCC, sham-challenged control; CC, challenged control; Met, Met-supplemented diet; Cys, Cys-supplemented diet; Met+Cys, Met and Cys-supplemented diet. GSH, glutathione; GSSG, oxidized GSH. *,† indicate the difference between SCC and CC ($P < 0.05$ and $P < 0.10$, respectively). Values are mean ± SEM, $n = 8$ per treatment.

The MAT1A catalyzes the conversion of Met to SAM (Martínez-López et al., 2008). Dietary Met supplementation with greater *MAT1A* gene expression may have increased the SAM. content of the liver. A growing body of evidence indicates that SAM modulates the hepatic inflammatory response. *In vitro* studies found that exogenous SAM supplementation increased IL-6 and IL-10 production in Kupffer cells of LPS-treated rats by activating adenosine receptors as a process of liver regeneration (Song et al., 2004; Song et al., 2005). This possibly explains the increase in the expression of genes encoding IL-6 and IL-10 in the livers of pigs that consumed the Met-supplemented diet. Considering that the LPS is mainly sequestered by Kupffer cells, the activated immune response in the liver by Met supplementation appeared to reduce the circulating inflammatory cytokines (e.g., IL-2, IL-4, and IL-8), suggesting ameliorated systemic inflammation.

However, antagonistic effects of Met + Cys supplementations were observed in inflammatory responses, redox status, and intestinal morphology. The independent benefits of either Met or Cys supplementation were not observed when both Met and Cys were supplemented together. The reason for this is unclear, but one possible explanation is that excessive Cys and its metabolites may have caused adverse effects in the intestine and liver. Cys can be easily oxidized to disulfide cystine in the extracellular matrix, which is reduced back by endogenous antioxidants (Bhattacharyya et al., 2014b). Thus, this suggests that excessive Cys paradoxically causes depletion of antioxidants, causing oxidative stress (Dilger and Baker, 2008). These toxic effects were usually observed at extreme levels, but also observed with a small quantity of Cys in a prooxidant-prevailing condition. Lin and Yin (2008) reported that Cys supplementation (1 g/L of water) increased the concentration of a lipid peroxidation marker, malondialdehyde, and decreased catalase activity in the liver of mice fed a high-fat diet, although it increased hepatic GSH synthesis. The Cys metabolism pathway is determined by its cellular concentration, which regulates the

activities of associated enzymes. Under a high Cys condition, cysteine dioxygenase is downregulated by increasing the Cys catabolism pathway, whereas γ-glutamyl cysteine synthetase is suppressed, which increases the catabolism of Cys and reduces GSH synthesis (Kwon and Stipanuk, 2001). In this regard, dietary Met + Cys supplementation may have increased the rate of Cys catabolism, generating NH_3 and H_2S that were relevant to systemic inflammation (Regina et al., 1993). However, further study is warranted to elucidate the antagonistic relations between Met and Cys supplementation.

In conclusion, the current study demonstrated the tissue-dependent benefits of dietary Met or Cys supplementation. Dietary Cys supplementation replenished the GSH depletion caused by LPS administration and enhanced the GSH/GSSH couple upregulating *GSR* gene expression in the jejunum. This benefit in the jejunum appeared to save the hepatic antioxidants, elevating the hepatic antioxidant capacity without alteration in the hepatic GSH level. Cys supplementation restored jejunal integrity that was impaired by LPS administration. By contrast, the dietary Met supplementation did not alter the jejunal GSH level, but it did enhance hepatic GSH levels and inflammatory responses against LPS injection, resulting in a reduction in serum pro-inflammatory cytokines. However, antagonistic relationships between Met and Cys supplementation were found with regard to inflammatory responses and redox status. Taken together, either Met or Cys should be carefully chosen with the consideration of target tissues when supplementing diets with SAA against inflammation and oxidative stress in an immune-stimulated condition in pigs.

6. MANUSCRIPT IV

Effects of Sanitary Conditions and Dietary Valine Supplementation on Growth Performance, Immune Response, Bacterial Profile, and Microbial Metabolites in Weaned Pigs

TRANSITION STATEMENT

Experiment 3 revealed that dietary sulfur amino acid supplementation can help weaned pigs recover from oxidative stress and systemic inflammation. Subsequent experiment was performed to test the efficacy of functional amino acid on microbial composition and metabolites. The change in gut microbiota after weaning is a common challenge in nursery pig production. Valine was selected as a functional amino acid in this experiment. Sanitary challenge model was applied in Experiment 4 to mimic commercial environment.

6.1. ABSTRACT

This study investigated the effects of dietary L-Val supplementation and sanitary conditions on growth performance, immune response, and intestinal bacterial profile and metabolites in weaned pigs. Thirty-two weaned pigs (6.98 ± 0.47 kg) were randomly assigned to treatments in a 2 × 2 factorial arrangement based on dietary Val level and sanitary condition (low or high). The pigs were fed either a basal diet containing the standard levels of Val suggested by NRC (2012) or the basal diet supplemented with 0.1% of L-Val. A room designated as the high sanitary room was washed weekly, whereas the designated low sanitary room was not washed throughout the experiment and 5 kg of manure from the nursery pig barn was spread on the pen floors on day 1. All data were analyzed using a mixed procedure of SAS, with the individual pen as the experimental unit. The pigs raised in low sanitary conditions exhibited a lower average daily gain,

average daily feed intake, and gain to feed ratio and a higher incidence of diarrhea than those raised in high sanitary conditions during the 14-day experimental period ($P < 0.05$). The pigs in the low sanitary group also had a lower concentration of butyrate in the jejunum and a higher concentration of NH_3-N in the colon than those in the high sanitary group ($P < 0.05$). Dietary Val supplementation reduced plasma interleukin (IL)-1β and IL-1 receptor antagonist concentrations as well as isovalerate and NH_3-N concentrations in the colon ($P < 0.05$), regardless of sanitary condition. Interactions between dietary Val supplementation and sanitary conditions were observed in the abundance of mRNA-encoding β-defensins 113, 125, and 129 ($P < 0.05$). In conclusion, dietary Val supplementation beneficially modulates inflammatory response and microbial metabolites regardless of sanitary condition, while transcriptional levels of β-defensins are regulated by dietary Val supplementation in a manner dependent on housing hygiene conditions.

Key words: bacterial profile, microbial metabolites, sanitary condition, valine, weaned pigs

6.2. INTRODUCTION

It is generally accepted that ISS realigns the metabolic pathways of nutrients, particularly AA from a growth (protein accretion) to an immune response (Le Floc'h et al., 2004). Thus, immune status should be considered for optimal performance in swine nutrition, particularly nursery pigs whose immune and intestinal microbiota homeostases are vulnerable. Of numerous immune system stimulants, sanitary stress is the most relevant to practical swine production because commercial pigs are usually exposed to low sanitary (**LS**) condition compared with experimental pigs (Adewole et al., 2016). Pigs under LS condition are more likely to have a high pathogen load and inflammation in the intestinal and respiratory tracts (van der Meer et al., 2016; van der Meer et al., 2020) than those under high sanitary (**HS)** condition. In this regard, our previous studies showed that LS conditions can increase AA requirement for optimal growth performance and gut morphology (Jayaraman et al., 2017a; Kahindi et al., 2017a). This shows that supplementation with specific FAA can increase immune status and thus growth performance in pigs.

Valine is the fourth or fifth limiting AA in nursery pig diets and as a BCAA. It plays important roles in nutrient metabolism and immunity (Nie et al., 2018). Previous studies showed that dietary Val levels are associated with intestinal barrier function and morphology (Luo et al., 2014; Xu et al., 2018). Chen et al. (2017a) found that exogenous Val promotes macrophage phagocytosis, thereby reducing pathogen loading in mice. Furthermore, BCAA supplementation can upregulate ileal expression of β-defensin, an antimicrobial peptide that plays a crucial role in innate immunity, which emphasizes the importance of dietary BCAA balance (Ren et al., 2016). Therefore, it was hypothesized that LS condition increases intestinal pathogen loading and stimulates immune response, and that L-Val supplementation would ameliorate the negative effects of LS condition by upregulating antimicrobial peptides expression. The objective of this study was

to investigate the effects of sanitary condition and dietary L-Val supplementation on growth performance, intestinal bacterial composition and metabolites, and antimicrobial peptide gene expression in nursery pigs.

6.3. MATERIALS AND METHOD

6.3.1 ANIMALS, EXPERIMENTAL DESIGN, AND DIET

Thirty-two female piglets (TN 70 × TN Tempo; Topigs Norsvin, Winnipeg, MB, Canada) with an initial BW of 7.1 ± 0.4 kg were obtained from Glenlea Research Station at the University of Manitoba. The pigs were randomly assigned to a 2 × 2 factorial arrangement based on sanitary condition and the levels of dietary SID Val, giving 8 replicates per treatment. Pigs were individually housed in pens (1.2 m × 1.5 m) either in a LS room and a HS room and fed ad libitum for 14 days. Corn-wheat-soybean meal-fish meal-dried whey-based standard Val (**STD Val**) diet was formulated to contain 0.86% of SID Val and 1.35% of SID Lys, which are equal to NRC (2012) requirements (Table 6.1). L-Val was supplemented to STD Val diet replacing 0.1% of cornstarch to make supplemental Val (**SUP Val**) diet containing 0.96% of SID Val and 0.71 of SID Val:SID Lys.

6.3.2. EXPERIMENTAL PROCEDURE, SAMPLING, AND MEASUREMENTS

A room designated as HS condition was washed and disinfected before the experiment commences and the room was washed on day 7. In contrast, a room designated as LS condition was not washed throughout the experimental period and 5 kg of manure from the nursery pig barn was spread on the pen floors in the LS room on day 1. Stainless-steel feeders for pigs in LS condition was not cleaned after previous group's use. STD diet was blown away using a fan for 20 mins in the morning and afternoon to create dusts in LS condition. A single dose of *Escherichia coli* O55:B5 LPS (100 μg/mL; Sigma-Aldrich) was administrated intramuscularly at 0.1 mL/kg BW to pigs in

Table 6.1 Diet composition and nutrients contents of experimental diets, % (as-fed basis)[1]

Item	STD Val	SUP Val
Ingredient		
Corn	39.74	39.74
Wheat	25.00	25.00
Cornstarch	0.10	-
Soybean meal	23.00	23.00
Fish meal	3.00	3.00
Dried whey	5.00	5.00
Vegetable oils	0.60	0.60
Limestone	1.20	1.20
Monocalcium phosphate	0.75	0.75
Salt	0.35	0.35
Vitamin-mineral premix[2]	0.16	0.16
$_{L}$-Lys·HCl	0.60	0.60
$_{DL}$-Met	0.20	0.20
$_{L}$-Thr	0.20	0.20
$_{L}$-Trp	0.02	0.02
$_{L}$-Val	0.08	0.18
Calculated nutrients, %		
NE, Mcal/kg	2.47	2.47
Crude protein	20.5	20.5
Ca	0.82	0.82
STTD[3] P	0.41	0.41
SID[4] Ile	0.70	0.70
SID[4] Leu	1.37	1.37
SID[4] Lys	1.35	1.35
SID[4] Val	0.86	0.96
SID Val:Lys	0.637	0.711

[1]STD Val, the standard NRC (2012) level of standardized ileal digestible Val (0.79%); SUP Val, 12% over the standardized ileal digestible Val requirement.
[2]Supplied per kilogram of diet: vitamins A, 2,200 IU; vitamin D_3, 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; thiamine, 1.5 mg; riboflavin, 4 mg; niacin, 30 mg; pantothenic acid, 12 mg; vitamin B_{12}, 0.02 mg; folic acid, 0.3 mg; 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; biotin 0.2 mg.
[3]Standardized total tract digestible.
[4]Standadized ileal digestible.

LS room, whereas pigs in HS room were intramuscularly injected with an equal volume of sterilized PBS on the first day of experiment. All pigs and feeders were weighed on days 7 and 14 to calculate BW gain and feed intake.

Rectal temperature was determined at 5 h, 24 h, and 48 h post-injection. Air quality indicators including particle matter (**PM**) 2.5, PM10, total particles, and concentrations of HCHO, total volatile organic compounds, and CO_2 were measured in the morning and afternoon, using an air quality monitor (Temtop M2000, Elitech Technology, Inc. San Jose, CA) (Table 6.2). Blood samples (5 mL) from the jugular veins of all pigs were collected into a vacutainer tubes (BD Vacutainer® sodium heparin spray-coated tube) to obtain plasma on day 14. Afterward, all pigs were euthanized by captive bolt following stresnil-xylazine (2:4 mg/kg) sedation to allow for tissue and digesta collection. A sample of jejunum was taken 4 m away from the ileocecal junction. The jejunum sample was rinsed with cold PBS. The mid-jejunum and proximal colon digesta samples were collected for analyses for bacterial profile and microbial metabolites. The tissue and digesta samples were snap-frozen in liquid N and then stored in a −80°C freezer.

6.3.3. GENOMIC DNA, TOTAL RNA EXTRACTION, COMPLEMENTARY DNA SYNTHESIS AND REAL-TIME QUANTITATIVE PCR

Genomic DNA from the jejunum and colon digesta were extracted for the determination of the relative abundance of target bacteria. Digesta samples were thawed before analyses, and DNA from 250-mg of digesta samples were extracted using QIAmp PowerFecal Pro DNA kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Total RNA was extracted from 80-mg jejunum samples using 1 mL TRIzol (Invitrogen Canada Inc., Burlington, ON, Canada) according to the manufacturer's protocol. The quantity and quality of isolated DNA and RNA were

determined using a Nanodrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE). The integrity of the total RNA was confirmed by agarose gel electrophoresis. A total of 2 μg of total RNA was used to synthesize cDNA using a high-capacity cDNA synthesis kit (Applied Biosystems, Waltham, MA) following the supplier's protocol. Quantitative real-time PCR was performed in duplicate reactions, using a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories), as described by Waititu et al. (2017). For qPCR amplification, negative controls were prepared by replacing the cDNA with nuclease-free water. A melt curve analysis was performed with a temperature gradient of 0.1°C/s from 70°C to 95°C to confirm that only specific products were amplified. Pairs of primers for mRNA gene expression were designed and checked for target identity using the National Center for Biotechnology Information database. Pairs of primers for bacteria (*Lactobacillus*/*Lactococcus*, Bifidobacterium, *Escherichia* coli/*Hafnia*/*Shigella*, *Clostridium perfringens*, *Salmonella* spp., and Butyryl-CoA:Acetate CoA-transferase) were obtained from previously published works (Remely et al., 2015; Pereira et al., 2020). All primers used for this study, information on their sequences, and reference are presented in Table 6.3.

6.3.4. SAMPLE PREPARATION AND CHEMICAL ANALYSES

Blood samples were centrifuged at 2,000 × g for 10 min at 4°C to recover plasma, which was immediately stored at –80˚C until required for cytokine analyses. Plasma samples were analyzed for the concentrations of cytokines, including TNF-α, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, and IL-18, using a commercial multiplex assay using laser bead technology at Eve Technologies Corporation. The volatile fatty acid (**VFA**) concentrations were determined by gas chromatography (Varian Chromatography System, model Star 3400; Varian Medical Systems, Palo Alto, CA) with a capillary column (30 × 0.5 mm; Restek Corp., Belefonte, PA), according to

the method described by Erwin et al. (1961). Briefly, 1 mL of 25% metaphosphoric acid was mixed with 5 mL of digesta fluid in a 15-mL centrifuge tube, and the mixture was frozen overnight. The frozen samples were then thawed centrifuged for 20 min at 3,000 × g at 4 °C, and 1 mL of the supernatant was transferred to a gas chromatography vial, using a syringe fitted with a filter.

The NH_3-N concentration in the jejunum and colon digesta samples were determined using the method described by Novozamsky et al. (1974). Briefly, 1.5 mL of a reagent containing 200 mL of 0.05% sodium nitroprusside and 10 mL of 4% ethylenediaminetetraacetic acid was added to 50 µL of sample in a 10-mL test tube. A solution containing 10% sodium hypochlorite (2.5 mL) was then added to the mixture. Test tubes containing the resulting mixture were placed in a test tube rack and incubated in complete darkness for 30 min, and then the optical density of the mixture was immediately read at 630 nm using a spectrophotometer (SoftMax Pro; Molecular Devices, Abingdon, Oxfordshire, UK).

6.3.5. CALCULATIONS AND STATISTICAL ANALYSES

The geometric means of glyceraldehyde 3-phosphate dehydrogenase and hypoxanthine-guanine phosphoribosyltransferase expression were used to normalize the transcriptional levels for antimicrobial peptides. The relative expression was expressed as a ratio of the target gene to the STD Val in HS group, using the formula $2^{-\Delta\Delta Ct}$ according to Livak and Schmittgen (2001), where $\Delta\Delta Ct = (Ct_{target} - Ct_{geometric\ mean})_{treatment} - (Ct_{target} - Ct_{geometric\ mean})_{STD\ Val\ (HS\ condition)}$.

The concentrations of NH_3-N were determined by calculating the concentration from a regression equation of the standard curves with R^2 values of 0.998. All data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Inst. Inc., Cary, NC) with each animal used as the experimental unit. The model included sanitary condition, dietary L-Val supplementation, and their interaction. The LSMEANS statement with the Tukey- adjusted PDIFF option was used to

Table 6.2 Air quality of high and low sanitary conditions

	Week 1		Week 2	
Item[1]	High sanitary	Low sanitary	High sanitary	Low sanitary
$PM_{2.5}$, $\mu g/m^3$	3.26	7.03	5.64	6.02
PM_{10}, $\mu g/m^3$	4.86	1.83	9.22	2.14
Particles, $\#/m^3$	660	1,161	864	1,020
HCHO, mg/m^3	0.001	0.167	0.003	0.027
TVOC, mg/m^3	0.042	3.28	0.14	1.57
CO_2, ppm	530	741	686	671
Temperature, °C	27.4	27.8	26.2	27.5
Humidity, %	45.5	43.3	44.2	40.9

[1]PM, particle matter; TVOC, total volatile organic compounds.

Table 6.3 Primers used for qPCR analyses of antimicrobial peptides and bacteria

Target[1]	Primer sequence (5' to 3')	Reference[2]
GAPDH	F: GTGAACGGATTTGGCCGC R: AAGGGGTCATTGATGGCGAC	NM_001206359.1
HPRT1	F: GGCTATGCCCTTGACTACAATG R: CAATGGGACTCCAGATGTTTCC	NM_001032376.2
DEFB1	F: CAG GAT TGA AGG GAC CTG TTA C R: TAC TTC ACT TGG CCT GTG TG	NM_214442.2
DEFB113	F: CTT CTG CTG GTT GCT GTT TC R: AAA GGA CAG AGA GGT GCT ATT C	XM_021100003.1
DEFB114	F: CCA GTA CAG AGA GGT GTA GGA R: GCT CCA GCT CAG AGA AGA TTA G	NM_001129973.1
DEFB125	F: CAG GGA GCC TGA TCT ACT GAT A R: AGG AAC TCG CTT GGT TCT TG	NM_001129974.1
DEFB129	F: GAA CTC TAC CAC CAC CAA CTC R: CCG TGG GTG AAT CTC TTC TTT	NM_001129975.1
PG1	F: GCT GTG TGA CTT CAA GGA GAA R: GCA GAA CCT ACG CCT ACA ATA G	NM_001123149.1
PG3	F: GCT GTG TGA CTT CAA GGA GAA R: GCA GAA CCT ACG CCT ACA ATA G	NM_001123150.1
PG4	F: GCT GTG TGA CTT CAA GGA GAA R: GAA ATC TCC TGA CAC CCT CAT T	NM_213863.1
PR39	F: GGT GAT GGA GTG GCT GAT AAC R: GAA GTC TGA GCC ACA ACA AAT AAG	NM_214450.1
Total bacteria	F: ACTCCTACGGGAGGCAG R: GTATTACCGCGGCTGCTG	Remely et al. (2015)
Lactobacillus/*Lactococcus*	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	Pereira et al. (2020)
Bifidobacterium	F: TCGCGTCYGGTGTGAAAG R: CCACATCCAGCRTCCAC	Pereira et al. (2020)
Escherichia coli subgroup[2]	F: GTTAATACCTTTGCTCATTGA R: ACCAGGGTATCTAATCCTGTT	Pereira et al. (2020)

Table 6.3 Primers used for qPCR analyses of antimicrobial peptides and bacteria (continued)

Target[1]	Primer sequence (5' to 3')	Reference[2]
Clostridium perfringens	F: CGCATAACGTTGAAAGATGG R: CCTTGGTAGGCCGTTACCC	Wu et al. (2011)
Salmonella spp.	F: CGAAGAGACCCCTGTCGTACT R: AAGTGGACGCATCGACCAA	Castaneda-Gulla et al. (2020)
Butyryl-CoA:acetate CoA-transferase	F: GCIGAICATTTCACITGGAAYWSITGGCAYATG R: CCTGCCTTTGCAATRTCIACRAANGC	Remely et al. (2015)

[1]GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPRT1, Hypoxanthine-guanine phosphoribosyltransferase 1; DFEB, defensin beta; PG, protegrin; PR39, proline-arginine-rich 39-amino acid peptide.
[2]Escherichia coli/Hafnia/Shigella.

calculate and separate the mean values for each treatment. Results were considered significant at $P < 0.05$ and tendencies were observed at $0.05 < P \leq 0.10$.

6.4. RESULTS

6.4.1. GROWTH PERFORMANCE, RECTAL TEMPERATURE, AND DIARRHEA INCIDENCE

Pigs housed in LS condition showed lower ($P < 0.05$) ADG during the first week and second week of experimental period than those housed in HS condition (Table 6.4). The LS-housed pigs had lower ($P < 0.05$) ADFI during the week 1 and overall experimental period than HS-housed pigs. LS condition decreased ($P < 0.05$) G:F during the week 1 and week 2 of experimental period. Pigs in HS condition had higher ($P < 0.05$) rectal temperature at 5 h post-injection than those in LS condition, but comparable rectal temperature was observed at 24 h and 48 h post-injection between HS and LS-housed pigs. Although there was no difference in diarrhea incidence in week 1, pigs in LS condition showed higher ($P < 0.05$) incidence of diarrhea in week 2 than those in HS condition. Dietary Val supplementation did not affect ADG, ADFI, G:F, and diarrhea incidence during the experimental period.

6.4.2. CONCENTRATIONS OF PLASMA CYTOKINES AND MICROBIAL METABOLITES IN THE JEJUNUM AND COLON

Pigs under LS condition tended to have higher ($P = 0.06$) plasma IL-1ra concentration than those under HS condition on day 14 (Table 6.5). Increased IL-10 concentration in plasma was observed ($P < 0.05$) in pigs under LS condition than in pigs under HS condition on day 14.

Table 6.4 Effect of dietary Val supplementation on growth performance of nursery pigs with different sanitary conditions

	High sanitary		Low sanitary		SEM	*P*-values		
Item	STD Val	SUP Val	STD Val	SUP Val		Clean	Val	C×V
Average daily gain, g/day								
d 0 – 7	123	115	44	34	27.56	< 0.01	0.71	0.97
d 7 – 14	204	261	136	115	55.82	0.04	0.71	0.43
d 0 – 14	163	188	90	75	37.24	0.01	0.88	0.54
Average daily feed intake, g/day								
d 0 – 7	194	184	142	121	28.38	0.03	0.54	0.83
d 7 – 14	320	369	255	249	52.13	0.05	0.64	0.56
d 0 – 14	257	276	199	185	38.56	0.04	0.93	0.63
Gain to feed ratio, g/g								
d 0 – 7	0.56	0.60	0.17	0.26	0.10	< 0.01	0.44	0.78
d 7 – 14	0.63	0.70	0.37	0.37	0.11	< 0.01	0.70	0.74
d 0 – 14	0.61	0.67	0.30	0.35	0.08	< 0.01	0.46	0.95
Rectal temperature, °C								
5 h	38.5	38.3	39.7	39.4	0.22	< 0.01	0.302	0.88
24 h	38.7	38.6	38.7	38.7	0.14	0.88	0.640	0.99
48 h	38.7	38.4	38.4	38.4	0.18	0.61	0.486	0.49
Diarrhea incidence[1], %								
d 0 – 7	13.9	11.1	8.3	25.0	-	0.44	0.34	0.67
d 7 – 14	14.8	3.7	29.6	35.2	-	< 0.01	0.51	0.13
d 0 – 14	14.4	6.7	21.1	31.1	-	< 0.01	0.99	0.45

[1]100 × number of pigs with diarrhea/(number of pigs × total experimental days); Means are different by a chi-square contingency test.

Dietary Val supplementation decreased ($P < 0.05$) IL-1β, and IL-1ra concentrations in the plasma, regardless of sanitary condition. In addition, tendencies toward the decreased concentrations of IL-1α, IL-4, IL-12, IL-18, and TNF-α concentrations were observed ($P < 0.10$) in the plasma of pigs fed SUP Val diet than those fed STD Val diet.

Pigs raised in LS condition had lower ($P < 0.05$) concentrations of acetate, butyrate, and total VFA in the jejunum and NH_3-N concentration in the colon than those raised in HS condition (Table 6.6). In contrast, trends for the increase ($P < 0.10$) in concentrations in acetate, propionate, and VFA were observed in LS-housed pigs than in HS-housed pigs. Although dietary Val supplementation did not affect jejunal microbial metabolites, it decreased ($P < 0.05$) isovalerate and NH_3-N concentrations in the colon, regardless of sanitary condition. In addition, a tendency towards the increase ($P = 0.05$) in butyrate concentration in the colon of pigs fed SUP Val diet than those fed STD Val diet.

6.4.3. RELATIVE ABUNDANCE OF SELECTED BACTERIA IN THE JEJUNUM AND COLON DIGESTA AND JEJUNAL ANTIMICROAL PEPTIDE GENE EXPRESSION

Pigs in LS condition tended to have ($P = 0.07$) lower abundance of Bifidobacterium and higher *Escherichia coli* subgroup ($P = 0.08$) in jejunum digesta than those in HS condition (Table 6.7). In addition, higher abundance of *Clostridium perfringens* in the colon was observed ($P < 0.05$) in pigs in LS condition than those in HS condition. A trend for the increase in Bifidobacterium in the jejunum digesta was observed ($P = 0.09$) in pigs fed SUP Val diet than those fed STD Val diet, regardless of sanitary condition. In the colon digesta, pigs fed SUP Val diet had higher ($P < 0.05$) abundance of Butyryl-CoA:acetate CoA-transferase gene than those fed STD Val diet. There was an interactive effect ($P < 0.05$) of sanitary condition and dietary Val supplementation

Table 6.5 Effect of dietary Val supplementation on plasma cytokines concentrations (ng/mL) of nursery pigs with different sanitary conditions[1]

	High sanitary		Low sanitary			*P*-values		
Item	STD Val	SUP Val	STD Val	SUP Val	SEM	Clean	Val	C×V
GM-CSF	0.94	0.23	0.60	0.58	0.39	0.987	0.275	0.307
IFNγ	27.42	12.73	31.51	22.10	7.54	0.357	0.105	0.716
IL-1α	0.18	0.09	0.21	0.15	0.04	0.231	0.067	0.678
IL-1β	1.48	0.66	1.78	1.08	0.37	0.323	0.041	0.872
IL-1ra	1.35	0.71	1.92	1.31	0.31	0.058	0.044	0.975
IL-2	1.79	0.98	2.45	1.70	0.50	0.164	0.115	0.949
IL-4	6.47	2.86	7.66	5.00	1.75	0.326	0.070	0.778
IL-6	0.61	0.29	0.75	0.46	0.16	0.324	0.058	0.921
IL-8	0.20	0.08	0.18	0.22	0.07	0.387	0.561	0.223
IL-10	2.14	1.94	4.52	3.17	0.80	0.028	0.325	0.718
IL-12	1.27	0.95	1.42	1.14	0.18	0.331	0.095	0.919
IL-18	6.07	3.22	7.18	4.87	1.34	0.287	0.053	0.835
TNFα	1.05	0.37	2.95	0.96	0.84	0.117	0.092	0.403

GM-CSF, granulocyte-macrophage colony-stimulating factor; IFNγ, interferon gamma; IL, interleukin; IL-1ra, interleukin 1 receptor antagonist; TNFα, tumor necrosis factor alpha.

on *Lactobacillus*/*Lactococcus* abundance in the colon. Dietary Val supplementation increased ($P < 0.05$) the abundance of *Lactobacillus*/*Lactococcus* in the colon of pigs under LS condition but did not affect in pigs under HS condition.

Pigs raised in LS condition tended to have lower abundance of mRNA encoding protegrin 4 ($P = 0.082$) and proline-arginine-rich 39-amino acid peptide (**PR39**) ($P = 0.08$) than those raised in HS condition, regardless of dietary Val supplementation (Table 6.8). There were interactive effects of sanitary condition and Val supplementation ($P < 0.05$) on mRNA abundance encoding β-defensin 113, β-defensin 125, β-defensin 129, and protegrin 1. SUP Val-fed pigs had the downregulated ($P < 0.05$) expressions of β-defensin 113, β-defensin 125, β-defensin 129, and protegrin 1, compared with STD Val-fed pigs in HS condition, but no difference was observed between SUP Val and STD Val in pigs raised in LS condition.

6.4.4. DISCUSSION

Of various immune challenge model, sanitary challenge model has been recently received great attention in swine nutrition because it best mimics the commercial swine barn environment, meaning that dietary intervention can be tested for the practical purpose. In this regards, sanitary challenge model was employed in the present study to investigate the effects of different dietary Val levels in a commercial barn environment in comparison with experimental environment. However, the model was combined with the low dose of LPS, an endotoxin commonly used for acute ISS in nutrition studies, to reflect the additional possible stressors in commercial environment for nursery pigs apart from housing hygiene (e.g., transportation, handling, social stress) and to maximize the impact of LS condition on the gut. The febrile response confirmed the acute and transient immune stimulation by LPS injection.

Table 6.6 Effect of dietary Val supplementation on microbial metabolites in the jejunum and colon of nursery pigs with different sanitary conditions

	High sanitary		Low sanitary			*P*-values		
Item[1]	STD Val	SUP Val	STD Val	SUP Val	SEM	Clean	Val	C×V
Jejunum[2]								
Acetate	3.94	4.78	2.02	2.31	0.860	0.010	0.484	0.729
Propionate	0.61	1.04	0.37	0.39	0.387	0.223	0.532	0.570
Isobutyrate	0.20	0.17	0.16	0.17	0.050	0.700	0.871	0.670
Butyrate	0.12	0.12	0.03	0.04	0.031	0.005	0.854	0.816
Valerate	0.17	0.21	0.14	0.14	0.036	0.147	0.587	0.611
VFA	5.10	6.34	2.72	3.05	1.154	0.013	0.467	0.673
NH_3-N	8.14	7.39	7.63	7.14	1.427	0.783	0.659	0.926
pH	6.63	6.46	6.35	6.18	0.209	0.172	0.405	0.997
Colon								
Acetate	18.89	25.41	27.80	34.85	5.044	0.071	0.176	0.957
Propionate	10.26	12.64	16.30	16.45	2.881	0.089	0.655	0.693
Isobutyrate	0.49	0.37	0.37	0.35	0.058	0.209	0.240	0.349
Butyrate	5.31	6.31	5.45	9.39	1.266	0.202	0.054	0.242
Isovalerate	0.61	0.33	0.45	0.37	0.088	0.514	0.041	0.271
Valerate	1.52	1.60	1.75	2.55	0.409	0.147	0.276	0.373
VFA	37.08	46.66	54.61	63.96	9.158	0.060	0.296	0.989
NH_3-N	15.80	11.36	19.50	16.09	1.877	0.028	0.040	0.780
pH	6.18	6.19	6.10	6.08	0.172	0.579	0.970	0.925

[1]BCFA, branched-chain fatty acids = the sum of isobutyrate, isovalerate, and valerate; VFA, total volatile fatty acids = acetate + propionate + butyrate + BCFA.
[2]Isovalerate was not detected in most of jejunal digesta samples.

The LS condition is typically created by the absence of cleaning, the addition of manure, and manure build up. This condition increases the chances of pigs to contact the manure and to ingest the pathogens residing manure. The pathogenic *E. coli* are facultative anaerobes and they can survive in the manure (Semenov et al., 2011), meaning that they can be orally transferred into the gut. This may explain our finding of tendency towards the increase in *E. coli* subgroup abundance in the jejunum of LS housed pigs compared with HS housed pigs. This is partially supported by Montagne et al. (2012) in which greater populations of Enterobacteriaceae, a family that includes *E. coli* in feces of LS-housed pigs compared with HS-housed pigs. In addition, the increase in *Clostridium perfringens* abundance in the colon, but not in the jejunum was observed in pigs housed in LS condition compared with HS condition. Considering that *C. perfringens* are the obligatory anaerobic and spore-forming bacteria (Brynestad and Granum, 2002), the *C. perfringens* were possibly present in the forms of spore in the manure and ingested spores may have been germinated and proliferated under favorable environment. Interestingly, apart from harmful bacteria, the abundance of *Bifidobacterium* spp. in the jejunum was suppressed by sanitary challenge. Similarly, our previous study (Waititu et al., 2017) found the lower abundance of *Bifidobacterium* spp. in the cecum and colon in pigs housed in LS room compared with those housed in HS room. This may be associated with the proliferation of harmful bacteria that can competitively suppress the beneficial bacteria (Mousavi Khaneghah et al., 2020).

Not surprisingly, the concentrations of intestinal microbial metabolites were influenced by sanitary challenge. Recent studies (Cho et al., 2020; Te Pas et al., 2020) have reported that butyrate concentrations in the colon digesta or feces decreased when pigs were housed in LS condition compared with HS condition. This is consistent with our results showing the higher concentrations in the jejunum of pigs housed in LS condition than those housed in HS condition. This seems to

Table 6.7 Effect of dietary Val supplementation on the relative abundance of bacteria on the jejunal mucosa of the pigs with sanitary condition

	High sanitary		Low sanitary			*P*-values		
Item	STD Val	SUP Val	STD Val	SUP Val	SEM	Clean	Val	C×V
Jejunum[1]								
Lactobacillus/*Lactococcus*	1.00	1.38	1.39	1.61	0.24	0.192	0.214	0.739
Bifidobacterium	1.00	2.24	0.46	0.91	0.53	0.065	0.094	0.421
Escherichia coli subgroup[2]	1.00	0.37	2.87	1.67	0.95	0.076	0.293	0.739
Clostridium perfringens	1.00	0.99	1.05	1.08	0.28	0.802	0.959	0.932
Colon								
Lactobacillus/*Lactococcus*	1.00^{xy}	0.65^{xy}	0.32^{x}	1.37^{y}	0.36	0.951	0.283	0.037
Bifidobacterium	1.00	0.79	0.97	0.42	0.25	0.423	0.139	0.504
Escherichia coli subgroup[2]	1.00	0.55	1.16	1.32	0.48	0.289	0.745	0.480
Clostridium perfringens	1.00	0.84	1.24	1.83	0.29	0.037	0.450	0.192
Salmonella spp.	1.00	0.82	1.15	1.01	0.32	0.586	0.612	0.943
Butyryl-CoA:acetate CoA-transferase	1.00	1.98	1.65	3.65	0.72	0.115	0.046	0.477

[1]Butyryl-CoA:acetate CoA-transferase and *Salmonella* spp. was not detected.

[2]Escherichia coli/Hafnia/Shigella.

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

Table 6.8 Effect of dietary Val supplementation on relative abundance of mRNA genes encoding antimicrobial peptides in the jejunum of nursery pigs with sanitary condition

	High sanitary		Low sanitary			*P*-values		
Item[1]	STD Val	SUP Val	STD Val	SUP Val	SEM	SC	Val	C×V
DEFB1	1.00	0.85	0.51	0.83	0.19	0.176	0.644	0.217
DEFB113	1.00^{x}	0.32^{y}	0.39^{y}	0.54^{y}	0.19	0.285	0.160	0.030
DEFB114	1.00^{X}	0.40^{Y}	0.38^{Y}	0.72^{XY}	0.24	0.512	0.582	0.051
DEFB125	1.00^{x}	0.37^{y}	0.35^{y}	0.64^{xy}	0.21	0.366	0.410	0.031
DEFB129	1.00^{x}	0.40^{y}	0.39^{y}	0.59^{xy}	0.20	0.284	0.311	0.049
PG1	1.00^{x}	0.46^{y}	0.44^{y}	0.68^{xy}	0.19	0.328	0.405	0.036
PG3	1.00^{x}	0.53^{xy}	0.33^{y}	0.69^{xy}	0.22	0.246	0.799	0.060
PG4	1.00	1.28	0.68	0.74	0.26	0.082	0.481	0.643
PR39	1.00	0.75	0.31	0.58	0.25	0.078	0.958	0.277

[1]DFEB, defensin beta; PG, protegrin; PR39, proline-arginine-rich 39-amino acid peptide.
^{x,y}Within a row, means with different superscripts differ ($P < 0.05$).
^{X,Y}Within a row, means with different superscripts differ ($P < 0.10$).

be associated with the abundance of butyrate-producing bacteria, such as *Clostridiales* family XIII incertae Sedis (Cho et al., 2020) and *Clostridium* IV and XIVa (Waititu et al., 2017). The trophic roles of butyric acid in the gut have been well documented. Because butyric acid has immunomodulatory properties and capability to stimulate enterocyte proliferation (Montagne et al., 2003), maintaining HS conditions for nursery pigs would be beneficial for their gut health. In addition, butyrate is known to have antidiarrheal function by promoting the absorption of sodium, potassium, and water (Leonel and Alvarez-Leite, 2012). Thus, the higher butyrate concentrations and lower abundance of harmful bacteria associated with diarrhea may contribute to the less incidence of diarrhea throughout the experimental period in the HS housed pigs compared with the LS housed pigs.

In contrast to the metabolite concentrations in the jejunum, higher VFA concentrations were observed in LS housed pigs compared with HS housed pigs. This is supported by previous studies in which sanitary challenge increased total VFA in the colon digesta (Te Pas et al., 2020) or the feces (Montagne et al., 2012). Te Pas et al. (2020) speculated that the increase in VFA may be associated with the increase in the abundance of *Lachnospiraceae* family, a major family involved in VFA synthesis from complex polysaccharides. In addition, Montagne et al. (2012) postulated that the increase in VFA in pigs housed in LS conditions may be explained by the concept of “hygiene hypothesis”, which means that the exposure to the microorganisms helps animals develop healthy immune system and intestinal microbiota (Stiemsma et al., 2015). However, in the present study, considering the lower VFA concentrations in the jejunum and the greater colonic concentrations of NH_3-N, a noxious product of protein fermentation, in LS-housed pigs, it is possible that the LS condition possibly increased the passage of carbohydrates and proteins from the small intestine to large intestine, promoting the fermentation of carbohydrates

and proteins and their metabolites in the large intestine. Furthermore, this shift of digestion site may be associated with the lower G:F in LS-housed pigs. In fact, it has been documented that the shift in digestion site from the foregut to hindgut reduces the energy efficiency (Koo and Nyachoti, 2021). However, no information is available whether LS condition can affect ileal digestibility.

In sanitary challenge model, it is assumed that the immune system of pigs is mildly and chronically stimulated by varied stressors including biologic compounds (e.g., pathogens) and non-biologic compounds (e.g., noxious gasses, dust). Because of warm and wet environment in swine facility, manure is easily fermented by microbes emitting various noxious gasses. As measured in the present study, formaldehyde and total volatile organic compounds concentrations were higher in LS room than in HS room. Other than organic compounds, despite they are not analyzed in the present study, our previous study (Jayaraman et al., 2017a) showed that the concentrations of NH_3 and H_2S, typical noxious compounds in swine barn, are higher in LS room than in HS room. In addition, in the present study, dust was generated by blowing away feed using a fan in LS room to mimic the commercial environment in which stock density and pigs' activity are high. This resulted in numerically higher PM 2.5, PM 10, and total particles. All these non-biologic and biologic stressors are well documented to be associated with chronic inflammation (van der Meer et al., 2016; Wolkoff, 2018; Tran et al., 2020). However, in contrast to our expectation, sanitary challenge did not elevate plasma pro-inflammatory cytokine concentrations, but did elevate anti-inflammatory cytokines: IL-1ra and IL-10. IL-ra binds IL-1 receptors located on various cells such as endothelium, immune cells, and muscle cells and they prevent IL-1 binding, thereby inhibiting pro-inflammatory responses of IL-1 (Arend et al., 1998). IL-10 is a potent immunosuppressive cytokine with various effects on innate and adaptive immune system (Jaffer et al., 2010). Thus, it seems that pigs under LS condition restored the inflammatory state by

upregulating anti-inflammatory cytokines at the time of sampling. This is partially supported by previous finding (van der Meer et al., 2016) in which pigs in LS condition had higher concentrations of APP (i.e., haptoglobin and PigMAP) in the serum, but the concentrations decreased as pigs grew older. Immune system burden together with harmful microbial load possibly contributed to the poor ADG in pigs raised under LS conditions compared with pigs raised under HS conditions in the present study. This is consistent with previous studies where pigs raised in LS condition showed compromised growth performance than those raised in HS condition (Zhao et al., 2007; Le Floc'h et al., 2009; van der Meer et al., 2016; Jayaraman et al., 2017a; Shin et al., 2017; Cho et al., 2020).

To investigate the effects of dietary L-Val supplementation on our measurements, 0.1% of L-Val was supplemented to STD Val diet. The STD Val diet was formulated to contain 1.37% of SID Lys and 0.86% of SID Val, which were precisely 100% of NRC (2012) requirement for 11 kg BW at 2,467 kcal/kg of diet. The L-Val supplementation level was based on the previous study (Xu et al., 2018) in which the diet with 70% of SID Val: SID Lys led to best results on growth performance and intestinal histomorphology.

Our finding suggests that dietary Val supplementation modulates bacterial composition. Dietary Val supplementation increased the abundance of *Lactobacillus*/*Lactococcus* when pigs were under LS condition. Pigs fed the SUP Val diet were more enriched with *Bifidobacterium* spp. in the jejunum and Butyryl-CoA:Acetate CoA-transferase in the colon regardless of sanitary condition, compared with pigs fed with STD Val diet. Similarly, it has been reported that dietary Leu and Val supplementation to a low protein diet significantly improved the abundance of Lactobacillales and Aeromonadales and suppressed the abundance of Gammaproteobacteria in the distal intestine (Yin et al., 2020). It is unclear how dietary AA supplementation can modify

microbial composition. However, given that L-Val is absorbed into the body at almost 100% at the small intestine, its effect on distal intestine may be associated with the enhanced host immune system (Qi et al., 2021). In fact, L-Val is known to regulate the function and maturation of monocyte-derived dendritic cells, which are antigen-presenting cells playing critical roles in linking innate and adaptive immunity (Kakazu et al., 2007). Furthermore, Chen et al. (2017a) reported that exogenous L-Val promoted macrophage phagocytosis, which reduced bacterial infection in mice.

Butyrly-CoA:acetate CoA-transferase is the enzyme that is involved in the final step of butyrate production in bacteria, by catalyzing the conversion of butyryl-CoA and acetate into butyrate and acetyl-CoA (Hippe et al., 2011). Thus, this gene is often targeted to quantify the bacteria that are capable to produce butyrate in the gut. In the present study, higher abundance of Butyryl-CoA:acetate CoA-transferase gene in SUP Val-fed pigs aligned with higher butyrate concentrations in the colon, reflecting that SUP Val promoted butyrate-producing bacteria in the colon.

Because intestinal branched chain fatty acids including iso-valerate and iso-butyrate are microbially produced from BCAA, the concentrations of intestinal branched chain fatty acids widely used as a protein fermentation indicator (Heo et al., 2010). NH_3-N is also an indicator of fermentation of N compounds including protein and urea (Nyachoti et al., 2006). Thus, the decreased colonial iso-valerate and NH_3-N concentrations with SUP Val suggests the decreased fermentation of N-containing compounds in the colon. The SUP Val may have reduced AA flow into the large intestine. This is partially supported by previous studies in which dietary BCAA supplementation upregulated the expression of mRNA encoding AA transporters including Na^+-neutral AA exchanger-2, cationic AA transporter-1 (**CAT1**), $b^{0,+}$ AA transporter (Zhang et al.,

2013). Another possible explanation is that L-Val supplementation may have balanced dietary AA and reduced the production of urea, a major AA metabolite (Wu, 2009). Although most of urea is excreted from the body through urine, blood urea can also be transferred into the intestinal lumen through urea transporter B system (Yu et al., 2019). Then, the urea is fermented by microbial urease, producing NH_3 and carbon dioxide (Barrett, 2014). This speculation can be partially supported by Spring et al. (2020) in which dietary BCAA supplementation can reduce urea concentrations in the plasma of nursery pigs. Similarly, dietary Val supplementation decreased the concentrations of NH_3 in the serum and litter of broiler chickens (Ospina-Rojas et al., 2014). However, it is uncertain whether SUP Val suppressed the abundance of the microbes that preferentially ferment protein or N-containing compounds. Thus, metagenomics and metabolomics would help elucidate the effect of SUP Val on intestinal metabolites.

Effects of SUP Val on plasma cytokine concentrations were noticeable by reducing concentrations of inflammatory cytokines, regardless of sanitary condition. The reason is not clear, but the beneficial modification of SUP Val on microbial metabolites and bacterial composition may be associated with suppressed inflammatory cytokines. In fact, protein fermentation products are known to irritate intestinal epithelial cells and provoke post-weaning diarrhea (Heo et al., 2013). Furthermore, there is growing evidence that BCAA balance seems to play important roles in immunity by serving as a fuel source for immune cells and modulating the functions of immune cells such as neutrophils, lymphocytes, and dendritic cells (Zhang et al., 2017).

Antimicrobial peptides are small peptides (typically 12-100 AA residues) that have potent antibacterial, antiviral, and antifungal properties in pigs (Zhang et al., 2000). Furthermore, accumulating evidence shows that antimicrobial peptides possess immunomodulatory activities (Giuliani et al., 2007). These antimicrobial peptides are synthesized in many cells in pigs,

including immune cells and epithelial cells and play important roles in maintaining a stable microbiota composition in the gut as a part of the innate immune system (Zhang et al., 2000). It has been generally believed that inflammatory (e.g., ulcerative colitis) and infectious stimuli can upregulate the expression of various antimicrobial peptides (Takahashi et al., 2001; Fahlgren et al., 2003; Campbell et al., 2012). Contradictorily, there is a consistent report that antimicrobial peptide expressions in the colon and ileum are attenuated in humans with Crohn's diseases (Wehkamp et al., 2007). To our best of knowledge, this is first report on the relation between sanitary condition and intestinal antimicrobial peptide expression in pigs. We found that sanitation degradation can decrease antimicrobial peptide expression in the jejunum, particularly Cathelicidin class which protegrin and PR39 belong to, regardless of dietary Val levels. Our results suggest that the antimicrobial deficiency in pigs under LS condition may be partly associated with poorer growth performance, compared with pigs under HS condition.

Ren et al. (2016) reported that dietary BCAA supplementation to protein restricted diet can promote the expression of β-defensins in epithelial cell IPEC-J2 and in vivo. In the present study, interactive effects of dietary Val supplementation and sanitary condition were noticeable on antimicrobial peptide expression in the jejunum. It seems that SUP Val diet suppresses antimicrobial peptide expression in HS condition while it upregulates the antimicrobial peptide expression in LS condition. The reason for the interaction is unclear. However, it is possible to postulate that the optimal BCAA ratio differs in different sanitary condition and thus SUP Val diet can be optimal for antimicrobial peptide expression in LS condition but surplus in HS condition. In fact, previous studies showed that sanitary condition can shift the requirements for AA (i.g., sulfur AA, Trp) for optimal growth and immune responses (Jayaraman et al., 2017a; Kahindi et al., 2017a). Because antimicrobial peptide syntheses are strictly regulated by complex stages at the

transcriptional, post-translational, and secretion levels (Mukherjee and Hooper, 2015), further studies should quantify their gene expression together with protein expression to investigate the relation between antimicrobial peptide and sanitary condition.

In conclusion, raising pigs in LS conditions can negatively impact intestinal microbial metabolites and bacterial composition in the jejunum and colon. This can partly contribute a higher diarrhea incidence and poorer growth performance in pigs under LS conditions compared with HS conditions. Although dietary Val supplementation could not ameliorate the negative effects of LS conditions, it suppressed inflammatory responses and beneficially modulated bacterial composition and microbial metabolites in the gut regardless of sanitary condition. Therefore, it is recommended that SUP Val diet is fed to nursery pigs for a fortified intestinal environment and suppressed inflammatory responses, regardless of sanitary condition. However, further studies are needed to confirm whether SUP Val diet is negatively impact on antimicrobial peptide expression in HS conditions.

7. MANUSCRIPT V

Effects of functional amino acid blend dietary supplementation with different feeding regimens on growth performance and protein utilization in weaned pigs

TRANSITION STATEMENT

Experiment 1 – 3 showed that dietary supplementation of Thr, sulfur amino acids, and Val can be a nutritional strategy to improve intestinal barrier function, microbial composition, inflammation, or redox status in nursery pigs. However, it was questioned that the benefits can enhance growth performance in nursery pig production. Furthermore, it should be investigated when the FAA are supplemented to maximize their efficacy. Therefore, Experiment 4 tested the FAA with a large number of animals and feeding regimen to commercially apply the nutritional strategy in nursery pig production

7.1. ABSTRACT

A study was conducted to investigate the effects of functional amino acid (AA) blend dietary supplementation with different feeding regimens on growth performance and protein utilization in weaned pigs. Ninety-six piglets (6.51 ± 0.34 kg) were randomly assigned to 1 of 4 feeding regimens, giving 8 replicates per treatment with 3 pigs per pen. Nursery diets were provided according to a 3-phase feeding program *ad libitum*: phase I (days 0 – 7), phase II (days 7 – 21), and phase III (days 21 – 35). Pigs were fed either a standard diet (STD) or a supplemental diet (SUP). The STD diet was formulated to meet NRC (2012) requirements for Lys, Met, Thr, Trp, and Val. An AA blend (Met, Thr, Trp, Val, and Ileu) was added to the STD diet to make the SUP diet. Pigs following the SUP7, SUP 21, and SUP35 regimens were fed a supplemental diet up to

phases I, II, and III, respectively. Pigs following the STD35 regimen were fed an STD diet throughout the experimental period. Fecal and blood samples were collected at the end of each phase to assess apparent total tract digestibility (ATTD) and blood chemistry profiles, respectively. On day 35, one pig per pen was euthanized to collect jejunum and muscle samples for gene expression analysis. All data were analyzed using a mixed SAS procedure, with the individual pen as the experimental unit. During phase I, pigs fed an SUP diet exhibited a greater ($P < 0.05$) ADG and gain:feed, as well as a greater γ-glutamyl transferase concentration in the plasma, than those fed the STD diet. During phase III, pigs following the SUP35 regimen tended to have higher ($P < 0.05$) ADG than those following the STD35 regimen. Pigs in the SUP35 group had a higher gain:feed ($P < 0.05$) than those in the SUP7 group. Pigs in the SUP21 and SUP35 groups had greater ($P < 0.05$) ATTD of N than those in the SUP7 group. Pigs in the SUP21 group had higher ($P < 0.05$) plasma urea concentrations than pigs in the STD35 or SUP7 group. Pigs in the SUP35 group had upregulated ($P < 0.05$) mRNA encoding mammalian target of rapamycin (mTOR) and cationic amino acid transporter 1 in their *longissimus dorsi* than those in the SUP21 group. In conclusion, feeding a SUP diet improved growth performance during phase I. Withdrawal of the SUP diet at phase II or phase III had a negative impact on subsequent gain:feed and protein utilization, lowering N digestibility. By contrast, continuation of the SUP diet throughout the 3-phase program led to higher ADG and G:F than STD group and SUP7 groups, respectively, with the greatest ATTD of N and *mTOR* expression in the muscle. It is recommended that feeding an SUP diet throughout 5-week nursery production period to achieve optimal growth performance and protein metabolism.

Keywords: feeding regimen, functional amino acids, nitrogen utilization, growth performance, weaned pigs

7.2. INTRODUCTION

The optimal dietary AA content has been an important topic in swine nutrition research because dietary AA are the building blocks of protein, which in turn becomes pork. However, over the past decade, the functionality of AA has received increasing attention (Wu, 2010; Le Floc'h et al., 2018) because synthetic AA become more popular and pigs' health has emerged as a vital issue in the pork industry (Pluske et al., 2018a). In fact, dietary Thr provision stimulates the synthesis of mucins, enhancing intestinal morphology, innate immunity, and intestinal barrier function (Chen et al., 2017b). Methionine—a SAA—undergoes transsulfuration and transmethylation pathways, which are intricately related to inflammation and oxidative balance (Bauchart-Thevret et al., 2009a). Dietary Trp is the precursor of kynurenine and serotonin, which are involved in neurotransmitter signaling and the modulation of inflammation and feed intake (Le Floc'h et al., 2011). The dietary balance of BCAA, including Leu, Ile, and Val, modulates the mTOR signaling pathway that mediates AA transporters and protein anabolism (Nie et al., 2018).

It is now generally accepted that ISS realigns AA utilization from growth toward immune response (Le Floc'h et al., 2004). However, conventional AA requirements were estimated for maximal protein accretion (NRC, 2012; van Milgen and Dourmad, 2015) and did not consider the role of AA metabolism in immune response. This is relevant to nursery pigs whose immune systems are often compromised by various types of weaning stress (Moeser et al., 2017). Growing evidence has shown that dietary FAA supplementation above the standard requirement can enhance the health status of weaned pigs, but information on how enhanced health status can lead to improved growth performance is limited. Furthermore, no information is available on how this strategy can be introduced via phase feeding in commercial nurseries. We therefore hypothesized that dietary supplementation with a FAA blend above the requirement would improve growth

performance and N utilization. The objective of this study was to investigate the effects of dietary supplementation of a FAA blend with different feeding regimens on growth performance and protein utilization in weaned pigs.

7.3. MATERIALS AND METHODS

7.3.1. ANIMALS, HOUSING, EXPERIMENTAL DESIGN, AND DIET

Ninety-six piglets (TN 70 × TN Tempo; Topigs Norsvin, Winnipeg, MB, Canada) with an initial BW of 6.51 ± 0.34 kg were obtained from Glenlea Research Station at the University of Manitoba. The pigs were randomly assigned to 1 of 4 feeding regimens, giving 8 replicates per treatment with 3 pigs per pen. Nursery diets were provided according to a 3-phase feeding program: phase I (days 0 to 7), phase II (days 7 to 21), and phase III (days 21 to 35). Pigs were fed either a standard diet (**STD**) or a supplemental diet (**SUP**). Pigs in the control group were fed the STD diet throughout the experimental period (**STD35**). The remaining pigs were fed the supplemental diet up to phases I (**SUP7**), II (**SUP21**), and III (**SUP35**), respectively. Pigs in the SUP7 and SUP21 groups were fed the STD diet for the remainder of the experimental period after the withdrawal of the SUP diet.

The STD diet was formulated to be equivalent to the NRC (2012) requirement for SID Lys, Thr, Met, Met + Cys, Trp, and Val for 7 kg (phase I), 7–11 kg (phase II), and 11–25 kg (phase III) of BW (Table 7.1). Feed-grade $_{L}$-Thr, $_{DL}$-Met, $_{L}$-Trp, $_{L}$-Val, and $_{L}$-Ile were added to the STD diet at the expense of cornstarch, and the ratios for SID Thr, Met + Cys, Trp, Val, and Ile to SID Lys were 0.67, 0.62, 0.24, 0.70, and 0.57. These ratios were maintained in diets across the phases. TiO_2 (Sigma Chemical Company, St. Louis, MO) was added at 0.3 % to the experimental diets as an indigestible marker to determine ATTD. All experimental diets were fed

Table 7.1 Ingredient and calculated nutrient composition of experimental diets (%, as-fed basis)[1]

	Phase I		Phase II		Phase III	
Item	STD	SUP	STD	SUP	STD	SUP
Ingredient						
Corn	29.00	29.00	38.51	38.51	44.88	44.88
Wheat	25.00	25.00	25.00	25.00	25.00	25.00
Cornstarch	0.51	–	0.49	–	0.42	–
Soybean meal (CP, 46%)	29.50	29.50	29.00	29.00	24.00	24.00
Fish meal	3.00	3.00	–	–	–	–
Dried whey	7.00	7.00	–	–	–	–
Vegetable oils	1.52	1.52	1.75	1.75	0.90	0.90
Limestone	1.13	1.13	1.33	1.33	1.24	1.24
Monocalcium phosphate	0.75	0.75	1.18	1.18	0.87	0.87
Salt	0.40	0.40	0.40	0.40	0.40	0.40
Vitamin mineral premix[2]	1.00	1.00	1.00	1.00	1.00	1.00
Lys•HCl	0.51	0.51	0.61	0.61	0.59	0.59
$_{DL}$-Met	0.19	0.29	0.19	0.29	0.17	0.25
$_{L}$-Thr	0.17	0.29	0.18	0.30	0.18	0.28
$_{L}$-Trp	–	0.10	–	0.10	–	0.09
$_{L}$-Val	0.02	0.12	0.06	0.15	0.05	0.13
$_{L}$-Ile	–	0.09	–	0.08	–	0.07
Titanium dioxide	0.30	0.30	0.30	0.30	0.30	0.30
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated Nutrient, %						
NE, Mcal/kg	2.45	2.45	2.45	2.46	2.45	2.45
Crude protein, %	23.0	23.3	21.0	21.3	19.2	19.5
Ca, %	0.83	0.83	0.80	0.80	0.70	0.70
STTD[3] P, %	0.43	0.43	0.40	0.40	0.33	0.33
SID[4] Ile, %	0.81	0.90	0.73	0.81	0.66	0.73
SID Leu, %	1.53	1.53	1.44	1.44	1.35	1.35
SID Lys, %	1.44	1.44	1.35	1.35	1.23	1.23
SID Met, %	0.49	0.59	0.46	0.55	0.42	0.50
SID Met + Cys, %	0.79	0.89	0.75	0.84	0.69	0.77
SID Thr, %	0.85	0.97	0.79	0.91	0.73	0.83
SID Trp, %	0.24	0.34	0.22	0.32	0.20	0.29
SID Val, %	0.91	1.01	0.87	0.95	0.78	0.86

[1]STD, the standard NRC (2012) level of standardized ileal digestible Lys, Met, Met + Cys, Thr, Trp, and Val; SUP, $_{L}$-Thr, $_{DL}$-Met, $_{L}$-Trp, $_{L}$-Val, and $_{L}$-Ile supplemented diet.
[2]Supplied per kilogram of diet: vitamins A, 2,200 IU; vitamin D3, 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; thiamine, 1.5 mg; riboflavin, 4 mg; niacin, 30 mg; pantothenic acid, 12 mg; vitamin B12, 0.02 mg; folic acid, 0.3 mg; 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; biotin 0.08 mg.
[3]Standardized total tract digestible.
[4]Standardized ileal digestible.

in mash form. Each pen (1.2 × 1.5 m) had a plastic-covered expanded metal floor, a stainless-steel feeder, and a low-pressure nipple drinker. Pigs had free access to feed and water throughout the experiment. Room temperature was maintained at 29 ± 1°C during the first week and then reduced by 1°C per week thereafter.

7.3.2. EXPERIMENTAL PROCEDURE, SAMPLING, AND MEASUREMENTS

BW and feed consumption were determined weekly, and the two variables were used to calculate ADG, ADFI, and G:F. Freshly voided feces were collected from each pen from 0800 to 1600 h by grab sampling for 2 days at the end of each phase (days 5, 6, 19, 20, 33, and 34). To mimic the commercial environment, pen floors remained unwashed throughout the experimental period, and room floors were washed only on day 21. Samples were pooled by pen, weighed, and frozen at –20 °C until required for further analyses. On day 7, after weighing the pigs, blood samples (6 mL) were collected in a vacutainer (BD Vacutainer® sodium heparin spray-coated tube) from the jugular vein of 1 pig (median BW) per pen, and the same pigs were sampled on days 24 and 35. The blood samples were immediately centrifuged at 2,000 × g for 10 min at 4 °C to recover plasma. The plasma samples were immediately stored at −80 °C until required for analysis. On day 35, after the blood collection, the same pigs (1 pig per pen) were euthanized by captive bolt following Stresnil® xylazine (2:4 mg/kg) sedation. Samples from the proximal jejunum were taken 4 m away from the pylorus. The right side of each *longissimus dorsi* between 6–7th rib was sampled. The jejunum and muscle samples were rinsed with cold phosphate-buffered saline, snap-frozen in liquid N, and then stored in a −80 °C freezer. The whole GIT was removed, emptied, and weighed to calculate the empty BW. Spleen, kidneys, pancreases, and livers were weighed. Thereafter, the carcasses and internal organs were frozen separately at –20 °C. Care was taken to ensure that all blood was collected.

7.3.3. TOTAL RNA EXTRACTION, COMPLEMENTARY DNA SYNTHESIS, AND REAL-TIME QUANTITATIVE PCR

Total RNA was extracted from 80 mg of jejunum and muscle using 1 mL TRIzol™ (Invitrogen Canada Inc., Burlington, ON, Canada) according to the manufacturer's protocol. The quantity and quality of isolated DNA and RNA were determined using a Nanodrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE). The integrity of the total RNA was confirmed by agarose gel electrophoresis. A total of 2 μg of total RNA was used to synthesize cDNA using a high-capacity cDNA synthesis kit (Applied Biosystems, Waltham, MA) following the supplier's protocol. qPCR was performed for duplicate reactions using a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories), as described by Waititu et al. (2017). For qPCR amplification, negative controls were prepared by replacing the cDNA with nuclease-free water. A melt curve analysis was performed with a temperature gradient of 0.1 °C/s from 70 °C to 95 °C to confirm that only specific products were amplified. Pairs of primers for the mTOR signaling pathway (4E-BP1, myocyte enhancer factor 2A [**MEF2A**], P70); mTOR, myogenic regulatory factors (MyoD1, MyoG); and AA transporters (Ala/Ser/Cys-like sodium dependent neutral amino acid transporter 2, CAT1, L-type AA transporter 1 [**LAT1A**], sodium-coupled neutral AA transporter 2, b(0,+)-type amino acid transporter 1, sodium- and chloride-dependent neutral and basic AA transporter B(0,+)), were designed and checked for target identity using the National Center for Biotechnology Information

Table 7.2 Primers used for qPCR analyses for housekeeping genes, protein metabolism, and amino acid transporters

Target[1]	Amplicon size, bp	Primer sequence (5' to 3')	Reference[2]
GAPDH	142	F: GTGAACGGATTTGGCCGC R: AAGGGGTCATTGATGGCGAC	NM_001206359.1
β-actin	131	F: CCA CGA AAC TAC CTT CAA CTC R: TGA TCT CCT TCT GCA TCC T	DQ845171
4E-BP1	100	F: CGG TGA AGA GTC ACA GTT T R: TGG GAT CCT TCT CCC AAA	NM_001244225.1
MEF2A	94	F: GCA GAA CCA ACT CGG ATA TT R: GGA CTG TGC TCA AGG TAA TC	NM_001097421.1
P70	95	F: GAC ATC CTT GCT GAC GTA AA R: CGC AGG AAG TCC AGA ATA AG	NM_001243214.1
mTOR	101	F: CGT CAA GAA GGA GAA GGA AC R: CCA GCA CAC GAG GTA AAT AG	XM_003127584.6
MyoD1	101	F: CTA TGA TGA CCC GTG TTT CG R: AGT GTT CCT CGG GCT TTA	NM_001002824.1
MyoG	102	F: CCT ACA GAT GCC CAC AAT C R: TCA GTT GGG CAT GGT TTC	NM_001012406.1
ASCT2	134	F: CCA GCA AGA TTG TGG AGA TG R: GCG AGT GAA GAG GAA GTA GA	XM_003127238.5
CAT1	100	F: GTG TCG TCA TCG CAG TTT R: GCA AAG GGC ACA GAA AGA	NM_001012613.1
LAT1	116	F: GAG CTG AAT CCT GGC ATA AC R: CGC CCT ACG ATA AAC AAA CA	XR_002344446.1
SNAT2	97	F: CTT GCC GAC CAC GTT ATT R: ATG GGA AGG ATA GCA GGA T	NM_001317081.1
$b^{0,+}AT$	110	F: CTG GCC CAA GGA AAT ACA A R: CAT CCA TCA TAT GCC CAG AG	NM_001110171.1

Table 7.2 Primers used for qPCR analyses for housekeeping genes, protein metabolism, and amino acid transporters (continued)

Target[1]	Amplicon size, bp	Primer sequence (5' to 3')	Reference[2]
B^0AT1	130	F: AGT GTG TTC GCT GGA TTT G R: GGG CTA GAG CTT CTG GAT AA	NM_001348402.1

[1]4E-BP1, eukaryotic initiation factor 4E-binding protein 1; MEF2A, myocyte enhancer factor 2A; P70, ribosomal protein S6 kinase A1; mTOR, mammalian target of rapamycin; MyoD1, myoblast determination protein 1; MyoG, myogenin; ASCT2, Ala/Ser/Cys-like sodium dependent neutral amino acid transporter 2 (SLC1A5); CAT1, cationic amino acid transporter 1 (SLC7A1); LAT1, L-type AA transporter 1 (SLC7A5); SNAT2, sodium-coupled neutral AA transporter 2 (SLC38A2); $b^{0,+}$AT, b(0,+)-type amino acid transporter 1 (SLC7A9); B^0AT1, sodium- and chloride-dependent neutral and basic amino acid transporter B(0,+) (SLC6A14).
[2]National Center for Biotechnology Information (NCBI) Accession number.

database. All primers used for this study, information on their sequences, and references are presented in Table 7.2.

7.3.4. SAMPLE PREPARATION AND CHEMICAL ANALYSES

The frozen plasma samples were thawed and analyzed to determine the blood chemistry profiles using the Ortho Clinical Vitros 250 Chemistry System (Johnson & Johnson®, New Brunswick, NJ). Fecal samples were dried in a forced-air oven (CDO-28, Cascade Sciences, Hillsboro, OR) at 60 °C for 3 days and weighed to calculate the fecal DM. Dried feces and diet samples were finely ground before chemical analysis with a grain miller (50−200 μm of fineness; HC-700, Boshi Electronic Instrument, Guangzhou, China). The ground samples were analyzed for DM, N, and Ti content. The DM was determined according to the methods recommended by the AOAC (2006). The N content was determined using a combustion analyzer (model CNC-2000; Leco Corporation, St. Joseph, MI; method 984.13A-D) and was used to calculate the crude protein concentration (N × 6.25). TiO_2 was analyzed using an inductively coupled plasma spectrometer (Vista-MPX; Varian Canada Inc., Mississauga, ON). Frozen carcasses, organs, and blood were ground as described by Ayoade et al. (2012) with minor modifications. In brief, carcasses and organs were cut into smaller pieces using an electric band saw and then ground in a meat grinder (model H600, Hobart Manufacturing Co. Ltd., Toronto, ON, Canada) to pass through a 12-mm die. The ground pieces were mixed in a mixer for 10 min to ensure even distribution and facilitate uniform sampling. Samples of approximately 500 g were taken from the mixtures and were later freeze-dried. The samples were weighed before and after freeze-drying to calculate the body water content.

7.3.5. CALCULATIONS AND STATISTICAL ANALYSES

The ATTD was calculated using the following equation:

ATTD (%) = {1- [(Td / Tf) × (Nf / Nd)]}×100 where Nf = nutrient concentration in feces (%), Nd = nutrient concentration in diet (%), Td = TiO_2 concentration in diet (% DM), and Tf = TiO_2 concentration in feces (%). The geometric means of *glyceraldehyde 3-phosphate dehydrogenase* and *β-actin* expressions were used to normalize the transcriptional levels. The relative expression was expressed as a ratio of the target gene to the STD group, using the formula $2^{-\Delta\Delta Ct}$ according to Livak and Schmittgen (2001), where $\Delta\Delta Ct = (Ct_{target} - Ct_{geometric\ mean})_{treatment} - (Ct_{target} - Ct_{geometric\ mean})_{STD}$.

All data were analyzed according to a randomized complete block design using the mixed SAS procedure (version 9.4; SAS Inst. Inc., Cary, NC) with the pen as the experimental unit. For evaluating the data for the blood chemistry profile, intestinal morphology and blood profile, body composition, organ weight, and gene expression, the individual pig (median BW in each pen) was used as the experimental unit. The feeding regimen was considered the main effect, with block and sex regarded as random effects. The LSMEANS statement with a Tukey-adjusted PDIFF option was used to calculate and separate the mean values for each treatment. Results were considered significant at $P < 0.05$ and tendencies were observed at $0.05 < P \leq 0.10$.

7.4. RESULTS

7.3.6. GROWTH PERFORMANCE, APPARENT TOTAL TRACT DIGESTIBILITY, AND FECAL DRY MATTER

During phase I, pigs fed the SUP diet had greater ($P < 0.05$) ADG and G:F than those fed the STD diet (Table 7.3). Similarly, there was a trend for the increase ($P = 0.06$) in ADFI in pigs fed the SUP diet compared to those fed the STD diet. The feeding regimen during phase II did not affect growth performance. By contrast, pigs fed the SUP diet throughout the experimental period tended

Table 7.3 Effect of functional amino acid supplementation duration on average daily gain (ADG), average daily feed intake (ADFI), and gain:feed in nursery pigs

	Feeding regimen[1]				SEM	*P*-value
Item	STD	SUP7	SUP21	SUP35		
Initial body weight (day 0), kg	6.51	6.51	6.51	6.52		
Final body weight (day 35), kg	21.66	22.39	22.33	23.39	0.632	0.232
Phase I (day 0 – 7)						
ADG, g/day	41	76	–	–	12.02	0.016
ADFI, g/day	109	133	–	–	11.20	0.062
Gain:feed, g/g	0.30	0.55	–	–	0.08	0.011
Phase II (day 7 – 21)						
ADG, g/day	374	385	384	–	19.4	0.899
ADFI, g/day	485	526	498	–	24.7	0.447
Gain:feed, g/g	0.77	0.74	0.77	–	0.026	0.516
Phase III (day 21 – 35)						
ADG, g/day	688^{Y}	706^{XY}	719^{XY}	777^{X}	24.0	0.057
ADFI, g/day	1,017	1,064	1,049	1,097	40.9	0.553
Gain:feed, g/g	0.68^{xy}	0.67^{y}	0.69^{xy}	0.71^{x}	0.013	0.037
Overall (day 0 – 35)						
ADG, g/day	433	454	452	482	16.2	0.201
ADFI, g/day	623	666	646	661	25.8	0.591
Gain:feed, g/g	0.70^{XY}	0.68^{Y}	0.70^{XY}	0.73^{X}	0.018	0.055

[1]STD, the standard NRC (2012) level of standardized ileal digestible Lys, Met, Met + Cys, Thr, Trp, and Val; amino acids ($_{L}$-Thr, $_{DL}$-Met, $_{L}$-Trp, $_{L}$-Val, and $_{L}$-Ile) were supplemented to STD diet (SUP) and fed for 7 days (SUP7), 21 days (SUP21), and 35 days (SUP35).

^{X,Y}Within a row, means with different superscripts differ ($P < 0.10$).

^{x,y}Within a row, means with different superscripts differ ($P < 0.05$).

to have greater ADG ($P = 0.06$) than those fed the STD diet throughout the experimental period. Pigs fed the SUP7 diet showed lower ($P < 0.05$) G:F than those fed the SUP35 diet during phase III. Similarly, the SUP7 regimen tended to reduce ($P = 0.04$) G:F compared with the SUP35 regimen.

The feeding regimens during phases I and II did not affect the ATTD of DM and N (Table 7.4). Pigs in the SUP7 group had lower ($P < 0.05$) ATTD of DM than those in SUP21 group. Similarly, pigs in the SUP7 group had lower ($P < 0.05$) ATTD of N than those in SUP21 and SUP35 groups. The feeding regimen did not affect fecal DM during the experimental period. Pigs following STD, SUP21, and SUP35 regimens had the greatest ($P < 0.05$) ATTD of DM in phase III, but pigs in the SUP7 group had an ATTD of DM in phase III comparable to that in phase II. Pigs following the SUP7 regimen had lower ($P < 0.05$) fecal DM in phase III than in phase I, whereas pigs following the SUP21 and SUP35 regimens had lower ($P < 0.05$) fecal DM in phase II than in phase I.

7.3.7. BLOOD CHEMISTRY PROFILE AND ORGAN WEIGHT

No differences were observed in the blood chemistry profiles on days 7 and 21, except for γ-glutamyl transferase (Table 7.5). Pigs fed the SUP diet during phase I had higher ($P < 0.05$) γ-glutamyl transferase concentrations in the plasma on day 7 than those fed the STD diet. Similarly, pigs fed the SUP diet for phase I and phase II tended to have higher ($P = 0.09$) γ-glutamyl transferase concentrations in the plasma on day 21 than those fed the STD diet. On day 35, pigs following the SUP21 regimen had greater ($P < 0.05$) PUN concentrations than those following the STD and SUP7 regimens. The feeding regimens did not affect organ weight (% of empty BW), except for the kidneys (Table 7.6). Pigs following the SUP21 regimen had lighter (*P*

Table 7.4 Effect of functional amino acid supplementation duration on fecal dry matter and apparent total tract digestibility (ATTD) in nursery pigs

Item	Feeding regimen[1]				SEM	*P*-value
	STD	SUP7	SUP21	SUP35		
ATTD of dry matter, %						
Phase I	79.1^{c}	78.8^{b}	–^{c}	–^{c}	1.10	0.804
Phase II	82.3^{b}	81.5^{a}	81.8^{b}	–^{b}	0.78	0.787
Phase III	85.5^{axy}	83.8^{ay}	86.0^{ax}	84.9^{axy}	0.54	0.039
ATTD of nitrogen, %						
Phase I	70.1^{c}	69.5^{c}	–^{c}	–^{c}	1.77	0.721
Phase II	76.3^{b}	76.0^{b}	75.6^{b}	–^{b}	1.18	0.895
Phase III	83.6^{axy}	81.6^{ay}	84.3^{ax}	84.7^{ax}	0.65	0.010
Fecal dry matter, %						
Phase I	34.5	34.1^{a}	–^{a}	–^{a}	1.66	0.807
Phase II	30.7	31.0^{ab}	30.1^{b}	–^{b}	0.77	0.530
Phase III	31.2	30.4^{b}	31.2^{ab}	31.2^{ab}	0.50	0.595

[1]STD, the standard NRC (2012) level of standardized ileal digestible Lys, Met, Met + Cys, Thr, Trp, and Val; amino acids ($_{L}$-Thr, $_{DL}$-Met, $_{L}$-Trp, $_{L}$-Val, and $_{L}$-Ile) were supplemented to STD diet (SUP) and fed for 7 days (SUP7), 21 days (SUP21), and 35 days (SUP35).

^{a,b,c}Within a column, means with different superscripts differ ($P < 0.05$).

^{x,y}Within a row, means with different superscripts differ ($P < 0.05$).

< 0.05) kidneys than pigs fed the STD diet throughout the experimental period.

7.3.8. BODY COMPOSITION AND RELATIVE ABUNDANCE OF mRNA EXPRESSION

No differences in body composition were observed across feeding regimens (Table 6). The relative abundance of mRNA expression in the muscles (*longissimus dorsi*) and jejunum is shown in Table 7. Feeding of an SUP diet throughout the experimental period upregulated mRNA encoding *mTOR* and *CAT1* compared with feeding of an SUP diet for phases I and II. By contrast, pigs fed the SUP diet through the experimental period had a suppressed ($P < 0.05$) abundance of mRNA encoding *CAT1* than those fed an SUP diet for phases I and II. Similarly, there was a trend ($P = 0.05$) of suppressed mRNA abundance of *LAT1A* in the SUP35 group compared with the SUP21 group.

7.5. DISCUSSION

Optimizing dietary AA content is extremely important for maximizing growth performance and minimizing feed costs and N excretion through swine nutrition; thus, numerous efforts have been made to establish a model that estimates protein accretion and AA requirements (NRC, 2012; van Milgen and Dourmad, 2015). These models, commonly generated using a factorial approach, provide the requirements in a dynamic way. A phase feeding program is generally applied to pigs to provide nutrients close to the requirement and reduce feed costs (Lenis and Jongbloed, 1999). Because dietary AA are not only the building blocks of proteins but are also mobilized for immune responses, it is generally believed that ISS increases some AA requirements, realigning AA metabolism from growth to immune response (Reeds and Jahoor, 2001; Obled, 2003); thus, there is a growing interest in dietary AA supplementation above the requirement for nursery pigs whose immune systems are often stimulated by various stressors to enhance immunity and

Table 7.5 Effect of functional amino acid supplementation duration on blood chemistry profile in nursery pigs

Item	Feeding regimen[1]				SEM	*P*-value
	STD	SUP7	SUP21	SUP35		
Day 7						
Urea, mmol/L	3.5	3.2	–	–	0.37	0.502
Creatinine, mmol/L	96.1	97.4	–	–	5.86	0.850
Alkaline phosphatase, U/L	357.6	342.4	–	–	32.79	0.690
γ-glutamyl transferase, U/L	25.0	32.4	–	–	2.98	0.040
Creatine kinase, U/L	358.4	365.9	–	–	48.04	0.893
Glucose, mmol/L	6.5	6.7	–	–	0.22	0.365
Total protein, g/L	49.9	49.1	–	–	1.55	0.679
Albumin, g/L	34.0	34.5	–	–	1.21	0.702
Globulin, g/L	15.9	14.6	–	–	1.47	0.454
Day 21						
Urea, mmol/L	2.4	3.0	3.3	–	0.63	0.521
Creatinine, mmol/L	63.9	67.0	68.2	–	3.66	0.633
Alkaline phosphatase, U/L	325.4	328.5	357.2	–	30.94	0.623
γ-glutamyl transferase, U/L	25.8^{Y}	33.4^{XY}	34.6^{X}	–	3.22	0.092
Creatine kinase, U/L	988.9	987.0	1132.9	–	380.93	0.930
Glucose, mmol/L	6.8	6.6	6.6	–	0.35	0.903
Total protein, g/L	48.0	47.6	47.3	–	1.59	0.927
Albumin, g/L	32.5	33.6	32.6	–	1.27	0.764
Globulin, g/L	15.5	14.0	14.7	–	1.10	0.607
Day 35						
Urea, mmol/L	2.4^{y}	2.7^{y}	3.7^{x}	3.0^{xy}	0.24	0.004
Creatinine, mmol/L	67.8	67.5	76.5	69.1	2.85	0.108
Alkaline phosphatase, U/L	336.0	308.1	374.3	306.5	24.24	0.188
γ-glutamyl transferase, U/L	28.1	36.8	37.1	32.9	3.14	0.174
Creatine kinase, U/L	2,914	2,249	1,522	1,258	607.8	0.227
Glucose, mmol/L	7.3	7.6	7.5	7.2	0.17	0.330
Total protein, g/L	54.4	54.3	53.6	52.0	1.52	0.675
Albumin, g/L	40.0	39.5	39.5	38.8	1.46	0.944
Globulin, g/L	14.4	14.8	14.1	13.3	1.06	0.720

[1]STD, the standard NRC (2012) level of standardized ileal digestible Lys, Met, Met + Cys, Thr, Trp, and Val; amino acids ($_{L}$-Thr, $_{DL}$-Met, $_{L}$-Trp, $_{L}$-Val, and $_{L}$-Ile) were supplemented to STD diet (SUP) and fed for 7 days (SUP7), 21 days (SUP21), and 35 days (SUP35).

^{X,Y}Within a row, means with different superscripts differ ($P < 0.10$).

^{x,y}Within a row, means with different superscripts differ ($P < 0.05$).

growth performance. This study therefore aimed to optimize dietary EAA supplementation in a nursery phase feeding program for growth and protein utilization.

At weaning, pigs face environmental, nutritional, and psychological stressors, causing post-weaning anorexia (Lallès et al., 2004). The SUP diet improved growth performance during phase I (days 0–7) compared with the STD diet. Supplemental EAA could have helped pigs recover from weaning stress by increasing protein metabolism and thus growth performance. In fact, Met, as a SAA, is a precursor of SAM, which becomes a donor of the methyl group and modulates immunity (Ding et al., 2015). Furthermore, dietary Met can be converted into Cys, which is a precursor of GSH—a major intracellular antioxidant in the body—to maintain the redox status (Bauchart-Thevret et al., 2009a). Our previous study showed that dietary $_{DL}$-Met supplementation above the requirement improved hepatic GSH concentrations and suppressed the hepatic inflammatory response in LPS-challenged pigs (Unpublished data). Furthermore, Trp is metabolized into kynurenines by scavenging free radicals, and their metabolites, including 5-hydroxyrtyptophan, 3-hydrpxyanthranilic acid, and 3-hydroxykynurenine, have antioxidant capacity (Le Floc'h et al., 2018). In fact, Mao et al. (2014b) reported that dietary Trp supplementation alleviated oxidative stress in diquat-challenged weaned pigs by increasing hepatic glutathione peroxidase and decreasing malondialdehyde concentrations. γ-glutamyl transferase is a cell surface enzyme that hydrolyzes the γ-glutamyl bond of extracellular reduced and oxidized GSH, maintaining Cys levels and transporting AA and peptides through cell membranes (Whitfield, 2001). Therefore, our finding of the increase in the plasma concentration of γ-glutamyl transferase on day 7 supports the postulation that an SUP diet led to elevated protein metabolism. It has been documented that dietary Trp levels modulate voluntary feed intake in pigs because Trp is a precursor of serotonin—a neurotransmitter that regulates appetite

Table 7.6 Effect of functional amino acid supplementation duration on body composition and organ weights of nursey pigs

Item	Feeding regimen[1]				SEM	*P*-value
	STD	SUP7	SUP21	SUP35		
Empty body weight	19.94	20.62	20.77	21.18	0.575	0.483
Organ weight, % of empty body weight						
Stomach	1.04	1.08	0.97	1.04	0.054	0.543
Small intestine	5.10	5.21	5.04	5.70	0.300	0.351
Large intestine	2.44	2.23	2.33	2.39	0.138	0.585
Gastrointestinal tract	8.58	8.52	8.34	9.14	0.344	0.315
Spleen	0.29	0.28	0.25	0.29	0.022	0.570
Kidneys	0.72^{x}	0.67^{xy}	0.58^{y}	0.67^{xy}	0.025	0.005
Pancreas	0.24	0.22	0.21	0.22	0.014	0.475
Liver	3.59	3.48	3.35	3.44	0.116	0.528
PDV[2]	9.11	9.02	8.80	9.65	0.345	0.273
Body composition, %						
Water	70.5	70.3	70.3	70.6	0.31	0.896
Protein	17.1	17.0	17.0	16.8	0.24	0.920
Fat	8.7	8.9	9.1	8.7	0.25	0.650
Ash	2.6	2.5	2.5	2.5	0.04	0.174

[1]STD, the standard NRC (2012) level of standardized ileal digestible Lys, Met, Met + Cys, Thr, Trp, and Val; amino acids (L-Thr, DL-Met, L-Trp, L-Val, and L-Ile) were supplemented to STD diet (SUP) and fed for 7 days (SUP7), 21 days (SUP21), and 35 days (SUP35).

[2]Portal drained viscera.

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

in the hypothalamus (Kwon et al., 2019). However, it is unclear whether the increase in feed intake in the present study was a consequence of improved growth or dietary Trp supplementation.

It is also possible that the NRC (2012) requirement for several AA has been underestimated, possibly causing the AA deficiency with the STD diet in the present study. For example, Soumeh et al. (2015) reported that dietary 0.70 SID Val:Lys was optimal for growth performance for 8–14-kg nursery pigs, which was higher than the NRC (2012) requirement. The poorer growth performance in pigs fed the STD diet in phase I may therefore have been due to a lack of Val supply. It is also worth noting that the phase I STD diet was formulated for 7-kg BW, but the actual experimental pigs weighed during phase I was 6.5–7.0 kg, meaning that the several AA concentrations of the STD diet were slightly lower than the requirement.

There is little information regarding dietary supplementation with an EAA blend above the requirement for growth performance in nursery pigs. Numerous studies using dietary supplementation with a single AA have supported our data showing improved growth performance with an SUP diet. Dietary Trp supplementation at 0.13 % or 0.28 % increased ADG, ADFI, and G:F for a week post-weaning (Mao et al., 2014b). Dietary Ile supplementation at 0.4 % improved ADG before and after *E. coli* K88 challenge in weaned pigs (Ren et al., 2019). Dietary AA blend (Met, Trp, Thr) supplementation above the requirement improved ADG and G:F in *Salmonella*-inoculated pigs (Rodrigues et al., 2021). Wellington et al. (2019) reported that supplemental Thr above the requirement increased ADG, ADFI, and G:F throughout the experimental period in *Salmonella*-challenged growing pigs.

To the best of our knowledge, no information is available on how long dietary AA supplementation can provide benefits during nursery production. The present study involved a 3-phase feeding program and tested the supplementation duration. The pigs showed improved

growth performance with the SUP diet during the overall experimental period, but this was not evident in phase II. Weaning stress (e.g., social mixing, weaner diet, new environment) may have impacted pigs' growth during phase I, but the pigs may have adapted to the stressors from phase II. This is in line with the increase in ATTD of DM and N during phase II compared with phase I. By contrast, the benefit of the SUP diet was clear in phase III. The SUP35 regimen led to the highest ADG and G:F in phase III, which may have been associated with deterioration of the sanitary environment in phase III. To mimic the commercial environment, the pen floor was not washed during the experimental period. Furthermore, as pigs grow old and bigger, the space allowance decreases, and manure excretion increases. In fact, it has been thoroughly documented that sanitary deterioration can mildly activate the immune system, and pigs reared in LS conditions have higher AA requirements (Jayaraman et al., 2017a; Kahindi et al., 2017a).

Pigs following the SUP7 regimen had the lowest G:F ratio during phase III. Numerically, the lowest G:F was also observed in the SUP7 group during phase II, reflecting the negative effect of withdrawal of supplemental AA at phase II on the subsequent G:F. There are limitations to the results based on our analyses; however, it seems clear that the continuous supply of additional EAA after phase I was important for the development of intestinal functioning, because pigs in the SUP7 group had lower ATTD of DM and N than those in the SUP21 and SUP35 groups. Furthermore, the SUP7 regimen led to lower fecal DM (%) during phase III than during phase I, which is contradictory to other feeding regimens in which comparable fecal DM during phase III compared with phase I was observed.

Dietary AA are moved into the bloodstream by different AA transporter systems that are located on the apical and basolateral membranes of intestinal epithelial cells (Broer, 2008). Furthermore, the AA transporters on cell membranes facilitate the efflux of free AA from the blood

into the cells (McGivan and Pastor-Anglada, 1994). This indicates the significance of AA transporter for the utilization of dietary AA from digestion to protein accretion. AA transporters also function as receptors by sensing the extracellular availability of AA and regulating protein synthesis or enzyme kinetics (Hyde et al., 2003). The protein synthesis in skeletal muscle is mediated by the mTOR signaling pathway, and protein kinase mTOR can promote the initiation of translation by the phosphorylation of downstream effectors such as 4E-BP1 and P70 (Wang and Proud, 2006). Myogenesis is the formation of skeletal muscle tissue, and myogenic regulatory factors, including MyoD, MyoG, and MEF2A, regulate myofiber differentiation and muscle growth (Asfour et al., 2018); thus, we analyzed the gene expression of the transporters and protein regulatory factors in the gut and muscle to investigate the impact of supplemental AA on AA transport and muscle accretion. LAT1 transports large neutral AA including BCAA and aromatic AA (e.g., Phe, Try, and Trp), by exchanging intracellular Gln (Broer, 2008), suggesting that most of the supplemental AA in the present study could have been transported via LAT1. In the present study, pigs in the SUP21 group had lower expression of LAT1A in the jejunum than those in the SUP35 group. The AA transporter possibly sensed the lower concentrations of EAA after the withdrawal of the SUP diet, and the intestine may have upregulated the expression of LAT1 to adapt to the discontinuation of supplemental AA and maintain EAA levels in the body, which is often called “adaptive derepression” (Hatzoglou et al., 2004). This speculation is further supported by our results for PUN and *mTOR* expression in the muscles of pigs in the SUP21 group, indicating higher protein degradation and suppressed protein anabolism. However, previous studies reported that the feeding of a lower protein diet compared with a higher protein diet downregulated *LAT1* expression in the small intestine (Qiu et al., 2016; Sun et al., 2020); thus, it seems that a downshift of dietary AA levels modulates LAT1 transporters in an opposite way to consistent provision of

Table 7.7 Effect of functional amino acid supplementation duration on relative mRNA abundance in muscle (*longissimus dorsi*) and jejunum of nursery pigs[1,2]

Item	Feeding regimen[3]				SEM	*P*-value
	STD	SUP7	SUP21	SUP35		
Muscle						
4E-BP1	1.00	1.42	1.33	1.63	0.250	0.356
MEF2A	1.00	1.00	1.10	1.16	0.210	0.902
P70	1.00	1.09	0.95	0.85	0.234	0.838
mTOR	1.00^{xy}	0.68^{xy}	0.48^{y}	1.39^{x}	0.269	0.034
MyoD1	1.00	1.46	1.40	1.12	0.401	0.795
MyoG	1.00	0.43	0.70	0.89	0.371	0.395
ASCT2	1.00	1.04	0.68	1.27	0.235	0.287
CAT1	1.00^{xy}	0.79^{xy}	0.58^{y}	2.19^{x}	0.625	0.044
LAT1	1.00	0.98	0.89	1.19	0.397	0.900
SNAT2	1.00	0.96	0.69	1.30	0.217	0.206
Jejunum						
ASCT2	1.00	1.86	2.30	0.82	0.518	0.112
$b^{0,+}AT$	1.00	0.94	0.99	1.22	0.193	0.679
$B^{0}AT1$	1.00	0.71	0.90	1.20	0.233	0.208
CAT1	1.00^{xy}	4.56^{xy}	6.26^{x}	0.31^{y}	3.318	0.039
LAT1A	1.00^{xy}	3.73^{xy}	4.85^{x}	0.57^{y}	2.398	0.050
SNAT2	1.00	1.48	1.04	0.59	0.345	0.219

[1]4E-BP1, eukaryotic initiation factor 4E-binding protein 1; MEF2A, myocyte enhancer factor 2A; P70, ribosomal protein S6 kinase A1; mTOR, mammalian target of rapamycin; MyoD1, myoblast determination protein 1; MyoG, myogenin; ASCT2, Ala/Ser/Cys-like sodium dependent neutral amino acid transporter 2 (SLC1A5); CAT1, cationic amino acid transporter 1 (SLC7A1); LAT1, L-type AA transporter 1 (SLC7A5); SNAT2, sodium-coupled neutral AA transporter 2 (SLC38A2); $b^{0,+}AT$, b(0,+)-type amino acid transporter 1 (SLC7A9); $B^{0}AT1$, sodium- and chloride-dependent neutral and basic amino acid transporter B(0,+) (SLC6A14).

[3]STD, the standard NRC (2012) level of standardized ileal digestible Lys, Met, Met + Cys, Thr, Trp, and Val; amino acids ($_{L}$-Thr, $_{DL}$-Met, $_{L}$-Trp, $_{L}$-Val, and $_{L}$-Ile) were supplemented to STD diet (SUP) and fed for 7 days (SUP7), 21 days (SUP21), and 35 days (SUP35).

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

lower dietary AA levels, which requires further studies. One of the main substrates of CAT1 is Arg (Broer, 2008), which is a key AA in the final step of the urea cycle, being converted into urea by arginase (Barrett, 2014). Higher urea production with the SUP21 regimen may have increased the need for Arg, thereby upregulating CAT1 to increase Arg uptake in the gut. This result is partially supported by the fact that increased protein catabolism led to the upregulation of CAT1 in various chicken tissues (Humphrey and Klasing, 2005; Humphrey et al., 2006). Similarly, Osmanyan et al. (2018) reported that lower dietary digestible AA led to higher *CAT1* gene expression in broiler chickens.

Pigs following the SUP35 regimen had the greatest expression of *mTOR* in their *longissimus dorsi* compared to any other group. EAA, particularly Leu, stimulate skeletal muscle protein synthesis (Wang and Proud, 2006). Although dietary Leu content was equal in the SUP and STD diets, L-Ile and L-Val supplementation may have improved the metabolic balance of BCAA, minimizing the oxidation of Leu and improving the activation of mTOR. In fact, balanced BCAA increased the activation of the mTOR pathway in skeletal muscle in growing pigs (Duan et al., 2016a). There is also evidence that Arg can activate mTOR signaling pathways by binding to cytosolic arginine sensors for mTOR complex 1 subunit 1 (Kamei et al., 2020). Upregulated *CAT1,* therefore, possibly increased intracellular Arg concentrations and stimulated mTOR. However, although some reports have claimed that supplemental AA upregulate the expression of AA transporters in muscles (Drummond et al., 2010; Hu et al., 2019), the mechanism has not been identified. In contrast to the upregulated *mTOR* expression in pigs fed the SUP35 diet, no differences in myogenic regulatory factors, body protein proportions, or protein masses were observed. Pigs may have reached the generic upper limit for protein accretion. However, the

stimulated mTOR pathway possibly contributed to the increased G:F throughout the experimental period by increasing protein synthesis efficiency.

Interestingly, pigs fed the STD diet throughout the experimental period had a lighter kidney weight than pigs following the SUP21 regimen. Kidneys are organs that are intricately linked with AA metabolism. Urea—a waste product of AA metabolism that is mainly synthesized in the liver—is mostly excreted by the kidneys in urine (Barrett, 2014). Furthermore, kidneys per se metabolize Glu vigorously as a means to maintain acid-base balance by producing bicarbonate and excreting ammonium directly into urine, which complements hepatic urea synthesis (van de Poll et al., 2004). Lack of analyses of kidneys limits the discussion of the present findings; however, it would be worthwhile for future studies to focus on the metabolism of dietary AA above the requirement in kidneys.

In conclusion, feeding a nursery diet supplemented with EAA including Met, Thr, Trp, Val, and Ile, above the NRC (2012) requirement for phase I improved growth performance. Withdrawal of the SUP diet at phase II or phase III had a negative impact on subsequent feed efficiency and protein utilization, lowering N digestibility. By contrast, continuation of the SUP diet throughout the 3-phase program led to higher ADG and G:F than STD group and SUP7 groups, respectively, with the greatest ATTD of N and *mTOR* expression in the muscle. Therefore, we recommend feeding an SUP diet throughout the 5-week nursery production period to maximize feed efficiency and N utilization. Future studies should focus on the effects of a SUP diet during nursery production on subsequent growth performance and protein metabolism.

8. GENERALL DISCUSSION

Weaning is an inevitable process in pork production because sows' reproductive cycle continues. At weaning, piglets are exposed to various stressors, and their health status is often compromised. Management at weaning is important because it determines the lifelong health and performance of pigs (Mahan and Lepine, 1991; Moeser et al., 2017). Although weaning is a common process in all farms, the intensity of weaning stress may differ depending on the facility (e.g., sanitary condition, temperature, pathogen prevalence, and space allowance) and management (e.g., diet composition, handling, transportation, and vaccination) (Kil and Stein, 2010; Jayaraman and Nyachoti, 2017). The development of a nutritional strategy against specific weaning stress enables users (farmers) to apply the strategy in the farm context. A series of studies were conducted to test the efficacy of FAA against post-weaning challenges, which were broadly divided into three categories: 1) dietary stress, 2) oxidative and inflammatory stress, and 3) poor sanitary condition (microbial infection). Based on the biological function, a single commercially available FAA was chosen for each post-weaning challenge.

After weaning, pigs must ingest nutrients and energy from a solid plant-based diet rather than sows' milk. The ingredient composition of a typical weaner diet is complex, containing dairy products (e.g., whey powder and skim milk powder) and animal-originated ingredients (e.g., blood meal and fish meal). Although the price unit of the weaner diet is the greatest among swine diets (Manitoba Agriculture, 2016), its high complexity has long been regarded as necessary for the optimal growth of nursery pigs (Mahan et al., 2004). However, the increased price of feedstuffs and the improved management and facility influenced the simplified weaner diet by replacing the dairy products and animal-originated ingredients with cereal and soybean meal. Previous studies have confirmed that pigs fed a simple diet show poorer growth performance for a short time (1–3

weeks) after weaning but afterward catch up with the growth lag compared with those fed a complex diet (Dritz et al., 1996; Mahan et al., 2004; Sulabo et al., 2010; Skinner et al., 2014; Koo et al., 2017). My previous study (Koo, 2017) confirmed that the total feed cost per pig fed a simple diet was 26.4% lower than that fed a complex diet. Furthermore, feeding a simple diet reduced the feed cost/kg of BW gain by 21 cents compared with feeding a complex diet. Despite these benefits, farmers are reluctant to feed a simple diet because of the concern that a high amount of antinutritional factors, including dietary fiber and antigenic compounds, in a simple diet can inflame the GIT and compromise growth performance in the commercial environment. In fact, our previous study found villus atrophy and lymphocyte proliferation in pigs fed a simple diet compared with pigs fed a complex diet (Koo et al., 2017). The biologic roles of Thr in GIT health have been well documented. Thus, the first study was performed to test the hypothesis that feeding a simple diet would activate the immune system and that additional dietary Thr above the standard requirement is needed to maintain intestinal function. As expected, in Manuscript I, pigs fed a simple diet showed poorer nutrient and energy utilization than pigs fed a complex diet. This is in accordance with the higher NSP content in a simple diet than in a complex diet. Furthermore, minerals such as Mn, Zn, and Mg seem to be present in more digestible forms in a complex diet than in a simple diet (Delaney, 1975; NRC, 2012). The DE and NE contents were lower in a simple diet than in a complex diet. By contrast, no difference in N retention was observed between pigs fed a simple diet and those fed a complex diet. Therefore, transient growth retardation with a simple diet seems to be associated with a lower energy content and mineral utilization rather than N utilization compared with a complex diet. Although dietary Thr supplementation did not seem to be effective in nutrient and energy digestibility, as shown in Manuscript II, its effect was apparent on intestinal integrity by increasing VH, the number of GC, and occludin gene expression,

regardless of diet complexity. Thr is a quantitatively major AA comprising mucin proteins and sIgA (Mukkur et al., 1985; Tang et al., 2021). It serves as an innate immune system that protects the gut from pathogen loading (Slack et al., 2012). Interestingly, dietary Thr supplementation appears to beneficially modulate microbial metabolites by increasing the total SCFA and reducing the NH_3-N concentration in the gut. This may be associated with the role of mucins as microbial substrates. Mucins are glycoproteins that can be fermented by intestinal microbes, and an increased mucin secretion can promote saccharolytic bacteria in other species (Faure et al., 2007; Chen et al., 2017b; Dong et al., 2017). This is supported by the finding in Manuscript I that greater ATTD of ADF and hemicellulose were found in pigs fed a simple diet than in pigs fed a complex diet. However, no information is available on the direct relationship between dietary Thr and microbial composition in pigs. The increase in SCFA levels and the decrease in NH_3-N levels in the gut may have reduced the expression of pro-inflammatory *IL-6* in the jejunum in SUP diet-fed pigs compared to STD diet-fed pigs. Dietary Thr supplementation, regardless of diet complexity, increased N retention and body protein mass without changing the body composition proportion. Zhao et al. (2020) recently reported that dietary Thr activates the Akt/mTOR signaling pathway, thereby upregulating the expression of muscle growth-associated genes, including growth hormone, insulin-like growth factor 1, MyoD1, and MyoG, and by increasing the muscle protein content in hybrid catfish. Thus, further studies should be performed to investigate whether Thr could have the same biologic function in pigs as in hybrid catfish to examine our finding that body protein mass increased with Thr supplementation. It is noteworthy that the effects of dietary Thr supplementation on intestinal integrity and microbial function were greater with a simple diet than with a complex diet. This suggests that a simple diet does not provide enough Thr to maintain optimal intestinal integrity compared with a complex diet. Moreover, dietary Thr supplementation

did not restore the IL-6 and IL-10 concentrations in plasma that were changed through the feeding of a simple diet. Thus, the benefits of dietary Thr appear to be limited in gut health. Overall, the L-Thr addition to a simple diet, which is considered to cause more stress to weaned pigs than a conventional complex diet, can be a nutritional strategy to fortify intestinal integrity, microbial metabolites, and protein mass. However, the systemic inflammation caused by the feeding of a simple diet should be taken into consideration even if L-Thr is supplemented to a simple diet.

Systemic inflammation is common in weaned pigs. Local inflammation caused by dietary composition (as shown in Manuscript II) and enteric and respiratory diseases can lead to systemic inflammation (Adewole et al., 2016; McGilvray et al., 2019). Furthermore, systemic inflammation is closely linked to oxidative stress (Gessner et al., 2017). Oxidative stress can be initiated in weaned pigs from various stressors, including immune system stimulation, housing conditions (e.g., hygiene and animal density) and management (e.g., handling and transport) (Adewole et al., 2016; Jayaraman and Nyachoti, 2017). The TLR family recognizes the damage-associated molecular pattern molecules produced from free radicals, which then activate the NF-κB signalling pathways, causing an inflammatory response (Gill et al., 2010). Thus, another study was designed to investigate SAA as FAA against systemic inflammation and oxidative stress in weaned pigs. To focus on the efficacy of SAA against systemic inflammation and oxidative stress, pigs were injected with LPS rather than with the pathogen. A 2 × 2 factorial arrangement was applied to compare the efficacy of individual Met and Cys supplementation and their interactive effects.

Dietary Cys supplementation replenished GSH levels in the jejunum depleted by LPS injection. The supplementation improved the intestinal morphology and barrier function, which were impaired by LPS injection. ISS is known to increase Cys metabolism, mainly for the synthesis of GSH (Rakhshandeh et al., 2019; Rakhshandeh et al., 2020). It seems that supplemental Cys is

necessary for the increased need for GSH synthesis. Interestingly, the increased Cys metabolism with Cys supplementation was accompanied by the upregulation of *GSR* and *GSS*. Growing evidence has shown that the fate of an enterocyte is associated with redox signaling (Vereecke et al., 2011; Circu and Aw, 2012). The improved redox potential of the GSH/GSSG couple could have enhanced intestinal integrity and barrier function.

Surprisingly, Met supplementation did not help weaned pigs recover from a depleted intestinal GSH level and impaired intestinal integrity. By contrast, pigs fed a Met-supplemented diet showed greater expressions of *MAT1A* and *CBS* than the other pigs, reflecting the increased transsulfuration and transmethylation rates. In the liver, Met supplementation elevated GSH levels and decreased inflammatory cytokine levels. Dietary Met seems to be prioritized for its metabolism in the liver, in which enzymes involved in transsulfuration and transmethylation are the most abundant (Riedijk et al., 2007). Furthermore, a racemic mixture of Met ($_{DL}$-Met) was supplemented with the MET diet. $_{D}$-Met must be converted to L-form to be metabolized in the body, and this process mostly occurs in the kidneys and liver (D'Mello, 2003). Thus, approximately half of Met supplementation could not be used in the intestine in the first pass. When both Met and Cys were supplemented together, antagonistic effects were observed on inflammatory responses, redox status, and intestinal morphology. According to a previous report, this may be due to excessive Cys concentrations that increase the extracellular disulfide bond in Cys–Cys, which requires antioxidants for the reduction (Dilger et al., 2007). Overall, Manuscript III implies that dietary $_{L}$-Cys supplementation can be added to a weaner pig diet to improve intestinal integrity, barrier function, and redox status, while $_{DL}$-Met can be added to improve the systemic redox status and inflammatory response. Any option should be chosen carefully because of its antagonistic effects.

Pathogen infection is common in piglets because of the fragile intestinal barrier functions of weaned pigs (Wijtten et al., 2011; Pluske et al., 2018a). This infection commonly accompanies post-weaning diarrhea, which leads to growth retardation and economic loss in nursery pig production (Heo et al., 2013). The ban on antibacterial growth promoters accelerated the development of a nutritional strategy to control post-weaning pathogen infection. Instead of the ETEC and ST challenges, which cause severe and acute infection, the sanitary challenge was applied in Study 4. The deterioration of sanitary conditions increases contact with exogenous pathogens and eventually causes mild inflammation; this best simulates the commercial environment (Waititu et al., 2017). In Study 4, dietary Val supplementation was tested against the bacterial composition and metabolites in the gut that were changed by the deterioration of sanitary conditions. To give weaned pigs additional stress that could not be simulated in an experimental environment, pigs were injected with a low dose of LPS at the beginning of the study. As expected, pigs housed in LS showed an increased abundance of harmful bacteria (e.g., *E. coli* subgroup and *Clostridium perfringens*) and a decreased abundance of beneficial bacteria (e.g., Bifidobacterium) in the jejunum or colon compared with those housed in HS. Furthermore, LS increased the production of NH_3-N in the colon. Although dietary Val supplementation could not restore all the negative changes, it promoted the abundance of Bifidobacterium and butyrate-producing bacteria. Microbial modification led to increased butyrate levels and decreased NH_3-N levels in the gut. Dietary Val supplementation downregulated the expression of intestinal antimicrobial peptide in pigs housed in the LS condition and numerically upregulated the expression in pigs housed in the HS condition. Thus, the bacteriostatic effect did not seem to be directly associated with intestinal antimicrobial peptide expression. Dietary Val supplementation could have enhanced the innate immune system and intestinal integrity, thereby changing the intestinal environment in which

microbes reside. Val is a major AA component of mucins, with 44% of mucin protein composed of Val and Thr (Mukkur et al., 1985). There is evidence that Val promotes macrophage phagocytosis and regulates the maturation of dendritic cells (Kakazu et al., 2007; Chen et al., 2017a). The microbial and metabolite modifications with dietary Val supplementation may explain the results of the suppressed concentrations of plasma inflammatory cytokines. Overall, Manuscript IV implies that L-Val can be added to a weaner pig diet to enhance the beneficial bacterial composition and microbial metabolites and to suppress the pro-inflammatory cytokines regardless of the sanitary condition.

Growth performance, such as feed intake, weight gain, and feed efficiency, is the most important response in pigs in terms of nutrition, as growth performance directly governs profit in pork production. Growth performance in nursery pig production is known to determine days up to market weight (Mahan and Lepine, 1991). However, the growth rates of pigs are the comprehensive consequence of numerous metabolisms in the digestive, immune, endocrine, and nervous systems (Pastorelli et al., 2012). Thus, individual variations in growth performance responses are greater than those in other responses (Aaron and Hays, 2004). To reduce the variance derived from the carryover effect of initial body weight and sex, pens are recommended to be blocked according to weight and sex (Aaron and Hays, 2004). Thus, with a pen housing 3 pigs serving as the experimental unit, 4 feeding regimens (treatments) were assigned in a randomized complete block design. To compare the FAA supplementation effect with the maximized statistical power, data from the SUP7, SUP21, and SUP35 groups were combined in phase I, while data from the SUP21 and SUP35 groups were combined in phase II.

Manuscripts III and IV found the challenge effect (LPS and sanitary challenge) but failed to find the treatment effect (FAA supplementation) on growth performance. This may be due to

the greater effect size of the immune challenge than that of FAA supplementation. Thus, Study 5 was designed to focus specifically on growth performance in response to dietary FAA supplementation. The supplementation doses of DL-Met, L-Thr, and L-Val were the same as those in Studies 1–4. Although the feed grade of L-Ile is not commonly used in swine diets at the moment, the recent release of a commercial product from CJ Bio and the growing need for synthetic L-Ile in a low-protein nursery diet will soon make the feed grade of L-Ile readily available. Thus, in Study 5, L-Ile was included in the FAA blend, and the supplementation doses were determined at 0.09% (phase I), 0.08 (phase II), and 0.07 (phase III), which were the levels that maintained the SID Ile:SID Val between the STD and SUP diets. The L-Trp supplementation level was determined based on previous studies (Jayaraman et al., 2017a; Sterndale et al., 2020). When the supplementation levels were determined, FAA doses above 0.2% were not considered from a practical point of view.

FAA supplementation improved ADG, ADFI, and G:F in the first week post-weaning, whereas no difference was observed during phase II (days 21–35). During phase III (days 21–35), pigs fed the SUP diet throughout the experimental period showed the highest ADG and G:F. This study is the first to report that FAA supplementation could eventually lead to growth performance during a five-week nursery production period. The results imply that pigs may require additional FAA for optimal growth during the first week of post-weaning and in phase III. Piglets are expected to be environmentally and immunologically stressed immediately after weaning and while adapting to a new environment. The pen floors where the experimental piglets were housed were not clean, and manures accumulated as the piglets grew. The sanitary condition could have deteriorated, possibly causing mild inflammation and requiring additional FAA for optimal growth. Interestingly, the withdrawal of the SUP diet after phase I was negative for the subsequent G:F.

The lowest G:F was observed in pigs in the SUP7 group. A continuous supply of a SUP diet to weaned pigs may be critical for the maintenance of intestinal digestive function. Pigs in the SUP7 group had a lower ATTD of DM and N than those in the SUP21 and SUP35 groups.

A general hypothesis on the use of FAA in nursery pigs states that FAA will balance the dietary AA profiles for optimal immune response and for minimizing protein loss. Surprisingly, however, no information was obtained on the effect of FAA supplementation on N utilization and body protein. Feeding the SUP diet throughout the experimental period upregulated the expression of *mTOR* and *CAT1* in the muscle. However, upregulating these genes failed to regulate the myogenic regulatory factors, body protein proportions, or protein masses. The pigs might have reached the generic upper limit for protein deposition. Nevertheless, the upregulation could have increased the protein synthesis efficiency, thereby increasing the G:F. Manuscript V suggests that an AA blend that includes Met, Thr, Trp, Val, and Ile supplementation to nursery diets can enhance growth performance in phases I and III, which recommend feeding the SUP diet throughout the five-week nursery pig production.

Thus, the main hypothesis that dietary FAA supplementation would enhance the immune system or fortify the health of weaned piglets, thereby improving protein accretion and growth performance, was partially accepted. Dietary FAA supplementation to a weaner pig diet can be a beneficial nutritional study to cope with post-weaning stress (e.g., dietary stress, oxidative stress, inflammation, and poor sanitary condition) by enhancing the intestinal integrity and barrier function, antioxidant defense system, and N utilization and by beneficially modulating the microbial profile and metabolites in the gut. However, although FAA supplementation improved growth performance, it failed to increase body protein accretion.

As shown in Manuscript II, single FAA (Thr) supplementation failed to restore systemic inflammation. However, Manuscripts III and IV showed that dietary Met or Val supplementation could successfully suppress the systemic inflammatory response. This indicates that the combination of FAA can complement the limitation of the biologic function of AA. However, the combination of Met and Cys supplementation has negative effects on the inflammatory response and gut health. Thus, supplemental FAA should be used with caution because of their interactions.

A series of studies revealed that the traditional AA requirement could be inaccurate when pigs undergo post-weaning stress. Therefore, the standard AA requirement should be subdivided depending on the possible physiological status of weaned pigs—1) normal physiological status (e.g., HS condition and good management) and 2) immune-challenged piglets (e.g., LS condition, high temperature, and poor management)—to beneficially apply dietary FAA as a nutritional strategy during the nursery production period.

9. CONCLUSIONS AND FUTURE STUDIES

CONCLUSIONS

The following conclusions can be drawn from the present study:

1. Dietary L-Thr supplementation (67% of SID Lys) improved intestinal integrity, microbial metabolites, and body protein mass. The benefits of supplementation were greater with a simple diet than with a complex diet. However, the inflammation caused by feeding with a simple diet could not be modulated with L-Thr supplementation.

2. The LPS challenge impaired intestinal morphology and permeability and caused hepatic GSH depletion. Dietary DL-Met supplementation at 0.1% (0.65 of SID Met:SID SAA and 0.48 of SID SAA:SID Lys) failed to restore the intestinal morphology, permeability, and redox status impaired by the LPS challenge, but it did improve hepatic GSH levels and the inflammatory response. By contrast, dietary L-Cys supplementation at 0.1% (0.52 of SID Met:SID SAA and 0.48 of SID SAA:SID Lys) improved the intestinal morphology, permeability, and redox status in LPS-challenged pigs, restoring them to a level comparable with that of the PBS-injected control. However, the L-Cys supplementation could not modulate hepatic GSH levels and circulating inflammatory cytokines. Furthermore, the supplementation of the combined DL-Met and L-Cys (0.57 of SID Met:SID SAA and 0.61 of SID SAA:SID Lys) showed antagonistic effects on the inflammatory response, intestinal morphology, and redox status.

3. Dietary L-Val supplementation (0.71 of SID Val:SID Lys) beneficially modulated the intestinal bacteria composition and microbial metabolites in the gut and suppressed the systemic inflammatory response, regardless of the sanitary condition. There are interactive effect of

sanitary conditions and dietary Val supplementation on the intestinal expression of antimicrobial peptide, including β-defensins, protegrin and PR39.

4. The supplementation of an AA blend, including Met, Thr, Trp, Val, and Ile, throughout a five-week nursery production period enhanced growth performance in phases I and III, with the upregulation of *mTOR* and an AA transporter in muscle. However, the withdrawal of the supplementation after phase I led to a lower G:F compared with the continuous feeding of the supplementation.

FUTURE STUDIES

Although the biologic roles of FAA have been well documented, limited information is available on their efficacy against weaning stress. Single AA supplementation to a nursery diet should be further tested to elucidate its mode of action against the immune response or intestinal microbiota. Future studies should be directed toward how FAA can be successfully applied in farms. Based on the above findings, further studies are required to

1. Investigate the effects of FAA, such as Thr and Trp, on intestinal microbiota and metabolites using advanced techniques (e.g., metagenomics and metabolomics).
2. Examine the effects of FAA supplementation on the growth performance of pigs with different weaning weights.
3. Explore the effects of FAA supplementation to nursery diet on subsequent growth performance up to market weight and meat quality.
4. Perform an economic analysis of the output of FAA supplementation.

10. LITERATURE CITED

Aaron, D. K., and V. W. Hays. 2004. How many pigs? Statistical power considerations in swine nutrition experiments. J. Anim. Sci. 82:E245-E254. doi: 10.2527/2004.8213_supplE245x

Abbas, A., A. Lichtman, and S. Pillai. 2017. Cellular and Molecular Immunology 9th Edition. Amsterdam, Netherlands: Elsevier.

Abdel-Salam, O. M. E., E. R. Youness, N. A. Mohammed, S. M. Y. Morsy, E. A. Omara, and A. A. Sleem. 2014. Citric acid effects on brain and liver oxidative stress in lipopolysaccharide-treated mice. J. Med. Food 17:588-598. doi: 10.1089/jmf.2013.0065

Adeola, O. 1995. Digestive utilization of minerals by weanling pigs fed copper- and phytase-supplemented diets. Can. J. Anim. Sci. 75:603-610. doi: 10.4141/cjas95-089

Adeola, O., P. C. Xue, A. J. Cowieson, and K. M. Ajuwon. 2016. Basal endogenous losses of amino acids in protein nutrition research for swine and poultry. Anim. Feed Sci. Technol. 221:274-283. doi: 10.1016/j.anifeedsci.2016.06.004

Adewole, D. I., I. H. Kim, and C. M. Nyachoti. 2016. Gut health of Pigs: Challenge models and response criteria with a critical analysis of the effectiveness of selected feed additives-A Review. Asian-Australas. J. Anim. Sci. 29:909-924. doi: 10.5713/ajas.15.0795

Agriculture, M. 2016. Guidelines for estimating swine farrow-finish costs. [Online] available: https://www.gov.mb.ca/agriculture/business-and-economics/financial-management/pubs/cop_swine_farrowfinish.pdf [2017 May 16].

Ai, Z., and I. A. Udalova. 2020. Transcriptional regulation of neutrophil differentiation and function during inflammation. J Leukoc Biol 107:419-430. doi: 10.1002/JLB.1RU1219-504RR

Akiho, H., E. Ihara, Y. Motomura, and K. Nakamura. 2011. Cytokine-induced alterations of gastrointestinal motility in gastrointestinal disorders. World J. Gastrointest. Pathophysiol. 2:72-81. doi: 10.4291/wjgp.v2.i5.72

Akira, S., and H. Hemmi. 2003. Recognition of pathogen-associated molecular patterns by TLR family. Immunol. Lett 85:85-95. doi: 10.1016/S0165-2478(02)00228-6

Al-Sadi, R., M. Boivin, and T. Ma. 2009. Mechanism of cytokine modulation of epithelial tight junction barrier. Front. Biosci 14:2765-2778. doi: 10.2741/3413

AOAC. 2006. Official Methods of Analysis. 18th ed. Assoc. Off. Anal. Chem., Washington, DC.

Arend, W. P., M. Malyak, C. J. Guthridge, and C. Gabay. 1998. Interleukin-1 receptor antagonist: Role in biology. Annu. Rev. Immunol. 16:27-55. doi: 10.1146/annurev.immunol.16.1.27

Asfour, H. A., M. Z. Allouh, and R. S. Said. 2018. Myogenic regulatory factors: The orchestrators of myogenesis after 30 years of discovery. Exp Biol Med (Maywood) 243:118-128. doi: 10.1177/1535370217749494

Attaix, D., S. Ventadour, A. Codran, D. Béchet, D. Taillandier, and L. Combaret. 2005. The ubiquitin–proteasome system and skeletal muscle wasting. Essays Biochem. 41:173-186. doi: 10.1042/bse0410173

Ayoade, D. I., E. Kiarie, M. A. Trinidade Neto, and C. M. Nyachoti. 2012. Net energy of diets containing wheat-corn distillers dried grains with solubles as determined by indirect calorimetry, comparative slaughter, and chemical composition methods. J. Anim. Sci. 90:4373-4379. doi: 10.2527/jas.2011-4858

Bach Knudsen, K. 2001. The nutritional significance of "dietary fibre" analysis. Anim. Feed Sci. Technol. 90:3-20. doi: 10.1016/S0377-8401(01)00193-6

Banskota, S., J. E. Ghia, and W. I. Khan. 2019. Serotonin in the gut: Blessing or a curse. Biochimie 161:56-64. doi: 10.1016/j.biochi.2018.06.008

Barrett, K. E. 2014. Gastrointestinal Physiology. 2nd ed. McGraw-Hill Education, New York, NY.

Bauchart-Thevret, C., J. Cottrell, B. Stoll, and D. G. Burrin. 2011. First-pass splanchnic metabolism of dietary cysteine in weanling pigs. J. Anim. Sci. 89:4093-4099. doi: 10.2527/jas.2011-3944

Bauchart-Thevret, C., B. Stoll, and D. G. Burrin. 2009a. Intestinal metabolism of sulfur amino acids. Nutr. Res. Rev. 22:175-187. doi: 10.1017/S0954422409990138

Bauchart-Thevret, C., B. Stoll, S. Chacko, and D. G. Burrin. 2009b. Sulfur amino acid deficiency upregulates intestinal methionine cycle activity and suppresses epithelial growth in neonatal pigs. J. Physiol. Endocrinol. Metab. 296:E1239-E1250. doi: 10.1152/ajpendo.91021.2008

Bhattacharyya, A., R. Chattopadhyay, S. Mitra, and S. E. Crowe. 2014a. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. Physiol. Rev. 94:329-354. doi: 10.1152/physrev.00040.2012

Bhattacharyya, A., R. Chattopadhyay, S. Mitra, and S. E. Crowe. 2014b. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. Physiol. Rev 94:329-354. doi: 10.1152/physrev.00040.2012

Blouet, C., F. Mariotti, D. Azzout-Marniche, V. Mathé, T. Mikogami, D. Tomé, and J.-F. Huneau. 2007. Dietary cysteine alleviates sucrose-induced oxidative stress and insulin resistance. Free Radic. Biol. Med. 42:1089-1097. doi: 10.1016/j.freeradbiomed.2007.01.006

Bogdan, C. 2001. Nitric oxide and the immune response. Nat. Immunol. 2:907-916. doi: 10.1038/ni1001-907

Bortoluzzi, C., S. J. Rochell, and T. J. Applegate. 2018. Threonine, arginine, and glutamine: Influences on intestinal physiology, immunology, and microbiology in broilers. Poult. Sci. 97:937-945. doi: 10.3382/ps/pex394

Broer, S. 2008. Amino acid transport across mammalian intestinal and renal epithelia. Physiol. Rev. 88:249-286. doi: 10.1152/physrev.00018.2006

Brown, J. A., and T. R. Cline. 1974. Urea excretion in the pig: An indicator of protein quality and amino acid requirements. J. Nutr. 104:542-545. doi: 10.1093/jn/104.5.542

Brubaker, S. W., K. S. Bonham, I. Zanoni, and J. C. Kagan. 2015. Innate immune pattern recognition: A cell biological perspective. Annu. Rev. Immunol. 33:257-290. doi: 10.1146/annurev-immunol-032414-112240

Brynestad, S., and P. E. Granum. 2002. Clostridium perfringens and foodborne infections. Int. J. Food Microbiol. 74:195-202. doi: 10.1016/S0168-1605(01)00680-8

Buchet, A., C. Belloc, M. Leblanc-Maridor, and E. Merlot. 2017. Effects of age and weaning conditions on blood indicators of oxidative status in pigs. PLoS. ONE 12:e0178487. doi: 10.1371/journal.pone.0178487

Calder, P. C. 2006. Branched-chain amino acids and immunity. J. Nutr. 136:288S-293S. doi: 10.1093/jn/136.1.288S

Campbell, J. M., J. D. Crenshaw, and J. Polo. 2013. The biological stress of early weaned piglets. J. Anim. Sci. Biotechnol. 4:19. doi: 10.1186/2049-1891-4-19

Campbell, Y., M. L. Fantacone, and A. F. Gombart. 2012. Regulation of antimicrobial peptide gene expression by nutrients and by-products of microbial metabolism. Eur. J. Nutr. 51:899-907. doi: 10.1007/s00394-012-0415-4

Capaldo, C. T., and A. Nusrat. 2009. Cytokine regulation of tight junctions. Biochim. Biophys. Acta 1788:864-871. doi: 10.1016/j.bbamem.2008.08.027

Capozzalo, M. M., J. C. Kim, J. K. Htoo, C. F. M. de Lange, B. P. Mullan, C. F. Hansen, J. W. Resink, and J. R. Pluske. 2017a. Pigs experimentally infected with an enterotoxigenic strain of Escherichia coli have improved feed efficiency and indicators of inflammation with dietary supplementation of tryptophan and methionine in the immediate post-weaning period. Anim. Prod. Sci. 57:935-947. doi: 10.1071/an15289

Capozzalo, M. M., J. W. Resink, J. K. Htoo, J. C. Kim, F. M. de Lange, B. P. Mullan, C. F. Hansen, and J. R. Pluske. 2017b. Determination of the optimum standardised ileal digestible sulphur amino acids to lysine ratio in weaned pigs challenged with enterotoxigenic Escherichia coli. Anim. Feed Sci. Technol. 227:118-130. doi: 10.1016/j.anifeedsci.2017.03.004

Castellano, R., M. H. Perruchot, J. A. Conde-Aguilera, J. van Milgen, A. Collin, S. Tesseraud, Y. Mercier, and F. Gondret. 2015. A methionine deficient diet enhances adipose tissue lipid metabolism and alters anti-oxidant pathways in young growing pigs. PLoS. ONE 10:e0130514. doi: 10.1371/journal.pone.0130514

CCAC. 2009. Guidelines on: the care and use of farm animals in research. In: Teaching and Testing. Canadian Council on Animal Care, Ottawa, ON Vol. II: 103-125.

Ceciliani, F., A. Giordano, and V. Spagnolo. 2002. The systemic reaction during inflammation: The acute-phase proteins. Protein Pept. Lett. 9:211-223. doi: 10.2174/0929866023408779

Cemin, H. S., C. M. Vier, M. D. Tokach, S. S. Dritz, K. J. Touchette, J. C. Woodworth, J. M. DeRouchey, and R. D. Goodband. 2018. Effects of standardized ileal digestible histidine to lysine ratio on growth performance of 7- to 11-kg nursery pigs. J. Anim. Sci. doi: 10.1093/jas/sky319

Chen, G., and J. Wang. 2014. Threonine metabolism and embryonic stem cell self-renewal. Current Opinion in Clinical Nutrition & Metabolic Care 17:80-85.

Chen, X. H., S. R. Liu, B. Peng, D. Li, Z. X. Cheng, J. X. Zhu, S. Zhang, Y. M. Peng, H. Li, T. T. Zhang, and X. X. Peng. 2017a. Exogenous L-valine promotes phagocytosis to kill multidrug-resistant bacterial pathogens. Front Immunol. 8:207. doi: 10.3389/fimmu.2017.00207

Chen, Y., D. Li, Z. Dai, X. Piao, Z. Wu, B. Wang, Y. Zhu, and Z. Zeng. 2014. L-Methionine supplementation maintains the integrity and barrier function of the small-intestinal mucosa in post-weaning piglets. Amino Acids 46:1131-1142. doi: 10.1007/s00726-014-1675-5

Chen, Y., H. Zhang, Y. Cheng, Y. Li, C. Wen, and Y. Zhou. 2018. Dietary L-threonine supplementation attenuates lipopolysaccharide-induced inflammatory responses and intestinal barrier damage of broiler chickens at an early age. Br. J. Nutr. 119:1254-1262. doi: 10.1017/S0007114518000740

Chen, Y. P., Y. F. Cheng, X. H. Li, W. L. Yang, C. Wen, S. Zhuang, and Y. M. Zhou. 2017b. Effects of threonine supplementation on the growth performance, immunity, oxidative status, intestinal integrity, and barrier function of broilers at the early age. Poult. Sci. 96:405-413. doi: 10.3382/ps/pew240

Cho, H. M., G. González-Ortiz, D. Melo-Durán, J. M. Heo, G. Cordero, M. R. Bedford, and J. C. Kim. 2020. Stimbiotic supplementation improved performance and reduced inflammatory response via stimulating fiber fermenting microbiome in weaner pigs housed in a poor sanitary environment and fed an antibiotic-free low zinc oxide diet. PLoS. ONE 15:e0240264. doi: 10.1371/journal.pone.0240264

Circu, M. L., and T. Y. Aw. 2012. Intestinal redox biology and oxidative stress. Semin. Cell Dev. Biol 23:729-737. doi: 10.1016/j.semcdb.2012.03.014

Clements, A., J. C. Young, N. Constantinou, and G. Frankel. 2012. Infection strategies of enteric pathogenic Escherichia coli. Gut microbes 3:71-87.

Corfield, A. P., N. Myerscough, R. Longman, P. Sylvester, S. Arul, and M. Pignatelli. 2000. Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. Gut 47:589-594. doi: 10.1136/gut.47.4.589

Cummins, P. M. 2012. Occludin: one protein, many forms. Mol. Cell Biol. 32:242-250. doi: 10.1128/MCB.06029-11

D'Mello, J. F. 2003. Amino acids in animal nutrition second edition. CABI.

de Ridder, K., C. L. Levesque, J. K. Htoo, and C. F. M. de Lange. 2012. Immune system stimulation reduces the efficiency of tryptophan utilization for body protein deposition in growing pigs1. J. Anim. Sci. 90:3485-3491. doi: 10.2527/jas.2011-4830

Delaney, R. A. M. 1975. The nutritive value of porcine blood plasma concentrates prepared by ultrafiltration and spray Drying. J. Sci. Food Agric. 26:303-310. doi: 10.1002/jsfa.2740260310

Dharmani, P., V. Srivastava, V. Kissoon-Singh, and K. Chadee. 2009. Role of intestinal mucins in innate host defense mechanisms against pathogens. J. Innate Immun. 1:123-135. doi: 10.1159/000163037

Diana, M., J. Quílez, and M. Rafecas. 2014. Gamma-aminobutyric acid as a bioactive compound in foods: a review. J. Funct. Foods 10:407-420. doi: 10.1016/j.jff.2014.07.004

Dilger, R. N., and D. H. Baker. 2008. Excess dietary L-cysteine causes lethal metabolic acidosis in chicks. J. Nutr. 138:1628-1633. doi: 10.1093/jn/138.9.1628

Dilger, R. N., S. Toue, T. Kimura, R. Sakai, and D. H. Baker. 2007. Excess dietary L-cysteine, but not L-cystine, is lethal for chicks but not for rats or pigs. J. Nutr. 137:331-338. doi: 10.1093/jn/137.2.331

Ding, W., Lorissa J. Smulan, Nicole S. Hou, S. Taubert, Jennifer L. Watts, and Amy K. Walker. 2015. S-adenosylmethionine levels govern innate immunity through distinct methylation-dependent pathways. Cell. Metab. 22:633-645. doi: 10.1016/j.cmet.2015.07.013

Ding, X., P. Bin, W. Wu, Y. Chang, and G. Zhu. 2020. Tryptophan metabolism, regulatory T cells, and inflammatory bowel disease: A mini review. Mediat. Inflamm. 2020:1-10. doi: 10.1155/2020/9706140

Domeneghini, C., A. Di Giancamillo, G. Bosi, and S. Arrighi. 2006. Can nutraceuticals affect the structure of intestinal mucosa? Qualitative and quantitative microanatomy in L -glutamine diet-supplemented weaning piglets. Vet. Res. Commun 30:331-342. doi: 10.1007/s11259-006-3236-1

Dong, X. Y., M. M. M. Azzam, and X. T. Zou. 2017. Effects of dietary threonine supplementation on intestinal barrier function and gut microbiota of laying hens. Poult. Sci. 96:3654-3663. doi: 10.3382/ps/pex185

Dréau, D., J. P. Lallès, V. Philouze-Romé, R. Toullec, and H. Salmon. 1994. Local and systemic immune responses to soybean protein ingestion in early-weaned pigs. J. Anim. Sci. 72:2090-2098. doi: 10.2527/1994.7282090x

Dritz, S. S., K. Q. Owen, R. D. Goodband, J. L. Nelssen, M. D. Tokach, M. M. Chengappa, and F. Blecha. 1996. Influence of lipopolysaccharide-induced immune challenge and diet complexity on growth performance and acute-phase protein production in segregated early-weaned pigs. J. Anim. Sci. 74:1620-1628. doi: 10.2527/1996.7471620x

Drummond, M. J., E. L. Glynn, C. S. Fry, K. L. Timmerman, E. Volpi, and B. B. Rasmussen. 2010. An increase in essential amino acid availability upregulates amino acid transporter expression in human skeletal muscle. J. Physiol. Endocrinol. Metab. 298:E1011-E1018. doi: 10.1152/ajpendo.00690.2009

Duan, Y., Q. Guo, C. Wen, W. Wang, Y. Li, B. Tan, F. Li, and Y. Yin. 2016a. Free Amino acid profile and expression of genes implicated in protein metabolism in skeletal muscle of growing pigs fed low-protein diets supplemented with branched-chain amino acids. J. Agric. Food Chem. 64:9390-9400. doi: 10.1021/acs.jafc.6b03966

Duan, Y., B. Tan, J. Li, P. Liao, B. Huang, F. Li, H. Xiao, Y. Liu, and Y. Yin. 2018. Optimal branched-chain amino acid ratio improves cell proliferation and protein metabolism of porcine enterocytesin in vivo and in vitro. Nutrition 54:173-181. doi: 10.1016/j.nut.2018.03.057

Duan, Y. H., L. M. Zeng, F. N. Li, Y. H. Li, B. E. Tan, Y. J. Ji, X. F. Kong, Y. L. Tang, Y. Z. Zhang, and Y. L. Yin. 2016b. Effects of dietary branched-chain amino acid ratio on growth performance and serum amino acid pool of growing pigs. J. Anim. Sci. 94:129-134. doi: 10.2527/jas.2015-9527

Ebisui, C., T. Tsujinaka, T. Morimoto, K. Kan, S. Iijima, M. Yano, E. Kominami, K. Tanaka, and M. Monden. 1995. Interleukin-6 induces proteolysis by activating intracellular proteases (cathepsins B and L, proteasome) in C2C12 myotubes. Clin. Sci. 89:431-439. doi: 10.1042/cs0890431

El-Merahbi, R., M. Löffler, A. Mayer, and G. Sumara. 2015. The roles of peripheral serotonin in metabolic homeostasis. FEBS Letters 589:1728-1734. doi: 10.1016/j.febslet.2015.05.054

Elango, R. 2020. Methionine nutrition and metabolism: Insights from animal studies to inform human nutrition. J. Nutr. 150:2518S-2523S. doi: 10.1093/jn/nxaa155

Englyst, H. N., and J. H. Cummings. 1988. Improved method for measurement of dietary fiber as non-starch polysaccharides in plant foods. J. Assoc. Off. Anal. 71:808-814.

Erwin, E. S., G. J. Marco, and E. M. Emery. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. J. Dairy Sci. 44:1768-1771. doi: 10.3168/jds.S0022-0302(61)89956-6

Ewaschuk, J. B., G. K. Murdoch, I. R. Johnson, K. L. Madsen, and C. J. Field. 2011. Glutamine supplementation improves intestinal barrier function in a weaned piglet model of Escherichia coli infection. Br. J. Nutr. 106:870-877. doi: 10.1017/S0007114511001152

Fàbrega, A., and J. Vila. 2013. Salmonella enterica Serovar Typhimurium skills to succeed in the host: Virulence and regulation. Clin. Microbiol. Rev. 26:308-341. doi: 10.1128/CMR.00066-12

Fahlgren, A., S. Hammarstrom, Å. Danielsson, and M. L. Hammarstrom. 2003. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. Clin. Exp. Immunol. 131:90-101. doi: 10.1046/j.1365-2249.2003.02035.x

Faure, M., F. Choné, C. Mettraux, J. P. Godin, F. Béchereau, J. Vuichoud, I. Papet, D. Breuillé, and C. Obled. 2007. Threonine utilization for synthesis of acute phase proteins, intestinal proteins, and mucins is increased during sepsis in rats. J. Nutr. 137:1802-1807. doi: 10.1093/jn/137.7.1802

Faure, M., C. Mettraux, D. Moennoz, J.-P. Godin, J. Vuichoud, F. Rochat, D. Breuillé, C. Obled, and I. n. Corthésy-Theulaz. 2006. Specific amino acids increase mucin synthesis and

microbiota in dextran sulfate sodium–treated rats. J. Nutr. 136:1558-1564. doi: 10.1093/jn/136.6.1558

Floc'h, N. L., and B. Sève. 2005. Catabolism through the threonine dehydrogenase pathway does not account for the high first-pass extraction rate of dietary threonine by the portal drained viscera in pigs. Br. J. Nutr. 93:447-456. doi: 10.1079/BJN20051375

Foley, S. L., A. M. Lynne, and R. Nayak. 2008. Salmonella challenges: Prevalence in swine and poultry and potential pathogenicity of such isolates. J. Anim. Sci. 86:E149-E162. doi: 10.2527/jas.2007-0464

Gessner, D. K., R. Ringseis, and K. Eder. 2017. Potential of plant polyphenols to combat oxidative stress and inflammatory processes in farm animals. J. Anim. Physiol. Anim. Nutr. 101:605-628. doi: 10.1111/jpn.12579

Gill, R., A. Tsung, and T. Billiar. 2010. Linking oxidative stress to inflammation: Toll-like receptors. Free Radic. Biol. Med. 48:1121-1132. doi: 10.1016/j.freeradbiomed.2010.01.006

Giuliani, A., G. Pirri, and S. Nicoletto. 2007. Antimicrobial peptides: an overview of a promising class of therapeutics. Open Life Sci. 2:1-33. doi: 10.2478/s11535-007-0010-5

Goering, H. K., and P. J. van Soest. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications) Agriculture Handbook No. 379. ARS, USDA, Washington, DC.

Gu, C., X. Mao, D. Chen, B. Yu, and Q. Yang. 2019. Isoleucine plays an important role for maintaining immune function. Curr. Protein Pept. Sci. 20:644-651. doi: 10.2174/1389203720666190305163135

Guo, P., X. Piao, D. Ou, D. Li, and Y. Hao. 2007. Characterization of the antigenic specificity of soybean protein beta-conglycinin and its effects on growth and immune function in rats. Arch. Anim. Nutr. 61:189-200. doi: 10.1080/17450390701318358

Haberecht-Muller, S., E. Kruger, and J. Fielitz. 2021. Out of control: the role of the ubiquitin proteasome system in skeletal muscle during inflammation. Biomolecules 11doi: 10.3390/biom11091327

Habibi, M., C. Shili, J. Sutton, P. Goodarzi, E. R. Maylem, L. Spicer, and A. Pezeshki. 2021. Branched-chain amino acids partially recover the reduced growth of pigs fed with protein-restricted diets through both central and peripheral factors. Anim. Nutr. 7:868-882. doi: 10.1016/j.aninu.2021.02.002

Han, J., Y. L. Liu, W. Fan, J. Chao, Y. Q. Hou, Y. L. Yin, H. L. Zhu, G. Q. Meng, and Z. Q. Che. 2009. Dietary L-arginine supplementation alleviates immunosuppression induced by cyclophosphamide in weaned pigs. Amino Acids 37:643-651. doi: 10.1007/s00726-008-0184-9

Hao, Y., Z. Zhan, P. Guo, X. Piao, and D. Li. 2009. Soybean β-conglycinin-induced gut hypersensitivity reaction in a piglet model. Arch. Anim. Nutr. 63:188-202. doi: 10.1080/17450390902860026

Hatzoglou, M., J. Fernandez, I. Yaman, and E. Closs. 2004. Regulation of cationic amino acid transport: the story of the CAT-1 transporter. Annu. Rev. Nutr. 24:377-399. doi: 10.1146/annurev.nutr.23.011702.073120

Have, G. A. M. T., M. P. K. J. Engelen, Y. C. Luiking, and N. E. P. Deutz. 2007. Absorption kinetics of amino acids, peptides, and intact proteins. Int. J. Sport Nutr. Exe. 17:23-36. doi: 10.1123/ijsnem.17.s1.s23

He, J., G. D. Feng, X. Ao, Y. F. Li, H. X. Qian, J. B. Liu, G. Y. Bai, and Z. Z. He. 2016. Effects of L-glutamine on growth performance, antioxidant ability, immunity and expression of

genes related to intestinal health in weanling pigs. Livest. Sci. 189:102-109. doi: 10.1016/j.livsci.2016.05.009

He, Q., H. Tang, P. Ren, X. Kong, G. Wu, Y. Yin, and Y. Wang. 2011. Dietary supplementation with L-arginine partially counteracts serum metabonome induced by weaning stress in piglets. J. Proteome Res. 10:5214-5221. doi: 10.1021/pr200688u

Heo, J. M., J. C. Kim, C. F. Hansen, B. P. Mullan, D. J. Hampson, and J. R. Pluske. 2010. Feeding a diet with a decreased protein content reduces both nitrogen content in the gastrointestinal tract and post-weaning diarrhoea, but does not affect apparent nitrogen digestibility in weaner pigs challenged with an enterotoxigenic strain of Escherichia coli. Anim. Feed Sci. Technol. 160:148-159. doi: 10.1016/j.anifeedsci.2010.07.005

Heo, J. M., F. O. Opapeju, J. R. Pluske, J. C. Kim, D. J. Hampson, and C. M. Nyachoti. 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J. Anim. Physiol. Anim. Nutr. 97:207-237. doi: 10.1111/j.1439-0396.2012.01284.x

Hippe, B., J. Zwielehner, K. Liszt, C. Lassl, F. Unger, and A. G. Haslberger. 2011. Quantification of butyryl CoA:acetate CoA-transferase genes reveals different butyrate production capacity in individuals according to diet and age. FEMS Microbiol. Lett. 316:130-135. doi: 10.1111/j.1574-6968.2010.02197.x

Hodo, T. W., M. T. P. de Aquino, A. Shimamoto, and A. Shanker. 2020. Critical Neurotransmitters in the Neuroimmune Network. Front. Immunol. 11:1-26. doi: 10.3389/fimmu.2020.01869

Hoffmann, E., O. Dittrich-Breiholz, H. Holtmann, and M. Kracht. 2002. Multiple control of interleukin-8 gene expression. J. Leukoc. Biol. 72:847-855. doi: 10.1189/jlb.72.5.847

Hou, Y., L. Wang, D. Yi, and G. Wu. 2015. N-acetylcysteine and intestinal health: a focus on its mechanism of action. Front. Biosci. 20:872-891 doi:10.2741/4342

Hou, Y., L. Wang, W. Zhang, Z. Yang, B. Ding, H. Zhu, Y. Liu, Y. Qiu, Y. Yin, and G. Wu. 2012. Protective effects of N-acetylcysteine on intestinal functions of piglets challenged with lipopolysaccharide. Amino Acids 43:1233-1242. doi: 10.1007/s00726-011-1191-9

Hu, C. J., F. N. Li, Y. H. Duan, T. Zhang, H. W. Li, Y. L. Yin, G. Y. Wu, and X. F. Kong. 2019. Dietary supplementation with arginine and glutamic acid alters the expression of amino acid transporters in skeletal muscle of growing pigs. Amino Acids 51:1081-1092. doi: 10.1007/s00726-019-02748-9

Humphrey, B. D., and K. C. Klasing. 2005. The acute phase response alters cationic amino acid transporter expression in growing chickens (Gallus gallus domesticus). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 142:485-494. doi: 10.1016/j.cbpa.2005.10.002

Humphrey, B. D., C. B. Stephensen, C. C. Calvert, and K. C. Klasing. 2006. Lysine deficiency and feed restriction independently alter cationic amino acid transporter expression in chickens (Gallus gallus domesticus). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 143:218-227. doi: 10.1016/j.cbpa.2005.11.019

Huntley, N. F., C. M. Nyachoti, and J. F. Patience. 2018. Lipopolysaccharide immune stimulation but not β-mannanase supplementation affects maintenance energy requirements in young weaned pigs. J. Anim. Sci. Biotechnol. 9:47. doi: 10.1186/s40104-018-0264-y

Hyde, R., P. M. Taylor, and H. S. Hundal. 2003. Amino acid transporters: roles in amino acid sensing and signalling in animal cells. Biochem. J. 373:1-18. doi: 10.1042/bj20030405

Jaffer, U., R. G. Wade, and T. Gourlay. 2010. Cytokines in the systemic inflammatory response syndrome: a review. HSR proceedings in intensive care & cardiovascular anesthesia 2:161-175.

Jayaraman, B., J. Htoo, and C. M. Nyachoti. 2015. Effects of dietary threonine:lysine ratioes and sanitary conditions on performance, plasma urea nitrogen, plasma-free threonine and lysine of weaned pigs. Anim. Nutr. 1:283-288. doi: 10.1016/j.aninu.2015.09.003

Jayaraman, B., J. K. Htoo, and C. M. Nyachoti. 2017a. Effects of different dietary tryptophan : lysine ratios and sanitary conditions on growth performance, plasma urea nitrogen, serum haptoglobin and ileal histomorphology of weaned pigs. Anim. Sci. J. 88:763-771. doi: 10.1111/asj.12695

Jayaraman, B., and C. M. Nyachoti. 2017. Husbandry practices and gut health outcomes in weaned piglets: A review. Anim. Nutr. 3:205-211. doi: 10.1016/j.aninu.2017.06.002

Jayaraman, B., A. Regassa, J. K. Htoo, and C. M. Nyachoti. 2017b. Effects of dietary standardized ileal digestible tryptophan:lysine ratio on performance, plasma urea nitrogen, ileal histomorphology and immune responses in weaned pigs challenged with *Escherichia coli* K88. Livest. Sci. 203:114-119. doi: 10.1016/j.livsci.2017.07.014

Ji, F. J., L. X. Wang, H. S. Yang, A. Hu, and Y. L. Yin. 2019. Review: The roles and functions of glutamine on intestinal health and performance of weaning pigs. Animal 13:2727-2735. doi: 10.1017/S1751731119001800

Jiménez-Escrig, A., M. D. Tenorio, I. Espinosa-Martos, and P. Rupérez. 2008. Health-promoting effects of a dietary fiber concentrate from the soybean byproduct okara in rats. J. Agric. Food Chem. 56:7495-7501. doi: 10.1021/jf800792y

Johnson, I. R., R. O. Ball, V. E. Baracos, and C. J. Field. 2006. Glutamine supplementation influences immune development in the newly weaned piglet. Dev. Comp. Immunol. 30:1191-1202. doi: 10.1016/j.dci.2006.03.003

Johnson, R. W. 1998. Immune and endocrine regulation of food intake in sick animals. Domest. Anim. Endocrinol. 15:309-319. doi: 10.1016/S0739-7240(98)00031-9

Kahindi, R., A. Regassa, J. Htoo, and M. Nyachoti. 2017a. Optimal sulfur amino acid to lysine ratio for post weaning piglets reared under clean or unclean sanitary conditions. Anim. Nutr. 3:380-385. doi: 10.1016/j.aninu.2017.08.004

Kahindi, R. K., J. K. Htoo, and C. M. Nyachoti. 2017b. Dietary lysine requirement for 7–16 kg pigs fed wheat–corn–soybean meal-based diets. J. Anim. Physiol. Anim. Nutr. 101:22-29. doi: 10.1111/jpn.12491

Kakazu, E., N. Kanno, Y. Ueno, and T. Shimosegawa. 2007. Extracellular branched-chain amino acids, especially valine, regulate maturation and function of monocyte-derived dendritic cells. J. Immunol. 179:7137-7146. doi: 10.4049/jimmunol.179.10.7137

Kamei, Y., Y. Hatazawa, R. Uchitomi, R. Yoshimura, and S. Miura. 2020. Regulation of skeletal muscle function by amino acids. Nutrients 12:261.

Kiela, P. R., and F. K. Ghishan. 2016. Physiology of intestinal absorption and secretion. Best Pract. Res. Clin. Gastroenterol. 30:145-159. doi: 10.1016/j.bpg.2016.02.007

Kil, D. Y., and H. H. Stein. 2010. Board Invited Review: Management and feeding strategies to ameliorate the impact of removing antibiotic growth promoters from diets fed to weanling pigs. Can. J. Anim. Sci. 90:447-460. doi: 10.4141/cjas10028

Kim, C. H., J. Park, and M. Kim. 2014. Gut microbiota-derived short-chain fatty acids, T cells, and inflammation. Immune Netw. 14:277-288.

Kim, M.-H., and H. Kim. 2017. The roles of glutamine in the intestine and its implication in intestinal diseases. Int. J. Mol. Sci. 18:1051.

Kimura, H. 2015. Physiological roles of hydrogen sulfide and polysulfides. In: P. K. Moore and M. Whiteman, editors, Chemistry, Biochemistry and Pharmacology of Hydrogen Sulfide. Springer International Publishing, Cham. p. 61-81.

Knudsen, K. E. B. 2014. Fiber and nonstarch polysaccharide content and variation in common crops used in broiler diets. Poult. Sci. 93:2380-2393. doi: 10.3382/ps.2014-03902

Kong, C., and O. Adeola. 2014. Evaluation of amino acid and energy utilization in feedstuff for swine and poultry diets. Asian-Australas. J. Anim. Sci. 27:917-925. doi: 10.5713/ajas.2014.r.02

Koo, B. 2017. A thesis: Nutritional strategies to control feed costs for nursery pigs without compromising gut health and growth performance. University of Manitoba. .

Koo, B., D. Bustamante-García, and C. M. Nyachoti. 2018. Energy content and nutrient digestibility of diets containing Lactobacillus-fermented barley or wheat fed to weaned pigs. J. Anim. Sci. 96:4802-4811. doi: 10.1093/jas/sky344

Koo, B., J. W. Kim, C. F. M. de Lange, M. M. Hossain, and C. M. Nyachoti. 2017. Effects of diet complexity and multicarbohydrase supplementation on growth performance, nutrient digestibility, blood profile, intestinal morphology, and fecal score in newly weaned pigs. J. Anim. Sci. 95:4060-4071. doi: 10.2527/jas2017.1760

Koo, B., and C. M. Nyachoti. 2021. Effect of oat particle size on energy and nutrient utilization in growing pigs. J. Anim. Sci. 99doi: 10.1093/jas/skab134

Koopmans, S. J., A. C. Guzik, J. van der Meulen, R. Dekker, J. Kogut, B. J. Kerr, and L. L. Southern. 2006. Effects of supplemental L-tryptophan on serotonin, cortisol, intestinal integrity, and behavior in weanling piglets. J. Anim. Sci. 84:963-971. doi: 10.2527/2006.844963x

Kwon, W. B., K. J. Touchette, A. Simongiovanni, K. Syriopoulos, A. Wessels, and H. H. Stein. 2019. Excess dietary leucine in diets for growing pigs reduces growth performance, biological value of protein, protein retention, and serotonin synthesis. J. Anim. Sci. 97:4282-4292. doi: 10.1093/jas/skz259

Kwon, Y. H., and M. H. Stipanuk. 2001. Cysteine regulates expression of cysteine dioxygenase and γ-glutamylcysteine synthetase in cultured rat hepatocytes. J. Physiol. Endocrinol. Metab. 280:E804-E815. doi: 10.1152/ajpendo.2001.280.5.E804

Lallès, J. P., G. Boudry, C. Favier, N. L. Floc'h, I. Luron, L. Montagne, I. P. Oswald, S. Pié, C. Piel, and B. Sève. 2004. Gut function and dysfunction in young pigs: physiology. Anim. Res. 53:301-316.

Lanas, A. 2008. Role of nitric oxide in the gastrointestinal tract. Arthritis research & therapy 10 Suppl 2:S4-S4. doi: 10.1186/ar2465

Le Floc'h, N., L. LeBellego, J. J. Matte, D. Melchior, and B. Sève. 2009. The effect of sanitary status degradation and dietary tryptophan content on growth rate and tryptophan metabolism in weaning pigs. J. Anim. Sci. 87:1686-1694. doi: 10.2527/jas.2008-1348

Le Floc'h, N., D. Melchior, and C. Obled. 2004. Modifications of protein and amino acid metabolism during inflammation and immune system activation. Livest. Prod. Sci. 87:37-45. doi: 10.1016/j.livprodsci.2003.09.005

Le Floc'h, N., and B. Seve. 2007. Biological roles of tryptophan and its metabolism: Potential implications for pig feeding. Livest. Sci. 112:23-32. doi: 10.1016/j.livsci.2007.07.002

Le Floc'h, N., W. Otten, and E. Merlot. 2011. Tryptophan metabolism, from nutrition to potential therapeutic applications. Amino Acids 41:1195-1205. doi: 10.1007/s00726-010-0752-7

Le Floc'h, N., A. Wessels, E. Corrent, G. Wu, and P. Bosi. 2018. The relevance of functional amino acids to support the health of growing pigs. Anim. Feed Sci. Technol. 245:104-116. doi: 10.1016/j.anifeedsci.2018.09.007

Lee, D. N., C. F. Wang, Y. H. Cheng, T. Y. Kuo, J. F. Wu, and H. T. Yen. 2003. Dietary glutamine supplementation enhances weaned pigs mitogen-induced lymphocyte proliferation. Asian-Australas. J. Anim. Sci. 16:1182-1187. doi: 10.5713/ajas.2003.1182

Lee, J., J. C. González-Vega, J. K. Htoo, and C. M. Nyachoti. 2021. Evaluating the standardized ileal digestible lysine requirement of 7- to 15-kg weanling pigs fed corn-soybean meal-based diets. Animal 15:100127. doi: 10.1016/j.animal.2020.100127

Lee, S., K. H. Han, Y. Nakamura, S. Kawakami, K. Shimada, T. Hayakawa, H. Onoue, and M. Fukushima. 2013. Dietary L-cysteine improves the antioxidative potential and lipid metabolism in rats fed a normal diet. Biosci. Biotechnol. Biochem. 77:1430-1434. doi: 10.1271/bbb.130083

Lenis, N. P., and A. W. Jongbloed. 1999. New Technologies in low pollution swine diets : Diet manipulation and use of synthetic amino acids, phytase and phase feeding for reduction of nitrogen and phosphorus excretion and ammonia emission - review. Asian-Australas. J. Anim. Sci. 12:305-327. doi: 10.5713/ajas.1999.305

Leonel, A. J., and J. I. Alvarez-Leite. 2012. Butyrate: implications for intestinal function. Curr. Opin. Clin. Nutr. Metab. Care 15:474-479. doi: 10.1097/MCO.0b013e32835665fa

Levrat, M. A., S. R. Behr, C. Rémésy, and C. Demigné. 1991. Effects of soybean fiber on cecal digestion in rats previously adapted to a fiber-free diet. J. Nutr. 121:672-678. doi: 10.1093/jn/121.5.672

Li, D. F., J. L. Nelssen, P. G. Reddy, F. Blecha, J. D. Hancock, G. L. Allee, R. D. Goodband, and R. D. Klemm. 1990. Transient hypersensitivity to soybean meal in the early-weaned pig. J. Anim. Sci. 68:1790-1799. doi: 10.2527/1990.6861790x

Li, H., J. P. Limenitakis, T. Fuhrer, M. B. Geuking, M. A. Lawson, M. Wyss, S. Brugiroux, I. Keller, J. A. MacPherson, S. Rupp, B. Stolp, J. V. Stein, B. Stecher, U. Sauer, K. D. McCoy, and A. J. MacPherson. 2015. The outer mucus layer hosts a distinct intestinal microbial niche. Nature Communications 6:8292. doi: 10.1038/ncomms9292

Li, P., Y. L. Yin, D. Li, S. W. Kim, and G. Wu. 2007. Amino acids and immune function. Br. J. Nutr. 98:237-252. doi: 10.1017/S000711450769936X

Li, Q., Y. Liu, Z. Che, H. Zhu, G. Meng, Y. Hou, B. Ding, Y. Yin, and F. Chen. 2012. Dietary L-arginine supplementation alleviates liver injury caused by Escherichia coli LPS in weaned pigs. Innate Immun. 18:804-814. doi: 10.1177/1753425912441955

Liang, H., Z. Dai, J. Kou, K. Sun, J. Chen, Y. Yang, G. Wu, and Z. Wu. 2019. Dietary L-tryptophan supplementation enhances the intestinal mucosal barrier function in weaned piglets: Implication of tryptophan-metabolizing microbiota. Int. J. Mol. Sci. 20:1-13.

Liang, H., Z. Dai, N. Liu, Y. Ji, J. Chen, Y. Zhang, Y. Yang, J. Li, Z. Wu, and G. Wu. 2018. Dietary L-tryptophan modulates the structural and functional composition of the intestinal microbiome in weaned piglets. Front. Microbiol. 9:1-12. doi: 10.3389/fmicb.2018.01736

Lin, C. C., and M. C. Yin. 2008. Effects of cysteine-containing compounds on biosynthesis of triacylglycerol and cholesterol and anti-oxidative protection in liver from mice consuming a high-fat diet. Br. J. Nutr. 99:37-43. doi: 10.1017/S0007114507793881

Litvak, N., A. Rakhshandeh, J. K. Htoo, and C. F. M. de Lange. 2013. Immune system stimulation increases the optimal dietary methionine to methionine plus cysteine ratio in growing pigs. J. Anim. Sci. 91:4188-4196. doi: 10.2527/jas.2012-6160

Litvak, Y., M. X. Byndloss, and A. J. Baumler. 2018. Colonocyte metabolism shapes the gut microbiota. Science 362:1017-1024. doi: 10.1126/science.aat9076

Liu, J., Y. Zhang, Y. Li, H. Yan, and H. Zhang. 2019. L-tryptophan enhances intestinal integrity in diquat-challenged piglets associated with improvement of redox status and mitochondrial function. Animals 9:266. doi: 10.3390/ani9050266

Liu, T., J. Peng, Y. Xiong, S. Zhou, and X. Cheng. 2002. Effects of dietary glutamine and glutamate supplementation on small intestinal structure, active absorption and DNA, rna concentrations in skeletal muscle tissue of weaned piglets during d 28 to 42 of age. Asian-Australas. J. Anim. Sci. 15:238-242. doi: 10.5713/ajas.2002.238

Liu, Y., J. Han, J. Huang, X. Wang, F. Wang, and J. Wang. 2009. Dietary L-arginine Supplementation Improves Intestinal Function in Weaned Pigs after an Escherichia coli Lipopolysaccharide Challenge. Asian-Australas. J. Anim. Sci. 22:1667-1675. doi: 10.5713/ajas.2009.90100

Liu, Y., J. Huang, Y. Hou, H. Zhu, S. Zhao, B. Ding, Y. Yin, G. Yi, J. Shi, and W. Fan. 2008. Dietary arginine supplementation alleviates intestinal mucosal disruption induced by Escherichia coli lipopolysaccharide in weaned pigs. Br. J. Nutr. 100:552-560. doi: 10.1017/S0007114508911612

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402-408. doi: 10.1006/meth.2001.1262

Luo, J.-B., L. Feng, W.-D. Jiang, Y. Liu, P. Wu, J. Jiang, S.-Y. Kuang, L. Tang, Y.-A. Zhang, and X.-Q. Zhou. 2014. The impaired intestinal mucosal immune system by valine deficiency for young grass carp (Ctenopharyngodon idella) is associated with decreasing immune status and regulating tight junction proteins transcript abundance in the intestine. Fish Shellfish Immunol. 40:197-207. doi: 10.1016/j.fsi.2014.07.003

Luo, J. L., F. Hammarqvist, K. Andersson, and J. Wernerman. 1998. Surgical trauma decreases glutathione synthetic capacity in human skeletal muscle tissue. J. Physiol. Endocrinol. Metab. 275:E359-E365. doi: 10.1152/ajpendo.1998.275.2.E359

Luppi, A. 2017. Swine enteric colibacillosis: diagnosis, therapy and antimicrobial resistance. Porc. Health Manag. 3:16. doi: 10.1186/s40813-017-0063-4

Ma, D., J. M. Guedes, A. W. Duttlinger, J. S. Johnson, S. M. Zuelly, D. C. Lay, B. T. Richert, and Y. H. B. Kim. 2021. Impact of L-glutamine as replacement of dietary antibiotics during post weaning and transport recovery on carcass and meat quality attributes in pigs. Livest. Sci. 244:104350. doi: 10.1016/j.livsci.2020.104350

Maenz, D. D., and J. F. Patience. 1992. L-threonine transport in pig jejunal brush border membrane vesicles. Functional characterization of the unique system B in the intestinal epithelium. J. Biol. Chem. 267:22079-22086.

Magierowski, M., K. Magierowska, S. Kwiecien, and T. Brzozowski. 2015. Gaseous mediators nitric oxide and hydrogen sulfide in the mechanism of gastrointestinal integrity, protection and ulcer healing. Molecules 20:9099-9123. doi: 10.3390/molecules20059099

Mahan, D. C., N. D. Fastinger, and J. C. Peters. 2004. Effects of diet complexity and dietary lactose levels during three starter phases on postweaning pig performance. J. Anim. Sci. 82:2790-2797. doi: 10.2527/2004.8292790x

Mahan, D. C., and A. J. Lepine. 1991. Effect of pig weaning weight and associated nursery feeding programs on subsequent performance to 105 kilograms body weight. J. Anim. Sci. 69:1370-1378. doi: 10.2527/1991.6941370x

Maletínská, L., A. Popelová, B. Železná, M. Bencze, and J. Kuneš. 2019. The impact of anorexigenic peptides in experimental models of Alzheimer's disease pathology. J. Endocrinol. 240:R47-R72. doi: 10.1530/joe-18-0532

Malmezat, T., D. Breuillé, P. Capitan, P. P. Mirand, and C. Obled. 2000a. Glutathione turnover is increased during the acute phase of sepsis in rats. J. Nutr. 130:1239-1246. doi: 10.1093/jn/130.5.1239

Malmezat, T., D. Breuillé, C. Pouyet, C. Buffière, P. Denis, P. P. Mirand, and C. Obled. 2000b. Methionine transsulfuration is increased during sepsis in rats. J. Physiol. Endocrinol. Metab. 279:E1391-E1397. doi: 10.1152/ajpendo.2000.279.6.E1391

Mao, X., C. Gu, M. Ren, D. Chen, B. Yu, J. He, J. Yu, P. Zheng, J. Luo, Y. Luo, J. Wang, G. Tian, and Q. Yang. 2018. L-Isoleucine administration alleviates rotavirus infection and immune response in the weaned piglet model. Front Immunol 9:1654. doi: 10.3389/fimmu.2018.01654

Mao, X., X. Lai, B. Yu, J. He, J. Yu, P. Zheng, G. Tian, K. Zhang, and D. Chen. 2014a. Effects of dietary threonine supplementation on immune challenge induced by swine Pseudorabies live vaccine in weaned pigs. Arch. Anim. Nutr. 68:1-15. doi: 10.1080/1745039X.2013.869988

Mao, X., M. Lv, B. Yu, J. He, P. Zheng, J. Yu, Q. Wang, and D. Chen. 2014b. The effect of dietary tryptophan levels on oxidative stress of liver induced by diquat in weaned piglets. J. Anim. Sci. Biotechnol. 5:49-56. doi: 10.1186/2049-1891-5-49

Mao, X., S. Qi, B. Yu, Z. Huang, H. Chen, Q. Mao, G. Han, and D. Chen. 2012. Dietary l-arginine supplementation enhances porcine β-defensins gene expression in some tissues of weaned pigs. Livest. Sci. 148:103-108. doi: 10.1016/j.livsci.2012.05.016

Martínez-López, N., M. Varela-Rey, U. Ariz, N. Embade, M. Vazquez-Chantada, D. Fernandez-Ramos, L. Gomez-Santos, Shelly C. Lu, Jose M. Mato, and Maria L. Martinez-Chantar. 2008. S-adenosylmethionine and proliferation: new pathways, new targets. Biochem. Soc. Trans. 36:848-852. doi: 10.1042/BST0360848

McCracken, B. A., M. E. Spurlock, M. A. Roos, F. A. Zuckermann, and H. R. Gaskins. 1999. Weaning anorexia may contribute to local inflammation in the piglet small intestine. J. Nutr. 129:613-619. doi: 10.1093/jn/129.3.613

McGilvray, W. D., D. Klein, H. Wooten, J. A. Dawson, D. Hewitt, A. R. Rakhshandeh, C. F. M. de Lange, and A. Rakhshandeh. 2019. Immune system stimulation induced by porcine reproductive and respiratory syndrome virus alters plasma free amino acid flux and dietary nitrogen utilization in starter pigs. J. Anim. Sci. doi: 10.1093/jas/skz120

McGivan, J. D., and M. Pastor-Anglada. 1994. Regulatory and molecular aspects of mammalian amino acid transport. The Biochemical journal 299(Pt 2):321-334. doi: 10.1042/bj2990321

McGuckin, M. A., R. Eri, L. A. Simms, T. H. Florin, and G. Radford-Smith. 2009. Intestinal barrier dysfunction in inflammatory bowel diseases. Inflamm. Bowel Dis. 15:100-113. doi: 10.1002/ibd.20539

Milner, J. A., and W. J. Visek. 1978. Dietary protein intake and arginine requirements in the rat. J. Nutr. 108:382-391. doi: 10.1093/jn/108.3.382

Min, Y. N., S. G. Liu, Z. X. Qu, G. H. Meng, and Y. P. Gao. 2017. Effects of dietary threonine levels on growth performance, serum biochemical indexes, antioxidant capacities, and gut morphology in broiler chickens. Poult. Sci. 96:1290-1297. doi: 10.3382/ps/pew393

Moeser, A. J., C. S. Pohl, and M. Rajput. 2017. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. Anim. Nutr. 3:313-321. doi: 10.1016/j.aninu.2017.06.003

Moffett, J. R., and M. A. Namboodiri. 2003. Tryptophan and the immune response. Immunol. Cell Biol. 81:247-265. doi: 10.1046/j.1440-1711.2003.t01-1-01177.x

Montagne, L., N. Le Floc'h, M. Arturo-Schaan, R. Foret, M. C. Urdaci, and M. Le Gall. 2012. Comparative effects of level of dietary fiber and sanitary conditions on the growth and health of weanling pigs. J. Anim. Sci. 90:2556-2569. doi: 10.2527/jas.2011-4160

Montagne, L., J. R. Pluske, and D. J. Hampson. 2003. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. Anim. Feed Sci. Technol. 108:95-117. doi: 10.1016/S0377-8401(03)00163-9

Mousavi Khaneghah, A., K. Abhari, I. Eş, M. B. Soares, R. B. A. Oliveira, H. Hosseini, M. Rezaei, C. F. Balthazar, R. Silva, A. G. Cruz, C. S. Ranadheera, and A. S. Sant'Ana. 2020. Interactions between probiotics and pathogenic microorganisms in hosts and foods: A review. Trends Food Sci. Techonol. 95:205-218. doi: 10.1016/j.tifs.2019.11.022

Mukherjee, S., and Lora V. Hooper. 2015. Antimicrobial defense of the intestine. Immunity 42:28-39. doi: 10.1016/j.immuni.2014.12.028

Mukkur, T. K. S., D. L. Watson, K. S. Saini, and A. K. Lascelles. 1985. Purification and characterization of goblet-cell mucin of high Mr from the small intestine of sheep. Biochem. J. 229:419-428. doi: 10.1042/bj2290419

Nichols, N. L., and R. F. Bertolo. 2008. Luminal threonine concentration acutely affects intestinal mucosal protein and mucin synthesis in piglets. J. Nutr. 138:1298-1303. doi: 10.1093/jn/138.7.1298

Nie, C., T. He, W. Zhang, G. Zhang, and X. Ma. 2018. Branched chain amino acids: Beyond nutrition metabolism. Int. J. Mol. Sci. 19:954. doi: 10.3390/ijms19040954

Noblet, J., H. Fortune, X. S. Shi, and S. Dubois. 1994. Prediction of net energy value of feeds for growing pigs. J. Anim. Sci. 72:344-354. doi: 10.2527/1994.722344x

Novozamsky, I., R. Eck, and J. C. van Schouwenburg. 1974. Total nitrogen determination in plant material by means of the indophenol-blue method. Neth. J. Agric. Sci. 22:3-5.

NRC. 2012. Nutrient Requirements of Swine 11th rev. ed. Natl. Acad. Press, Washigton, DC.

Nyachoti, C. M., C. F. M. de Lange, B. W. McBride, and H. Schulze. 1997. Significance of endogenous gut nitrogen losses in the nutrition of growing pigs: A review. Can. J. Anim. Sci. 77:149-163. doi: 10.4141/A96-044

Nyachoti, C. M., F. O. Omogbenigun, M. Rademacher, and G. Blank. 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. J. Anim. Sci. 84:125-134. doi: 10.2527/2006.841125x

O'Mahony, S. M., G. Clarke, Y. E. Borre, T. G. Dinan, and J. F. Cryan. 2015. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav. Brain Res. 277:32-48. doi: 10.1016/j.bbr.2014.07.027

Obled, C. 2003. Amino acid requirements in inflammatory states. Can. J. Anim. Sci. 83:365-373. doi: 10.4141/A03-021

Obled, C., I. Papet, and D. Breuille. 2002. Metabolic bases of amino acid requirements in acute diseases. Curr. Opin. Clin. Nutr. Metab. Care 5:189-197.

Osmanyan, A. K., S. Ghazi Harsini, R. Mahdavi, V. I. Fisinin, A. L. Arkhipova, T. T. Glazko, S. N. Kovalchuk, and G. Y. Kosovsky. 2018. Intestinal amino acid and peptide transporters

in broiler are modulated by dietary amino acids and protein. Amino Acids 50:353-357. doi: 10.1007/s00726-017-2510-6

Ospina-Rojas, I. C., A. E. Murakami, C. R. A. Duarte, C. Eyng, C. A. L. Oliveira, and V. Janeiro. 2014. Valine, isoleucine, arginine and glycine supplementation of low-protein diets for broiler chickens during the starter and grower phases. Br. Poult. Sci. 55:766-773. doi: 10.1080/00071668.2014.970125

Parker, A., L. Vaux, A. M. Patterson, A. Modasia, D. Muraro, A. G. Fletcher, H. M. Byrne, P. K. Maini, A. J. M. Watson, and C. Pin. 2019. Elevated apoptosis impairs epithelial cell turnover and shortens villi in TNF-driven intestinal inflammation. Cell Death Dis. 10:108. doi: 10.1038/s41419-018-1275-5

Parra, M. D., P. Fuentes, F. Tecles, S. Martínez-Subiela, J. S. Martínez, A. Muñoz, and J. J. Cerón. 2006. Porcine acute phase protein concentrations in different diseases in field conditions. J. Vet. Med. B Infect. Dis. Vet. Public Health 53:488-493. doi: 10.1111/j.1439-0450.2006.01002.x

Pastorelli, H., J. van Milgen, P. Lovatto, and L. Montagne. 2012. Meta-analysis of feed intake and growth responses of growing pigs after a sanitary challenge. Animal 6:952-961. doi: 10.1017/S175173111100228X

Perez-Vilar, J., and R. L. Hill. 1999. The Structure and Assembly of Secreted Mucins. J. Biol. Chem. 274:31751-31754. doi: 10.1074/jbc.274.45.31751

Peterson, L. W., and D. Artis. 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat. Rev. Immunol. 14:141-153. doi: 10.1038/nri3608

Pluske, J. R., D. J. Hampson, and I. H. Williams. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest. Prod. Sci. 51:215-236. doi: 10.1016/S0301-6226(97)00057-2

Pluske, J. R., D. L. Turpin, and J. C. Kim. 2018a. Gastrointestinal tract (gut) health in the young pig. Anim. Nutr. 4:187-196. doi: 10.1016/j.aninu.2017.12.004

Pluske, J. R., D. L. Turpin, and J. C. Kim. 2018b. Gastrointestinal tract (gut) health in the young pig. Anim. Nutr. 4:187-196. doi: 10.1016/j.aninu.2017.12.004

Pomar, C., I. Kyriazakis, G. C. Emmans, and P. W. Knap. 2003. Modeling stochasticity: Dealing with populations rather than individual pigs. J. Anim. Sci. 81:E178-E186. doi: 10.2527/2003.8114_suppl_2E178x

Qi, M., B. Tan, J. Wang, S. Liao, Y. Deng, P. Ji, T. Song, A. Zha, and Y. Yin. 2021. The microbiota–gut–brain axis: A novel nutritional therapeutic target for growth retardation. Crt. Rev. Food Sci. Nutr.:1-26. doi: 10.1080/10408398.2021.1879004

Qi, M., J. Wang, B. Tan, J. Li, S. Liao, Y. Liu, and Y. Yin. 2020. Dietary glutamine, glutamate, and aspartate supplementation improves hepatic lipid metabolism in post-weaning piglets. Anim. Nutr. 6:124-129. doi: 10.1016/j.aninu.2019.12.002

Qin, G., E. R. ter Elst, M. W. Bosch, and A. F. B. van der Poel. 1996. Thermal processing of whole soya beans: Studies on the inactivation of antinutritional factors and effects on ileal digestibility in piglets. Anim. Feed Sci. Technol. 57:313-324. doi: 10.1016/0377-8401(95)00863-2

Qiu, K., C. F. Qin, M. Luo, X. Zhang, W. J. Sun, N. Jiao, F. Li de, and J. D. Yin. 2016. Protein restriction with amino acid-balanced diets shrinks circulating pool size of amino acid by decreasing expression of specific transporters in the small intestine. PLoS. ONE 11:e0162475. doi: 10.1371/journal.pone.0162475

Rakhshandeh, A., C. F. M. de Lange, J. K. Htoo, A. Gheisari, and A. R. Rakhshandeh. 2019. Immune system stimulation increases the plasma cysteine flux and whole-body glutathione synthesis rate in starter pigs. J. Anim. Sci. 97:3871-3881. doi: 10.1093/jas/skz211

Rakhshandeh, A., C. F. M. de Lange, J. K. Htoo, and A. R. Rakhshandeh. 2020. Immune system stimulation increases the irreversible loss of cysteine to taurine, but not sulfate, in starter pigs. J. Anim. Sci. 98doi: 10.1093/jas/skaa001

Rakhshandeh, A., J. C. M. Dekkers, B. J. Kerr, T. E. Weber, J. English, and N. K. Gabler. 2012. Effect of immune system stimulation and divergent selection for residual feed intake on digestive capacity of the small intestine in growing pigs1. J. Anim. Sci. 90:233-235. doi: 10.2527/jas.53976

Rakhshandeh, A., J. K. Htoo, N. Karrow, S. P. Miller, and C. F. M. de Lange. 2014. Impact of immune system stimulation on the ileal nutrient digestibility and utilisation of methionine plus cysteine intake for whole-body protein deposition in growing pigs. Br. J. Nutr. 111:101-110. doi: 10.1017/S0007114513001955

Rao, R. 2008. Oxidative stress-induced disruption of epithelial and endothelial tight junctions. Front. Biosci 13:7210-7226. doi: 10.2741/3223

Reeds, P. J., C. R. Fjeld, and F. Jahoor. 1994. Do the differences between the amino acid compositions of acute-phase and muscle proteins have a bearing on nitrogen loss in traumatic states? J. Nutr. 124:906-910. doi: 10.1093/jn/124.6.906

Reeds, P. J., and F. Jahoor. 2001. The amino acid requirements of disease. Clin. Nutr. 20:15-22. doi: 10.1054/clnu.2001.0402

Regina, M., V. P. Korhonen, T. K. Smith, L. Alakuijala, and T. O. Eloranta. 1993. Methionine toxicity in the rat in relation to hepatic accumulation of S-adenosylmethionine: Prevention by dietary stimulation of the hepatic transsulfuration pathway. Arch. Biochem. Biophys. 300:598-607. doi: 10.1006/abbi.1993.1083

Rémond, D., C. Buffière, J.-P. Godin, P. P. Mirand, C. Obled, I. Papet, D. Dardevet, G. Williamson, D. Breuillé, and M. Faure. 2009. Intestinal inflammation increases gastrointestinal threonine uptake and mucin synthesis in enterally fed minipigs. J. Nutr. 139:720-726. doi: 10.3945/jn.108.101675

Rémond, D., C. Buffière, C. Pouyet, I. Papet, D. Dardevet, I. Savary-Auzeloux, G. Williamson, M. Faure, and D. Breuillé. 2011. Cysteine fluxes across the portal-drained viscera of enterally fed minipigs: effect of an acute intestinal inflammation. Amino Acids 40:543-552. doi: 10.1007/s00726-010-0672-6

Ren, M., S. Cai, T. Zhou, S. Zhang, S. Li, E. Jin, C. Che, X. Zeng, T. Zhang, and S. Qiao. 2019. Isoleucine attenuates infection induced by E. coli challenge through the modulation of intestinal endogenous antimicrobial peptide expression and the inhibition of the increase in plasma endotoxin and IL-6 in weaned pigs. Food Funct. 10:3535-3542. doi: 10.1039/c9fo00218a

Ren, M., X. T. Liu, X. Wang, G. J. Zhang, S. Y. Qiao, and X. F. Zeng. 2014. Increased levels of standardized ileal digestible threonine attenuate intestinal damage and immune responses in Escherichia coli K88+ challenged weaned piglets. Anim. Feed Sci. Technol. 195:67-75. doi: 10.1016/j.anifeedsci.2014.05.013

Ren, M., S. Zhang, X. Liu, S. Li, X. Mao, X. Zeng, and S. Qiao. 2016. Different Lipopolysaccharide Branched-Chain Amino Acids Modulate Porcine Intestinal Endogenous β-Defensin Expression through the Sirt1/ERK/90RSK Pathway. J. Agric. Food Chem. 64:3371-3379. doi: 10.1021/acs.jafc.6b00968

Ren, M., S. H. Zhang, X. F. Zeng, H. Liu, and S. Y. Qiao. 2015. Branched-chain amino acids are beneficial to maintain growth performance and intestinal immune-related function in weaned piglets fed protein restricted diet. Asian-Australas. J. Anim. Sci. 28:1742-1750. doi: 10.5713/ajas.14.0131

Rezaei, R., D. A. Knabe, C. D. Tekwe, S. Dahanayaka, M. D. Ficken, S. E. Fielder, S. J. Eide, S. L. Lovering, and G. Wu. 2013. Dietary supplementation with monosodium glutamate is safe and improves growth performance in postweaning pigs. Amino Acids 44:911-923. doi: 10.1007/s00726-012-1420-x

Riedijk, M. A., B. Stoll, S. Chacko, H. Schierbeek, A. L. Sunehag, J. B. van Goudoever, and D. G. Burrin. 2007. Methionine transmethylation and transsulfuration in the piglet gastrointestinal tract. PNAS 104:3408-3413. doi: 10.1073/pnas.0607965104

Rodrigues, L. A., M. O. Wellington, J. C. Gonzalez-Vega, J. K. Htoo, A. G. Van Kessel, and D. A. Columbus. 2021. Functional amino acid supplementation, regardless of dietary protein content, improves growth performance and immune status of weaned pigs challenged with Salmonella Typhimurium. J. Anim. Sci. 99:1-13. doi: 10.1093/jas/skaa365

Sbodio, J. I., S. H. Snyder, and B. D. Paul. 2019. Regulators of the transsulfuration pathway. Br. J. Pharmacol. 176:583-593. doi: 10.1111/bph.14446

Schaart, M. W., H. Schierbeek, S. R. D. van der Schoor, B. Stoll, D. G. Burrin, P. J. Reeds, and J. B. van Goudoever. 2005. Threonine utilization is high in the intestine of piglets. J. Nutr. 135:765-770. doi: 10.1093/jn/135.4.765

Schroeder, B. O. 2019. Fight them or feed them: how the intestinal mucus layer manages the gut microbiota. Gastroenterol. Rep. 7:3-12. doi: 10.1093/gastro/goy052

Schulze, H., P. van Leeuwen, M. W. A. Verstegen, J. Huisman, W. B. Souffrant, and F. Ahrens. 1994. Effect of level of dietary neutral detergent fiber on ileal apparent digestibility and ileal nitrogen losses in pigs. J. Anim. Sci. 72:2362-2368. doi: 10.2527/1994.7292362x

Semenov, A. V., L. van Overbeek, A. J. Termorshuizen, and A. H. van Bruggen. 2011. Influence of aerobic and anaerobic conditions on survival of Escherichia coli O157:H7 and Salmonella enterica serovar Typhimurium in Luria-Bertani broth, farm-yard manure and slurry. J. Environ. Manage. 92:780-787. doi: 10.1016/j.jenvman.2010.10.031

Shah, A. M., Z. Wang, and J. Ma. 2020. Glutamine metabolism and its role in immunity, a comprehensive review. Animals 10:326.

Shan, Y., A. Shan, J. Li, and C. Zhou. 2012. Dietary supplementation of arginine and glutamine enhances the growth and intestinal mucosa development of weaned piglets. Livest. Sci. 150:369-373. doi: 10.1016/j.livsci.2012.10.006

Shen, Y. B., M. T. Coffey, and S. W. Kim. 2015. Effects of short term supplementation of l-tryptophan and reducing large neutral amino acid along with L-tryptophan supplementation on growth and stress response in pigs. Anim. Feed Sci. Technol. 207:245-252. doi: 10.1016/j.anifeedsci.2015.06.020

Shen, Y. B., G. Voilque, J. Odle, and S. W. Kim. 2012. Dietary L-tryptophan supplementation with reduced large neutral amino acids enhances feed efficiency and decreases stress hormone secretion in nursery pigs under social-mixing stress. J. Nutr. 142:1540-1546. doi: 10.3945/jn.112.163824

Shen, Y. B., A. C. Weaver, and S. W. Kim. 2014. Effect of feed grade L-methionine on growth performance and gut health in nursery pigs compared with conventional DL-methionine. J. Anim. Sci. 92:5530-5539. doi: 10.2527/jas.2014-7830

Shin, T. K., Y. J. Yi, J. C. Kim, J. R. Pluske, H. M. Cho, S. S. Wickramasuriya, E. Kim, S. M. Lee, and J. M. Heo. 2017. Reducing the dietary omega-6 to omega-3 polyunsaturated fatty acid ratio attenuated inflammatory indices and sustained epithelial tight junction integrity in weaner pigs housed in a poor sanitation condition. Anim. Feed Sci. Technol. 234:312-320. doi: 10.1016/j.anifeedsci.2017.04.022

Skinner, L. D., C. L. Levesque, D. Wey, M. Rudar, J. Zhu, S. Hooda, and C. F. M. de Lange. 2014. Impact of nursery feeding program on subsequent growth performance, carcass quality, meat quality, and physical and chemical body composition of growing-finishing pigs. J. Anim. Sci. 92:1044-1054. doi: 10.2527/jas.2013-6743

Slack, E., M. L. Balmer, J. H. Fritz, and S. Hapfelmeier. 2012. Functional flexibility of intestinal IgA - broadening the fine line. Front. Immunol. 3:100. doi: 10.3389/fimmu.2012.00100

Slominski, B. A., and L. D. Campbell. 1990. Non-starch polysaccharides of canola meal: Quantification, digestibility in poultry and potential benefit of dietary enzyme supplementation. J. Sci. Food Agric. 53:175-184. doi: 10.1002/jsfa.2740530205

Soler-Rodriguez, A. M., H. Zhang, H. S. Lichenstein, N. Qureshi, D. W. Niesel, S. E. Crowe, J. W. Peterson, and G. R. Klimpel. 2000. Neutrophil activation by bacterial lipoprotein versus lipopolysaccharide: Differential requirements for serum and CD14. J. Immunol. Res. 164:2674-2683. doi: 10.4049/jimmunol.164.5.2674

Song, Z., T. Chen, I. V. Deaciuc, S. Uriarte, D. Hill, S. Barve, and C. J. McClain. 2004. Modulation of endotoxin stimulated interleukin-6 production in monocytes and Kupffer cells by S-adenosylmethionine (SAMe). Cytokine 28:214-223. doi: 10.1016/j.cyto.2004.08.004

Song, Z., S. Uriarte, R. Sahoo, T. Chen, S. Barve, D. Hill, and C. McClain. 2005. S-adenosylmethionine (SAMe) modulates interleukin-10 and interleukin-6, but not TNF, production via the adenosine (A2) receptor. Biochim. Biophys. Acta Mol. Cell. Res. 1743:205-213. doi: 10.1016/j.bbamcr.2004.12.001

Song, Z. h., G. Tong, K. Xiao, L. f. Jiao, Y. l. Ke, and C. h. Hu. 2016. L-Cysteine protects intestinal integrity, attenuates intestinal inflammation and oxidant stress, and modulates NF-κB and Nrf2 pathways in weaned piglets after LPS challenge. Innate Immun. 22:152-161. doi: 10.1177/1753425916632303

Souffrant, W. B. 2001. Effect of dietary fibre on ileal digestibility and endogenous nitrogen losses in the pig. Anim. Feed Sci. Technol. 90:93-102. doi: 10.1016/S0377-8401(01)00199-7

Soumeh, E. A., J. van Milgen, N. M. Sloth, E. Corrent, H. D. Poulsen, and J. V. Norgaard. 2015. Requirement of standardized ileal digestible valine to lysine ratio for 8- to 14-kg pigs. Animal 9:1312-1318. doi: 10.1017/S1751731115000695

Specian, R. D., and M. G. Oliver. 1991. Functional biology of intestinal goblet cells. Am. J. Physiol. Cell Physiol. 260:C183-C193. doi: 10.1152/ajpcell.1991.260.2.C183

Spring, S., H. Premathilake, C. Bradway, C. Shili, U. DeSilva, S. Carter, and A. Pezeshki. 2020. Effect of very low-protein diets supplemented with branched-chain amino acids on energy balance, plasma metabolomics and fecal microbiome of pigs. Sci. Rep. 10:15859. doi: 10.1038/s41598-020-72816-8

Stasi, C., S. Sadalla, and S. Milani. 2019. The relationship between the serotonin metabolism, gut-microbiota and the gut-brain axis. Curr. Drug. Metab. 20:646-655. doi: 10.2174/1389200220666190725115503

Stein, H. H., B. Sève, M. F. Fuller, P. J. Moughan, and C. F. M. de Lange. 2007. Invited review: Amino acid bioavailability and digestibility in pig feed ingredients: Terminology and application. J. Anim. Sci. 85:172-180. doi: 10.2527/jas.2005-742

Sterndale, S. O., D. W. Miller, J. P. Mansfield, J. C. Kim, and J. R. Pluske. 2020. Increasing dietary tryptophan in conjunction with decreasing other large neutral amino acids increases weight gain and feed intake in weaner pigs regardless of experimental infection with enterotoxigenic Escherichia coli. J. Anim. Sci. 98doi: 10.1093/jas/skaa190

Stiemsma, L. T., L. A. Reynolds, S. E. Turvey, and B. B. Finlay. 2015. The hygiene hypothesis: current perspectives and future therapies. ImmunoTargets Ther. 4:143-157. doi: 10.2147/ITT.S61528

Stipanuk, M. H. 2004. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. Annu. Rev. Nutr. 24:539-577. doi: 10.1146/annurev.nutr.24.012003.132418

Stipanuk, M. H. 2020. Metabolism of sulfur-containing amino acids: How the body copes with excess methionine, cysteine, and sulfide. J. Nutr. 150:2494S-2505S. doi: 10.1093/jn/nxaa094

Stoll, B., J. Henry, P. J. Reeds, H. Yu, F. Jahoor, and D. G. Burrin. 1998. Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. J. Nutr. 128:606-614. doi: 10.1093/jn/128.3.606

Strange, K. 1992. Regulation of solute and water balance and cell volume in the central nervous system. J. Am. Soc. Nephrol. 3:12.

Su, W., H. Zhang, Z. Ying, Y. Li, L. Zhou, F. Wang, L. Zhang, and T. Wang. 2018. Effects of dietary L-methionine supplementation on intestinal integrity and oxidative status in intrauterine growth-retarded weanling piglets. Eur. J. Nutr. 57:2735-2745. doi: 10.1007/s00394-017-1539-3

Sulabo, R. C., M. D. Tokach, J. M. DeRouchey, S. S. Dritz, R. D. Goodband, and J. L. Nelssen. 2010. Influence of feed flavors and nursery diet complexity on preweaning and nursery pig performance. J. Anim. Sci. 88:3918-3926. doi: 10.2527/jas.2009-2724

Sun, P., D. Li, B. Dong, S. Qiao, and X. Ma. 2008. Effects of soybean glycinin on performance and immune function in early weaned pigs. Arch. Anim. Nutr. 62:313-321. doi: 10.1080/17450390802066419

Sun, Y., T. Teng, G. Bai, S. Qiu, B. Shi, D. Ju, and X. Zhao. 2020. Protein-restricted diet balanced for lysine, methionine, threonine, and tryptophan for nursery pigs elicits subsequent compensatory growth and has long term effects on protein metabolism and organ development. Anim. Feed Sci. Technol. 270:114712. doi: 10.1016/j.anifeedsci.2020.114712

Takahashi, A., A. Wada, K.-i. Ogushi, K. Maeda, T. Kawahara, K. Mawatari, H. Kurazono, J. Moss, T. Hirayama, and Y. Nakaya. 2001. Production of β-defensin-2 by human colonic epithelial cells induced by Salmonella enteritidis flagella filament structural protein. FEBS Lett. 508:484-488. doi: 10.1016/S0014-5793(01)03088-5

Takeuchi, O., and S. Akira. 2010. Pattern recognition receptors and inflammation. Cell 140:805-820. doi: 10.1016/j.cell.2010.01.022

Tang, Q., P. Tan, N. Ma, and X. Ma. 2021. Physiological functions of threonine in animals: Beyond nutrition metabolism. Nutrients 13:2592. doi: 10.3390/nu13082592

Te Pas, M. F. W., A. J. M. Jansman, L. Kruijt, Y. van der Meer, J. J. M. Vervoort, and D. Schokker. 2020. Sanitary conditions affect the colonic microbiome and the colonic and systemic metabolome of female pigs. Front Vet. Sci. 7:585730. doi: 10.3389/fvets.2020.585730

Tian, Q. Y., Z. K. Zeng, Y. X. Zhang, S. F. Long, and X. S. Piao. 2016. Effect of L- or DL-methionine Supplementation on Nitrogen Retention, Serum Amino Acid Concentrations

and Blood Metabolites Profile in Starter Pigs. Asian-Australas. J. Anim. Sci. 29:689-694. doi: 10.5713/ajas.15.0730

Tran, V. V., D. Park, and Y.-C. Lee. 2020. Indoor Air Pollution, Related Human Diseases, and Recent Trends in the Control and Improvement of Indoor Air Quality. Int. J. Environ. Res. Public Health 17:2927-2954. doi: 10.3390/ijerph17082927

Trevisi, P., D. Melchior, M. Mazzoni, L. Casini, S. De Filippi, L. Minieri, G. Lalatta-Costerbosa, and P. Bosi. 2009. A tryptophan-enriched diet improves feed intake and growth performance of susceptible weanling pigs orally challenged with Escherichia coli K881. J. Anim. Sci. 87:148-156. doi: 10.2527/jas.2007-0732

Tripathi, P., P. Tripathi, L. Kashyap, and V. Singh. 2007. The role of nitric oxide in inflammatory reactions. FEMS Immunol. Med. Microbiol. 51:443-452. doi: 10.1111/j.1574-695X.2007.00329.x

van de Poll, M. C., P. B. Soeters, N. E. Deutz, K. C. Fearon, and C. H. Dejong. 2004. Renal metabolism of amino acids: its role in interorgan amino acid exchange. Am. J. Clin. Nutr. 79:185-197. doi: 10.1093/ajcn/79.2.185

van der Meer, Y., A. J. M. Jansman, and W. J. J. Gerrits. 2020. Low sanitary conditions increase energy expenditure for maintenance and decrease incremental protein efficiency in growing pigs. Animal 14:1811-1820. doi: 10.1017/S1751731120000403

van der Meer, Y., A. Lammers, A. J. M. Jansman, M. M. J. A. Rijnen, W. H. Hendriks, and W. J. J. Gerrits. 2016. Performance of pigs kept under different sanitary conditions affected by protein intake and amino acid supplementation. J. Anim. Sci. 94:4704-4719. doi: 10.2527/jas.2016-0787

van Milgen, J. 2002. Modeling biochemical aspects of energy metabolism in mammals. J. Nutr. 132:3195-3202. doi: 10.1093/jn/131.10.3195

van Milgen, J., and J. Y. Dourmad. 2015. Concept and application of ideal protein for pigs. J. Anim. Sci. Biotechnol. 6:15. doi: 10.1186/s40104-015-0016-1

van Milgen, J., A. Valancogne, S. Dubois, J.-Y. Dourmad, B. Sève, and J. Noblet. 2008. InraPorc: A model and decision support tool for the nutrition of growing pigs. Anim. Feed Sci. Technol. 143:387-405. doi: 10.1016/j.anifeedsci.2007.05.020

Vereecke, L., R. Beyaert, and G. van Loo. 2011. Enterocyte death and intestinal barrier maintenance in homeostasis and disease. Trends Mol. Med. 17:584-593. doi: 10.1016/j.molmed.2011.05.011

Vidovic, N., and S. Vidovic. 2020. Antimicrobial resistance and food animals: Influence of livestock environment on the emergence and dissemination of antimicrobial resistance. Antibiotics 9:52. doi: 10.3390/antibiotics9020052

Vince, A., A. M. Dawson, N. Park, and F. O'Grady. 1973. Ammonia production by intestinal bacteria. Gut 14:171-177. doi: 10.1136/gut.14.3.171

Waititu, S. M., F. Yin, R. Patterson, J. C. Rodriguez-Lecompte, and C. M. Nyachoti. 2016. Short-term effect of supplemental yeast extract without or with feed enzymes on growth performance, immune status and gut structure of weaned pigs challenged with *Escherichia coli* lipopolysaccharide. J. Anim. Sci. Biotechnol. 7:64. doi: 10.1186/s40104-016-0125-5

Waititu, S. M., F. Yin, R. Patterson, A. Yitbarek, J. C. Rodriguez-Lecompte, and C. M. Nyachoti. 2017. Dietary supplementation with a nucleotide-rich yeast extract modulates gut immune response and microflora in weaned pigs in response to a sanitary challenge. Animal 11:2156-2164. doi: 10.1017/S1751731117001276

Wang, H., C. Zhang, G. Wu, Y. Sun, B. Wang, B. He, Z. Dai, and Z. Wu. 2015a. Glutamine enhances tight junction protein expression and modulates corticotropin-releasing factor signaling in the jejunum of weanling piglets. J. Nutr. 145:25-31. doi: 10.3945/jn.114.202515

Wang, J., L. Chen, P. Li, X. Li, H. Zhou, F. Wang, D. Li, Y. Yin, and G. Wu. 2008. Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. J. Nutr. 138:1025-1032. doi: 10.1093/jn/138.6.1025

Wang, L., Y. Hou, D. Yi, Y. Li, B. Ding, H. Zhu, J. Liu, H. Xiao, and G. Wu. 2015b. Dietary supplementation with glutamate precursor alpha-ketoglutarate attenuates lipopolysaccharide-induced liver injury in young pigs. Amino Acids 47:1309-1318. doi: 10.1007/s00726-015-1966-5

Wang, Q., Y. Hou, D. Yi, L. Wang, B. Ding, X. Chen, M. Long, Y. Liu, and G. Wu. 2013. Protective effects of N-acetylcysteine on acetic acid-induced colitis in a porcine model. BMC Gastroenterol 13:133. doi: 10.1186/1471-230x-13-133

Wang, W. W., S. Y. Qiao, and D. F. Li. 2009. Amino acids and gut function. Amino Acids 37:105-110. doi: 10.1007/s00726-008-0152-4

Wang, X., and C. G. Proud. 2006. The mTOR pathway in the control of protein synthesis. Physiology 21:362-369. doi: 10.1152/physiol.00024.2006

Wang, Y., L. Su, M. D. Morin, B. T. Jones, L. R. Whitby, M. M. R. P. Surakattula, H. Huang, H. Shi, J. H. Choi, K.-w. Wang, E. M. Y. Moresco, M. Berger, X. Zhan, H. Zhang, D. L. Boger, and B. Beutler. 2016. TLR4/MD-2 activation by a synthetic agonist with no similarity to LPS. PNAS 113:E884-E893. doi: 10.1073/pnas.1525639113

Wassenaar, T. M., and K. Zimmermann. 2018. Lipopolysaccharides in food, food supplements, and probiotics: Should we be worried? Eur. J. Microbiol. Immunol. 8:63-69. doi: 10.1556/1886.2018.00017

Webel, D. M., B. N. Finck, D. H. Baker, and R. W. Johnson. 1997. Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. J. Anim. Sci. 75:1514-1520. doi: 10.2527/1997.7561514x

Wehkamp, J., M. Schmid, and E. F. Stange. 2007. Defensins and other antimicrobial peptides in inflammatory bowel disease. Curr. Opin. Gastroenterol. 23:370-378. doi: 10.1097/MOG.0b013e328136c580

Wellington, M. O., A. K. Agyekum, K. Hamonic, J. K. Htoo, A. G. Van Kessel, and D. A. Columbus. 2019. Effect of supplemental threonine above requirement on growth performance of Salmonella typhimurium challenged pigs fed high-fiber diets. J. Anim. Sci. 97:3636-3647. doi: 10.1093/jas/skz225

Wellington, M. O., J. K. Htoo, A. G. Van Kessel, and D. A. Columbus. 2018. Impact of dietary fiber and immune system stimulation on threonine requirement for protein deposition in growing pigs. J. Anim. Sci. 96:5222-5232. doi: 10.1093/jas/sky381

Whitfield, J. B. 2001. Gamma Glutamyl Transferase. Crit. Rev. Clin. Lab. Sci. 38:263-355. doi: 10.1080/20014091084227

Wijtten, P. J. A., J. v. d. Meulen, and M. W. A. Verstegen. 2011. Intestinal barrier function and absorption in pigs after weaning: a review. Br. J. Nutr. 105:967-981. doi: 10.1017/S0007114510005660

Williams, B. A., M. W. A. Verstegen, and S. Tamminga. 2001. Fermentation in the large intestine of single-stomached animals and its relationship to animal health. Nutr. Res. Rev. 14:207-228. doi: 10.1079/NRR200127

Wolkoff, P. 2018. Indoor air humidity, air quality, and health – An overview. Int. J. Hyg. Environ. Health 221:376-390. doi: 10.1016/j.ijheh.2018.01.015

Wolter, B. F., M. Ellis, B. P. Corrigan, J. M. DeDecker, S. E. Curtis, E. N. Parr, and D. M. Webel. 2003. Impact of early postweaning growth rate as affected by diet complexity and space allocation on subsequent growth performance of pigsin a wean-to-finish production system. J. Anim. Sci. 81:353-359. doi: 10.2527/2003.812353x

Woyengo, T. A., and C. M. Nyachoti. 2013. Review: Anti-nutritional effects of phytic acid in diets for pigs and poultry - current knowledge and directions for future research. Can. J. Anim. Sci. 93:9-21. doi: 10.1139/CJAS2012-017

Wu, G. 2009. Amino acids: metabolism, functions, and nutrition. Amino Acids 37:1-17. doi: 10.1007/s00726-009-0269-0

Wu, G. 2010. Functional amino acids in growth, reproduction, and health. Adv. Nutr. 1:31-37. doi: 10.3945/an.110.1008

Wu, G. 2014. Dietary requirements of synthesizable amino acids by animals: a paradigm shift in protein nutrition. J. Anim. Sci. Biotechnol. 5:34. doi: 10.1186/2049-1891-5-34

Wu, G. 2018. Principles of Animal Nutrition. CRC Press. Boca Raton, FL.

Wu, G., F. W. Bazer, G. A. Johnson, and Y. Hou. 2018. Board-invited review: Arginine nutrition and metabolism in growing, gestating, and lactating swine. J. Anim. Sci. 96:5035-5051. doi: 10.1093/jas/sky377

Wu, G., Y.-Z. Fang, S. Yang, J. R. Lupton, and N. D. Turner. 2004. Glutathione metabolism and its implications for health. J. Nutr. 134:489-492. doi: 10.1093/jn/134.3.489

Wu, G., S. A. Meier, and D. A. Knabe. 1996. Dietary glutamine supplementation prevents jejunal atrophy in weaned pigs. J. Nutr. 126:2578-2584. doi: 10.1093/jn/126.10.2578

Wu, G., Z. Wu, Z. Dai, Y. Yang, W. Wang, C. Liu, B. Wang, J. Wang, and Y. Yin. 2013. Dietary requirements of "nutritionally non-essential amino acids" by animals and humans. Amino Acids 44:1107-1113. doi: 10.1007/s00726-012-1444-2

Wu, J. J., C. M. Cao, D. D. Ren, Y. Zhang, Y. N. Kou, L. Y. Ma, S. B. Feng, Y. Li, and X. C. Wang. 2016. Effects of soybean antigen proteins on intestinal permeability, 5-hydroxytryptamine levels and secretory IgA distribution in the intestine of weaned piglets. Ital. J. Anim. Sci. 15:174-180. doi: 10.1080/1828051x.2016.1148559

Wu, L., P. Liao, L. He, Z. Feng, W. Ren, J. Yin, J. Duan, T. Li, and Y. Yin. 2015. Dietary L-arginine supplementation protects weanling pigs from deoxynivalenol-induced toxicity. Toxins 7:1341-1354.

Xiao, Y. P., X. Y. Li, T. X. Wu, L. Yang, Q. H. Hong, C. M. Yang, and A. G. Chen. 2012a. Effects of dietary glutamine supplementation on nutrient absorption and activity of enzymes involved in glutamine metabolism and energy production in the jejunum of weaned piglets. J. Anim. Vet. Adv. 11:1441-1449.

Xiao, Y. P., T. X. Wu, J. M. Sun, L. Yang, Q. H. Hong, A. G. Chen, and C. M. Yang. 2012b. Response to dietary L-glutamine supplementation in weaned piglets: A serum metabolomic comparison and hepatic metabolic regulation analysis. J. Anim. Sci. 90:4421-4430. doi: 10.2527/jas.2012-5039

Xing, S., B.-L. Zhang, M. Lin, J. Li, L. Zhang, F. Gao, and G. Zhou. 2017. Effects of alanyl-glutamine supplementation on the small intestinal mucosa barrier in weaned piglets challenged with lipopolysaccharide. Can. J. Anim. Sci. doi: 10.1139/cjas-2016-0039

Xu, B., Y. Yan, B. Yin, L. Zhang, W. Qin, Y. Niu, Y. Tang, S. Zhou, X. Yan, and L. Ma. 2021. Dietary glycyl-glutamine supplementation ameliorates intestinal integrity, inflammatory

response, and oxidative status in association with the gut microbiota in LPS-challenged piglets. Food Funct. 12:3539-3551. doi: 10.1039/D0FO03080E

Xu, Y. T., X. K. Ma, C. L. Wang, M. F. Yuan, and X. S. Piao. 2018. Effects of dietary valine:lysine ratio on the performance, amino acid composition of tissues and mRNA expression of genes involved in branched-chain amino acid metabolism of weaned piglets. Asian-Australas. J. Anim. Sci. 31:106-115. doi: 10.5713/ajas.17.0148

Yan, Y., B. Xu, B. Yin, X. Xu, Y. Niu, Y. Tang, X. Wang, C. Xie, T. Yang, S. Zhou, X. Yan, and L. Ma. 2020. Modulation of gut microbial community and metabolism by dietary glycyl-glutamine supplementation may favor weaning transition in piglets. Front. Microbiol. 10:1-14. doi: 10.3389/fmicb.2019.03125

Yang, R., X. Han, T. Uchiyama, S. K. Watkins, A. Yaguchi, R. L. Delude, and M. P. Fink. 2003. IL-6 is essential for development of gut barrier dysfunction after hemorrhagic shock and resuscitation in mice. Am. J. Physiol. Gastrointest. Liver Physiol. 285:G621-G629. doi: 10.1152/ajpgi.00177.2003

Yang, Z., M. S. Hasan, J. K. Htoo, D. D. Burnett, J. M. Feugang, M. A. Crenshaw, and S. F. Liao. 2019. Effects of dietary supplementation of L-methionine vs. DL-methionine on performance, plasma concentrations of free amino acids and other metabolites, and myogenesis gene expression in young growing pigs. Transl. Anim. Sci. 3:329-339. doi: 10.1093/tas/txy109

Yao, Z., J. M. Mates, A. M. Cheplowitz, L. P. Hammer, A. Maiseyeu, G. S. Phillips, M. D. Wewers, M. V. S. Rajaram, J. M. Robinson, C. L. Anderson, and L. P. Ganesan. 2016. Blood-borne lipopolysaccharide is rapidly eliminated by liver sinusoidal endothelial cells via high-density lipoprotein. J. Immunol. Res. 197:2390-2399. doi: 10.4049/jimmunol.1600702

Yi, D., Y. Hou, H. Xiao, L. Wang, Y. Zhang, H. Chen, T. Wu, B. Ding, C. A. Hu, and G. Wu. 2017. N-Acetylcysteine improves intestinal function in lipopolysaccharides-challenged piglets through multiple signaling pathways. Amino Acids 49:1915-1929. doi: 10.1007/s00726-017-2389-2

Yi, D., B. Li, Y. Hou, L. Wang, D. Zhao, H. Chen, T. Wu, Y. Zhou, B. Ding, and G. Wu. 2018. Dietary supplementation with an amino acid blend enhances intestinal function in piglets. Amino Acids 50:1089-1100. doi: 10.1007/s00726-018-2586-7

Yin, J., J. Ma, Y. Li, X. Ma, J. Chen, H. Zhang, X. Wu, F. Li, Z. Liu, T. Li, and Y. Yin. 2020. Branched-chain amino acids, especially of leucine and valine, mediate the protein restricted response in a piglet model. Food Funct. 11:1304-1311. doi: 10.1039/c9fo01757g

Yin, J., W. Ren, G. Yang, J. Duan, X. Huang, R. Fang, C. Li, T. Li, Y. Yin, Y. Hou, S. W. Kim, and G. Wu. 2016. L-Cysteine metabolism and its nutritional implications. Mol. Nutr. Food Res. 60:134-146. doi: 10.1002/mnfr.201500031

Yoon, S. Y., S. J. Sa, E. S. Cho, H. S. Ko, J. W. Choi, and J. S. Kim. 2020. Effects of zinc oxide and arginine on the intestinal microbiota and immune status of weaned pigs subjected to high ambient temperature. Animals 10:1537. doi: 10.3390/ani10091537

Yoshimura, A., Y. Hara, T. Kaneko, and I. Kato. 1997. Secretion of IL-1β, TNF-α, IL-8 and IL-1ra by human polymorphonuclear leukocytes in response to lipopolysaccharides from periodontopathic bacteria. J. Periodontal Res. 32:279-286. doi: 10.1111/j.1600-0765.1997.tb00535.x

Yu, L., T. Liu, S. Fu, L. Li, X. Meng, X. Su, Z. Xie, J. Ren, Y. Meng, X. Lv, and Y. Du. 2019. Physiological functions of urea transporter B. Pflügers Arch. 471:1359-1368. doi: 10.1007/s00424-019-02323-x

Zervas, S., and R. T. Zijlstra. 2002a. Effects of dietary protein and fermentable fiber on nitrogen excretion patterns and plasma urea in grower pigs. J. Anim. Sci. 80:3247-3256. doi: 10.2527/2002.80123247x

Zervas, S., and R. T. Zijlstra. 2002b. Effects of dietary protein and oathull fiber on nitrogen excretion patterns and postprandial plasma urea profiles in grower pigs. J. Anim. Sci. 80:3238-3246. doi: 10.2527/2002.80123238x

Zhan, Z., D. Ou, X. Piao, S. W. Kim, Y. Liu, and J. Wang. 2008. Dietary Arginine Supplementation Affects Microvascular Development in the Small Intestine of Early-Weaned Pigs. J. Nutr. 138:1304-1309. doi: 10.1093/jn/138.7.1304

Zhang, G., C. R. Ross, and F. Blecha. 2000. Porcine antimicrobial peptides: new prospects for ancient molecules of host defense. Vet. Res 31:277-296. doi: 10.1051/vetres:2000121

Zhang, H., Y. Chen, Y. Li, T. Zhang, Z. Ying, W. Su, L. Zhang, and T. Wang. 2019. L-threonine improves intestinal mucin synthesis and immune function of intrauterine growth-retarded weanling piglets. Nutrition 59:182-187. doi: 10.1016/j.nut.2018.07.114

Zhang, S., E. R. Gilbert, K. J. T. Noonan, B. Saremi, and E. A. Wong. 2018. Gene expression and activity of methionine converting enzymes in broiler chickens fed methionine isomers or precursors. Poult. Sci. 97:2053-2063. doi: 10.3382/ps/pey037

Zhang, S., S. Qiao, M. Ren, X. Zeng, X. Ma, Z. Wu, P. Thacker, and G. Wu. 2013. Supplementation with branched-chain amino acids to a low-protein diet regulates intestinal expression of amino acid and peptide transporters in weanling pigs. Amino Acids 45:1191-1205. doi: 10.1007/s00726-013-1577-y

Zhang, S., M. Ren, X. Zeng, P. He, X. Ma, and S. Qiao. 2014. Leucine stimulates ASCT2 amino acid transporter expression in porcine jejunal epithelial cell line (IPEC-J2) through PI3K/Akt/mTOR and ERK signaling pathways. Amino Acids 46:2633-2642. doi: 10.1007/s00726-014-1809-9

Zhang, S., X. Zeng, M. Ren, X. Mao, and S. Qiao. 2017. Novel metabolic and physiological functions of branched chain amino acids: a review. J. Anim. Sci. Biotechnol. 8:10. doi: 10.1186/s40104-016-0139-z

Zhao, J., A. F. Harper, M. J. Estienne, K. E. Webb, A. P. McElroy, and D. M. Denbow. 2007. Growth performance and intestinal morphology responses in early weaned pigs to supplementation of antibiotic-free diets with an organic copper complex and spray-dried plasma protein in sanitary and nonsanitary environments. J. Anim. Sci. 85:1302-1310. doi: 10.2527/jas.2006-434

Zhao, Y., Q. Jiang, X. Q. Zhou, S. X. Xu, L. Feng, Y. Liu, W. D. Jiang, P. Wu, J. Zhao, and J. Jiang. 2020. Effect of dietary threonine on growth performance and muscle growth, protein synthesis and antioxidant-related signalling pathways of hybrid catfish Pelteobagrus vachellifemale symbol x Leiocassis longirostrismale symbol. Br. J. Nutr. 123:121-134. doi: 10.1017/S0007114519002599

Zhao, Y., G. Qin, R. Han, J. Wang, X. Zhang, and D. Liu. 2014. beta-Conglycinin reduces the tight junction occludin and ZO-1 expression in IPEC-J2. Int. J. Mol. Sci. 15:1915-1926. doi: 10.3390/ijms15021915

Zheng, L., H. Wei, C. Cheng, Q. Xiang, J. Pang, and J. Peng. 2016. Supplementation of branched-chain amino acids to a reduced-protein diet improves growth performance in piglets: involvement of increased feed intake and direct muscle growth-promoting effect. Br. J. Nutr. 115:2236-2245. doi: 10.1017/S0007114516000842

Zheng, P., B. Yu, J. He, G. Tian, Y. Luo, X. Mao, K. Zhang, L. Che, and D. Chen. 2013. Protective effects of dietary arginine supplementation against oxidative stress in weaned piglets. Br. J. Nutr. 109:2253-2260. doi: 10.1017/S0007114512004321

Zheng, P., B. Yu, J. He, J. Yu, X. Mao, Y. Luo, J. Luo, Z. Huang, G. Tian, Q. Zeng, L. Che, and D. Chen. 2017. Arginine metabolism and its protective effects on intestinal health and functions in weaned piglets under oxidative stress induced by diquat. Br. J. Nutr. 117:1495-1502. doi: 10.1017/S0007114517001519

Zhong, X., W. Li, X. Huang, Y. Wang, L. Zhang, Y. Zhou, A. Hussain, and T. Wang. 2012. Effects of glutamine supplementation on the immune status in weaning piglets with intrauterine growth retardation. Arch. Anim. Nutr. 66:347-356. doi: 10.1080/1745039X.2012.683325

Zhu, H. L., Y. L. Liu, X. L. Xie, J. J. Huang, and Y. Q. Hou. 2013. Effect of L-arginine on intestinal mucosal immune barrier function in weaned pigs after Escherichia coli LPS challenge. Innate Immun. 19:242-252. doi: 10.1177/1753425912456223

Zhu, J. W., J. F. Yuan, H. M. Yang, S. T. Wang, C. G. Zhang, L. L. Sun, H. Yang, and H. Zhang. 2012. Extracellular cysteine (Cys)/cystine (CySS) redox regulates metabotropic glutamate receptor 5 activity. Biochimie 94:617-627. doi: 10.1016/j.biochi.2011.09.013