Assessment of standardized ileal digestible lysine and sulfur amino acids to lysine ratio for weaned piglets fed antibiotic-free diets

by

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ABSTRACT

Amino acids (AA) are required for protein accretion and the need for a specific AA depends on the physiological status, breed, and the health of the pig. Inasmuch as the AA requirements for growing pigs are given in an ideal AA ratio for protein accretion, the utilization of all AA is beyond growth and at least 30% of the total dietary AA will be used by the splanchnic tissue. A ban in the use of antimicrobial growth promoters (AGP) in piglets' diets is likely to increase incidences of disease occurrence and exert additional AA requirements. Immune challenge models were used to determine standardized ileal digestible (SID) Lys and sulfur amino acids (SAA):Lys requirements for piglets under an antibiotic-free feeding regime. The first objective was to establish the dietary Lys requirement for piglets raised under both clean and unclean sanitary conditions. The Lys requirement could not be determined in the first experiment. However, from the second and third experiments the dietary SID Lys content for optimal growth of 7 to 16 kg weaned piglets was estimated to be 1.32%. The objective of fourth experiment was to determine the optimum SID SAA:Lys ratio in piglets when reared under clean or unclean conditions. Based on performance parameters, the optimum SAA:Lys ratios were 58 and 61 for piglets raised under clean and unclean conditions, respectively. However, VH estimates were 60 and 66 SAA:Lys under clean and unclean sanitary conditions, respectively. The objective for the fifth experiment was to determine SID SAA:Lys ratio of piglets under an enterotoxigenic Escherichia coli challenge using genes for expression of key products in the Met metabolic pathway. Gene expressions of methionine adenosyltransferase 1 and 2-α, 5-methyltetrahydrofolate-homocysteine methyltransferase, and cystathionine γ-lyase was done for liver and ileal tissue. The gene expressions indicates that the dietary SAA:Lys ratio of 60 was enough to support piglet's immune response and performance during an immune challenge.

Therefore, under an antibiotic-free feeding regime, the Lys requirement recommended by NRC (2012) is sufficient, however, the SAA:Lys should be raised to 60 in diets of both healthy and immune challenged piglets.

DEDICATION

To my parents, William and Hilda Kahindi

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FOREWORD

This thesis was written in a manuscript format and it is composed of four manuscripts. All manuscripts were written according to the Journal of Animal Science format. The first manuscript has been published in Canadian Journal of Animal Science. The second, third, and fourth manuscripts are to be submitted to Journal of Animal Physiology and Nutrition, Animal, and British Journal of Nutrition, respectively.

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LIST OF ABBREVIATIONS

AA	Amino acids
ACTH	Adenocorticotropic hormone
ADFI	Average daily feed intake
ADG	Average daily gain
AGP	Antibiotic growth promoters
ANS	Anthranillic acid
APP	Acute phase protein
BW	Body weight
CCAC	Canadian council of animal care
CD	Crypt depth
CP	Crude protein
CRH	Corticotropic hormone
CSC	Clean sanitation condition
CT	Cycle number at an amplification threshold
CTH	Cystathionine γ-lyase
DCSAM	Decarboxylated S-adenosylmethionine
DLC	Dietary lysine content
DM	Dry matter
DE	Digestible energy
DNA	Deoxyribonucleic acid
EAA	Essential amino acids

ENL	Endogenous nutrient losses
EPN	Epinephrine
ETEC	Enterotoxigenic *Escherichia coli*
GABA	Gamma aminobutyrate
G:F	Gain to feed ratio
GIT	Gastrointestinal tract
GSC	Glutamate cysteine synthase
GSH	Glutathione
HMB	β-Hydroxyl-β-methylbutyrate
IFN-γ	Interferon gamma
IAAO	Indicator amino acid oxidation
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
Hcy	Homocysteine
ME	Metabolizable energy
MAT 1 & 2	Methionine adenosyltransferase 1 & 2
MTR	Methyltetrahydrofolate-homocysteine methyltransferase
mTOR	Mammalian target of rapamycin
mRNA	Messenger ribonucleic acid
NAS	N-acetylserotonin
NE	Net energy
NEAA	Non-essential amino acids

NEPN	Norepinephrine
NFkB	Nuclear factor kappa-B
NR	Nitrogen retention
NRC	National research council
P5C	Pyrroline-5-carboxylate
PUN	Plasma urea nitrogen
PWD	Post weaning diarrhea
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
SAA	Sulfur amino acids
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SID	Standardized ileal digestible
USC	Unclean sanitation condition
VH	Villus height
ΔCT	Normalized CT value
ΔΔCT	Comparative CT value

CHAPTER ONE

GENERAL INTRODUCTION

Amino acids are organic compounds that have amine and carboxylic group. Twenty amino acids (**AA**) are required to synthesize a protein and of these 10 AA are considered essential (**EAA**) and 10 are non-essential AA (**NEAA**). The EAA cannot be synthesized by the body and these are: lysine, methionine, threonine, tryptophan, phenyalanine, isoleucine, leucine, histidine, and valine (Reeds, 2000). The NEAA are: alanine, aspartate, cysteine, glutamate, glutamine glycine, proline, serine, tyrosine, and asparagine (Reeds, 2000). Although the NEAA can be synthesized by the body there are situation where their metabolic demand is greater than the synthesis rate, in such cases they will be termed as conditional EAA. The conditional AA are: glutamatine, cycteine, proline, arginine and glycine. Of the EAA, lysine (**Lys**) and methionine (**Met**) are the first and second limiting AA, respectively, in cereal-based diets fed to pigs. Lysine is a ketogenic AA and its metabolism gives rise to acetyl-CoA. The Lys acetyl residue is the location of acetylation and deacetylation reactions in a protein and these reactions are important in regulating cellular transcription factors and recruitment of effector proteins (Choudhary et al., 2009). Methionine contains a methyl group that makes it hydrophobic and sulfur that is readily oxidized by reactive oxygen species in the body. The genetic codon sequence for Met also forms the signal for protein synthesis initiation, making it a unique AA necessary for both protein structure synthesis and translation initiation.

The amino acid requirements are expressed in amounts and ratios appropriate for maintenance, muscle protein or milk synthesis (NRC, 2012). The growing pigs' EAA recommendation are given in an ideal AA ratio for protein accretion (Fuller et al., 1989; Chung and Baker, 1992; NRC, 1998) where the Lys content is expressed as 100 and the EAA as a ratio

to Lys. However, not all ingested AA are digested and utilized by the pigs, therefore supplying AA on standardized ileal digestible (**SID**) basis is considered as the closest estimate of the AA requirement. The SID coefficient is calculated by subtracting the amount of AA lost that is of endogenous origin from the difference between total AA intake and ileal output (Stein et al. 2007). In pigs, the digestibility of an AA in an ingredient is calculated from digesta at the terminal ileum rather than feces, because of the great contribution to protein synthesis by microorganisms in the large intestine.

The ideal AA ratio and AA recommendations given in NRC (2012) are made for healthy pigs and do not take into account any changes like environmental stressors or immune system stimulation that would occur during the pig's life. One of the changes in pig production is the ban in the use of sub-therapeutic levels of antibiotics as antimicrobial growth promoter (**AGP**). The ban in the use of AGP in livestock started from Sweden in 1986 and was adopted by the European Union since January 2006 (Directive 1831/2003/CEE, European Commission 2003). Health Canada Veterinary Drug Directorate (2014) has recommended use of AGP based on risk analysis principles. Thus there is a regulatory guide on the antibiotic acceptable as growth promoters in animals' diets. The antibiotics included in a diets are different from those used for treatment of human diseases. However, there is an ongoing debate on whether to completely eradicate AGP from animal's diets due to great concerns on the effects of AGP on human health. It has been noted that incidences of diseases like the post weaning diarrhea (**PWD**) has been on the rise since the AGP ban (Callesen, 2002; Dibner and Richards, 2005; Vigre et al., 2008). The PWD has been implicated with decreased performance (Pluske et al., 1997) that has a negative effect on time to market, consequently production cost. A potentially important means to minimize the negative impact of immune system stimulation, due to disease, is the fortification

of piglets' diets with nutrients at levels exceeding recommendations that have been established in pigs fed AGP.

Lysine is required mainly for protein accretion and a small portion is directed towards obligatory oxidation (Klasing, 2009). In the event of immune system activation loss of appetite and muscle catabolism are prominent. Studies using different immune stressors like *Escherichia coli* LPS, environmental stressors, and circovirus did not increase Lys requirement in pigs (Lindermann et al. 1993; Williams et al. 1997; Shelton et al. 2011). Addition of Lys in pigs' diets was not able to neutralize the depressing effect of immune challenge on growth. Since the efficiency of Lys utilization does not change with the immune system activation, the Lys requirement therefore follows suit. However, AGP-free feeding resulted in a 6% increase in Lys requirement of growing pigs (Bikker et al., 2006). Thus, it was possible that some of the dietary Lys is utilized by gut microbes whose population is reduced by use of AGP.

The dietary SAA support the innate immune system through the action of Cys that is necessary for, synthesis of mucin, acute phase proteins (**APP**), glutathione (**GSH**) and Tau. The mucin serves as a protective barrier to modulate the intestinal function and health (Bauchart-Thevret et al., 2009). Rats with damaged colon mucosa experienced a restoration after being supplemented with a combination of AA that included Cys (Faure et al., 2006). Glutathione is an antioxidant and plays an important role in immunomodulation by inhibiting prostaglandin production thereby having anti-inflammatory effects (Nimni et al., 2007). Additionally, nuclear factor kappa-B (**NFkB**), a transcription gene for pro-inflammatory cytokines is activated by oxidants and its activity is decreased by high levels of glutathione and other sulfur containing compounds (Staal et al., 1990).

Pigs fed graded SAA content with 7 days continuous injection with LPS had reduced urinary sulfur excretion, indicating that sulfur-containing AA were preferentially conserved or repartitioned (Rakhshandeh et al., 2010) thus suggesting a higher requirement during immune activation. The SAA requirement expressed as SAA:Lys ratio and based on protein deposition and plasma urea nitrogen, was increased from 0.55 in healthy to 0.75 in LPS injected grower pigs (Kim et al., 2012). It was also noted in the same study that increasing SAA:Lys ratio increased protein deposition in g/day in the LPS injected pigs. Therefore, the SAA requirement recommended for growth of healthy pigs would not be enough during immune system activation. Even though it is clear that increasing dietary SAA is beneficial during immune system activation, the amount of SAA that should be supplemented is not yet known. It is also possible that different type of immune system activation would vary the requirement level of supplementation.

Researchers are also challenged on the ideal response criteria for determining AA requirement in piglets during periods of environmental stress and immune system stimulation. The current recommendations for AA requirement in piglets are based on performance parameters. However, for SAA 30% of the total dietary Met is utilized for growth and 30% is used by the splanchnic tissue, as a source of energy, for synthesis of mucoproteins and mucin, antioxidant and maintenance of redox potential (Stipanuk, 2004; Stoll and Burrin, 2006). It is therefore necessary to include non-performance response criteria that consume a lot of the SAA when determining its requirement. These non-performance response criteria would be of importance in times of exposure to environmental stressors or change of feeding regimen for example, a ban in the use of in-feed antibiotics.

It was hypothesized that AA requirement under AGP-free regime would be higher. Also, the requirements for the AA involved in the immune system maintenance are higher when piglets are subjected to an immunological challenge.

CHAPTER TWO

LITERATURE REVIEW

2.1 Amino acids

Amino acids are not only important for protein synthesis, they are also substrates for synthesis of substances like, nucleic acids, neurotransmitters, and hormones and they are an energy source. As stated earlier, the AA are grouped into essential (**EAA**) and non-essential AA (**NEAA)** based on whether or not they can be synthesized by the body. Although the NEAA can be synthesized by the body there are situation where their metabolic demand is greater than the synthesis rate, in such cases they will be termed as conditional EAA as given in Table 2.1. For example, in suckling pigs Glu is conditionally EAA and supplementation of Glu to weaned piglets improved feed efficiency and prevented jejunal atrophy (Wu et al., 1996). The factors leading to an AA becoming conditional essential include, age or maturity of the animal, illness, and stress (Reeds, 2000). The AA are also categorized into 6 groups according to their chemical structure as influences by their side chains.

1. Aliphatic AA: Gly, Ala, Val, Leu, and Ile.
2. Hydroxylic and sulfur AA: Ser, Thr, Met, and Cys.
3. Aromatic: Phe, Tyr, Trp
4. Cyclic: Pro
5. Acidic and amide group: Asp, Glu, Asn, and Gln
6. Basic: Arg, Lys, His

The side chains also known as R-group are important in determining their polarity and interaction between AA.

Table 2.1 Amino acids classification in pigs; essential, non-essential and conditional essential

Essential amino acids	Non-essential amino acid	Conditional essential amino acid
Lysine	Alanine	Arginine
Methionine	Aspartate	Cysteine
Threonine	Asparagine	Glutamine
Tryptophan	Glutamate	Proline
Phenylalanine	Glycine	Tyrosine
Isoleucine	Serine	
Histidine		
Valine		

Since the provision of EAA in the diet is mandatory, they should be supplied as per body's requirement to avoid excesses or deficiencies. Hence recommendations on AA requirements for pigs of various age and production stages are given in NRC (2012).

Currently, AA requirements for maintenance or production are expressed in an ideal protein ratio, which is the AA profile for a specific function such as body maintenance, growth, milk production or gestation. Lysine is usually the reference AA because it is the first limiting AA and most of it is used for protein accretion. Under the ideal protein concept it is assumed that even though requirement of individual amino acids may vary at different ages, the ratio between essential and non-essential AA remains constant (Wang and Fuller, 1989). In pigs, the ideal protein ratio was considered as the minimum AA balance that would result in the highest N retention. The ideal protein concept was adopted to reduce excess dietary supply of AA and N excretion that causes pollution. Excess supply of an AA can cause antagonism for example with the branched-chain AA (**BCAA**), excess dietary Leu depressed feed intake and growth of pigs due to antagonism the other BCAA (Wiltafsky et al., 2010). The antagonism was a result of competition for the absorption sites in the gut and renal reabsorption. The ideal protein ratio developed by different researchers is close but not similar for all AA as shown in Table 2.2. The

differences are mainly attributed to breed and age of pigs used in determining the ideal protein ratio. Besides the ideal protein ratio AA requirement are given in mg per day or as percentage of feed.

Table 2.2 Ideal protein ratios for maintenance, milk synthesis and protein accretion

		NRC, 1998		NRC, 2012	Wang and Fuller, 1989	Chung and Baker, 1992
Amino acid	Maintenance	Milk synthesis	Protein accretion	Protein accretion	Protein accretion	Protein accretion
Lys	100	100	100	100	100	100
Met	28	26	27	27	-	-
Met+Cys	123	45	55	55	63	60
Thr	151	58	60	59	72	65
Trp	26	18	18	16	18	18
Ile	75	55	54	51	60	60
Leu	70	112	102	100	110	100
Val	67	85	68	64	75	68
Phe+Tyr	121	112	93	93	120	95
His	32	40	32	34	-	32
Arg	-200	66	48	45	-	42

2.2 Factors leading to changes in AA requirements of piglets

The AA requirement differs based on age, sex, species, other nutrients, environment and health status (Wang et al., 1998; Lumpkins et al., 2007) as briefly described below.

2.2.1 Dietary factors

The utilization of AA for a production function is dependent on other nutrients like vitamins, minerals and energy. A diet with sufficient energy content from carbohydrates and fat will reduce AA utilization for provision of total body energy, allowing for most of the AA to be used for protein synthesis. The animals are able to adjust their feed intake in order to meet their daily energy requirement, in cases energy deficiency the pigs will tend to eat more to compensate

for the shortage (Morris, 2004). The ADFI linearly decreased in growing pigs fed diets containing 8.1 to 11.1 MJ NE/kg (Quiniou and Noblet, 2012) and even though the NE intake was linearly increasing, it was not similar across diets. A growing pig needs energy for maintenance and protein accretion, the reduced dietary energy content would result in less protein accretion, hence increased AA degradation and requirement for an AA. For example, the Lys requirement was 0.61 and 0.63 SID Lys/MJ DE for diets with 3.0 or 2.5 times maintenance energy, respectively, in 20 to 45 gilts (Bikker et al., 1994).

An example of AA interacting with other nutrients is of Met and choline. Choline, its metabolite betaine and Met are methyl donors hence play an important role in methylation reactions. The need of Met for methylation reactions may alter its requirement for growth. Choline as a methyl donor can spare Met from the transmethylation pathway for use in protein accretion and consequently, reduce Met requirement. However, unless supplemented in Met deficient diets, choline seems to have little Met sparing effects (Schutte et al., 1997; Richard et al., 2011).

2.2.2 Age and sex of the animal

The age of the animal will influence AA needs in that young animals with a high growth rate will require more AA for muscle, as compared to older with lower growth rates. The Lys content as a % in diets of weaned pigs (7-11 kg) is 27% higher than for 25 to 50 kg growers (NRC, 2012). The sulfur AA (SAA; Met and Cys):Lys ratio for weaned pigs is 55 and 59 for 80 kg and heavier pigs. The changes in the ratio are mainly due to differences in the utilization of the SAA, that is for protein accretion vs. maintenance because the percentage of SAA needed for maintenance requirement increases as pig age.

The whole body protein composition between animals differs between sexes and so does the AA requirement. (Warnants et al., 2008) reported that increasing dietary lysine level resulted in higher feed efficiency in gilts compared to barrows. However, although the gilts were more efficient in diet utilization they tended to grow at slower compared to barrows. Thus the lysine requirement for gilts was 20% lower than to barrows. Cromwell et al. (1993) reported increasing growth rates, feed efficiency of gain and carcass leanness in gilts with increasing dietary protein and Lys contents but these effects were less in barrows. Contrary to Warnants et al. (2008), requirements were lower (13% CP and 0.60% Lys) for barrows than (17.2% CP and 0.90% Lys) for gilts. Therefore, to attain similar growth rates the gilts would require higher concentrations of dietary amino acids than barrows.

2.2.3 Health status

A disease incidence in pigs would increase the demand for AA such as Glu, Trp, Cys, Gly, Pro in order to support the immune system. The ideal SID Trp:Lys ratio for protein accretion recommended by NRC (1998 and 2012) is 0.18 and 0.16 respectively. Li et al. (2007), observed the ideal SID Trp:Lys ratio to be elevated from 0.21 to 0.29 following exposure of piglets to *Escherichia coli* LPS injection. Capozallo et al. (2012) infected weanlings with enterotoxigenic *Escherichia coli* and reported increased feed efficiency and reduced diarrhea in piglets fed diets containing SID Trp:Lys ratio of 0.26. Additionally, SID Trp:Lys ratio of 0.22 was reported by Trevisi et al. (2009) to be the requirement of enterotoxigenic *Escherichia coli* challenged piglets. The need for more Trp has also been affiliated to reduced Trp utilization efficiency (de Ridder et al., 2012). The latter challenged piglets with *Escherichia coli* LPS, fed them graded levels of Trp and observed increased protein deposition with increasing Trp level

but there was reduced efficiency of Trp for protein utilization. However, for the moderately inflamed piglets, the Trp requirement was 0.21 and similar to the healthy piglets' requirement (Le Floc'h et al., 2010). Thus the moderate inflammation induced by unsanitary condition only dropped the piglets' performance but not the Trp requirement.

The ideal threonine to Lys ratio is 0.6 for healthy pigs (NRC, 1998 and 2012). In piglets injected with *Pseudorabies* live vaccine were fed diets containing SID Thr content ranging of 0.74 to 1.11%, the diet with 0.89% Thr improved feed efficiency and pro-inflammatory cytokines produced by Th1 cells (Mao et al., 2014). Further, anti-inflammatory cytokine, IL-10 concentration in the serum was increased with Thr content of or greater than 0.89%. Thus, after *Pseudorabies* virus vaccination, the piglets need 20% more the recommended dietary Thr content. Similarly, Ren et al. (2014) challenged piglets with *Escherichia coli* and reported reduced IL 1β and increased jejunal villus height with 0.75 and 1.11% dietary SID Thr content, corresponding to 11 to 150% more dietary Thr. Therefore, these examples confirm that the health status of the pigs would affect the AA requirement.

2.3 Approaches, methods and response criteria for amino acid requirements

The amino acids requirements can be determined using either factorial or empirical approach. The factorial approach is where the daily requirements are obtained for an individual animal at a specific point in time by combining the estimated requirements for maintenance and production. Whereas in the empirical approach, the nutritional requirements are defined as the minimal amount of nutrients needed to maximize or minimize population responses for one or several performance criteria during a given period (Pomar et al., 2003).

Mathematical models have been developed under factorial approach using AA requirements determined through comparative slaughter method, which encompass the differences in AA composition for maintenance and production, for example protein accretion. Thus the protein and AA composition of various body tissues from representative animals can be determined before the start that can be compared those on a test diet at the end of the experiment. The body composition is meant to reflect the AA requirement of an animal. The advantages of the method is firstly, that one can measure nutrient utilization efficiency. Secondly, several AA requirements can be determined at a time. Thirdly, modelling allows one to simulate the impact of factors that AA requirements and can give the cost benefit analysis. However, the method is expensive due to the slaughters and that the estimated requirements based on a single animal at one point in time does not necessarily correspond to the level of nutrients that will optimize population responses (Hauschild et al., 2010).

The empirical approach is the most common where a dose-response technique is used and a response curve is given. To get the optimal value the responses are fitted to linear or curve-linear plateau models (Robbins et al., 2006). The response criteria used to estimate AA requirements are different and may give different requirements (Baker, 1986). The method has been criticized for being time consuming expensive and laborious as each AA requirement had to be determined separately. Also, in case of an antagonistic AA, its requirement must be satisfied for the test AA optimal values to be acceptable (D'Mello, 2003). Below are some of the empirical methods used in AA requirement determination with different response criteria.

The common response criteria used in determining AA requirements include; animal performance (growth and feed efficiency), nitrogen balance, plasma urea nitrogen, plasma AA concentrations and direct or indirect oxidation indicator methods. In pigs, performance and

nitrogen retention are commonly used methods for grower pigs and sows, respectively, in determining AA requirements mainly due to the simplicity of the methods. The usage of animal performance criteria in AA requirement determination would give reliable results as long as there are no limiting nutrients. The methods of AA determination, however, will create a difference in the requirement because the accuracy of the methods differs. For example, in humans, Lys as determined by nitrogen balance was 12 mg/kg/day while indirect AA oxidation indicator method was 30 mg/kg/day (Kurpad and Thomas, 2011).

Table 2.3 A comparison of amino acid requirement determination methods; lysine and sulfur amino acids as examples

Amino acid	Pig body weight, kg	Method[1]	Requirement estimate,%	Source
Lysine (SID)[2]				
	11 to 25	ADG, GF	1.23	NRC, 2012
	10 to 20	ADG, GF	1.01	NRC, 1998
	11 to 25	ADG	1.30	Yi et al., 2006
	19	PUN	0.92	Coma et al., 1995
	19	NR	0.91	Coma et al., 1995
	16 to 24	IAAO	0.91	Bertolo et al., 2005
Sulfur amino acids (SID)				
	11 to 25	ADG and G:F	0.68	NRC, 2012
	10 to 20	ADG and G:F	0.58	NRC, 1998
	13 to 25	ADG	0.77	Gaines et al., 2004
	11 to 25	G:F	0.83	Yi et al., 2006
	11 to 22	G:F	0.74	Gaines et al., 2003
	9 to 11	IAAO	0.73	Moehn et al., 2008

[1]Method: ADG = average daily gain; G:F = gain to feed ratio; PUN = plasma urea nitrogen; NR = nitrogen retention; IAAO = indicator amino acid oxidation. [2]SID = standardized ileal digestible

2.3.1 Performance indicators

For years, AA requirements have been determined based on growth, feed intake and feed efficiency. The underlying principle is that weight gain and feed efficiency are maximized with optimum supply of AA and any level below or above leads to inefficiency of AA utilization. Energy should also be provided at adequate level since it has been established that energy intakes limits protein synthesis and metabolism (Duffy et al., 1981). In principal, the animals are provided with increasing level of the limiting AA in diets while crude protein and other nutrients are similar across dietary treatments (Loughmiller et al., 1998; Viera et al., 2004). It is expected that the effects of increasing dietary AA content should be reflected on the growth and feed efficiency. The optimum level is either determined using non-linear regression or responses are fitted to a broken-line model (Robbins et al., 1979).

As a part of growth as a response criteria the carcass characteristics and muscle tissue protein can be measured and are an index of feed efficiency. Also, besides determining the AA requirement, the carcass characteristics link animal's performance to its economic value. For example, longissimus muscle area linearly increased for 55 kg gilts, whereas the back fat linearly decreased for 72 kg fed diets with graded digestible lysine (Friesen et al., 1994). Therefore, the fast growing lean pigs would have high Lys requirements based on their carcass characteristics. This principle was confirmed by Taylor et al. (2012) who fed 3 pig genotypes diets with increasing Lys content. It was found that the Lys requirement was similar for 3 pig genotypes in the first 2 weeks following weaning, however, afterwards the Large white and Hampshire grew faster that Pietrain and similarly their Lys requirement was higher than for the Pietrain.

The main disadvantages of the performance parameters are: 1) the technique is only useful in rapidly growing young animals but not in adults. 2) The technique cannot be used for

immune challenged animals. 3) The technique is time consuming, laborious because it involves a large number of animals. 4) Only one AA can be studied at a time. 5) Carcass characteristic as a response criteria can only be used for Lys whose utilization in the body is mainly for protein accretion.

2.3.2 Nitrogen balance technique

The basic concept for N balance technique is that, protein is the major N containing substance in the body, so that gain or loss of N from the body can be regarded as synonymous with gain or loss of protein (WHO, 2007). A diet deficient in EAA leads to high N losses as compared to sufficient diet. This is because protein can only be synthesized up to the level of the limiting AA. Thus the rest of the AA not used for protein synthesis will be degraded resulting in N production. The N retention is measured as the difference between dietary N intake and losses through urine and feces. The AA requirement is established by having dietary treatments with graded levels of the AA in question. The treatment with the lowest positive N production as well as highest growth for young pigs or maintains body condition for adults is the requirement.

The advantages of this method are firstly; animal performance can be observed concurrently with N balance, as done in pigs (Chung and Baker, 1992) and broilers (Pope et al., 2004). Secondly, the procedure is non-invasive therefore has been preferred in human studies. However, the N balance technique is criticized for underestimating AA requirements due urea recycling. Further, the fecal N includes contribution from microbial protein synthesis in the large intestine leading to inappropriate estimates. Metges et al. (1999) confirmed the contribution of the microbial protein to the total protein pool by orally infusing a ^{15}N source to human diet, which was absorbed both in the small intestine and colon and supplied 1 to 20% of the total Lys.

Therefore, this method of determining AA requirement should always be used in comparison to another.

2.3.3 Plasma amino acids

The concentration of plasma AA with changing dietary level of the test AA is measured. The plasma AA content is stagnant in case of a limiting EAA but rise steadily once requirement is met. Mclaughlan and Illmanthe (1967), showed that free plasma AA can be used to predict AA requirement in an animal, where they determined Trp, Thr, Leu and Ile and requirements of rats. The method has an advantage of being able to use a short time to get reliable data (Bae et al., 2011). Watanabe et al. (1998) tested the method on rats fed diets containing from 60 to 120% of the requirement of AA; Ser, Gly, and Thr for a 7 d period. Blood samples were collected from the rats from d 2 to 7 and they reported that the d 2 samples gave reliable data that was as good as d 7's. Hence the method allows for repeatability of the sample collections, use of few animals, and the requirement of several AA can be done within a short period.

Unfortunately, the presence of related AA in the diet could influence the requirement of a test AA. For instance, high dietary concentration of Cys reduced Met requirement in pigs (Shoveller et al., 2003), a principal known as Met sparing effect. The branched chain AA share a degradation and absorption pathway, thus excess of one would antagonize another consequently affect their plasma concentration. Leucine supplementation led to reduced Ile but not Val content in the piglets' plasma (Elango et al., 2004). To overcome the antagonism, there is addition of the antagonized AA in the test diets. Another disadvantage with the method is that there is a lot of

blood samplings which would normally distress the animal. Further, the plasma AA content is different between the fed and post fed state, thus the blood sampling is done several times in the day, to get a representative sample.

2.3.4 Plasma urea nitrogen

The underlying principle for plasma urea nitrogen (PUN) is that urea is produced from AA catabolism if the AA were not used for protein synthesis. With a deficit of an EAA the PUN concentration is high as a result of catabolism of excess AA not used in protein synthesis, however, PUN concentration decreases with increasing AA till a plateau is reached once a requirement is met. This method is used as a short term response criteria and has been efficient in determining Lys (Coma et al., 1995), Ile (Parr, et al., 2003), Thr (Pedersen et al., 2003) in pigs. However, the method depends on an indirect parameter to determine AA requirements thus it is considered insensitive (Pencharz and Ball, 2003). Most of the limitations to the method are similar to plasma amino acids.

2.3.5 Isotope tracer technique

The isotope tracer technique uses radioactive or stable isotopes to label substrates whose metabolism will be monitored. An isotope is infused for a period of time till a steady state within the body is attained. All isotope-dilution techniques measure the degree of isotopic enrichment of an AA after a challenge dose of labeled tracer. Oxidation is measured by using isotopically labeled AA. The technique is based on the principle that if an EAA is deficient in the diet the amount of protein that can be synthesized is limited, creating an excess of the rest of the AA which will be oxidized. Thus the oxidation of the indicator AA would decrease with increasing

test AA until the optimum requirement level is reached, where thereafter oxidation is constant (Zello et al., 1995). Subjects are given infusions of the indicator amino acid either orally or intravenously. The indicator AA can be phenylalanine, leucine, isoleucine and lysine offered on a diet with adequate quantity of the labeled AA that will be oxidized to CO_2. Among the isotope tracer methods are; direct AA oxidation and balance, and indirect AA oxidation and balance. The indicator amino acid method has been used in humans (Di Buono et al., 2001; Kurpad et al., 2006; Elango et al., 2007), neonatal pigs (House et al,. 1997; Shoveller et al., 2003), growing pigs (Bertolo et al. 2005; Moehn et al. 2008) with an advantage of being non-invasive in nature and with no need for adaptation before taking oxidation measurements (Zello et al., 1995).

Using the direct indicator AA method, Young et al. (1991) determined Met requirement of men who were given a 5 d diet adaptation period to attain a steady state of AA and N. A priming dose of tracers (L-$^{2}H_3$-methyl1-^{13}C methionine and ^{13}C bicarbonate) were given intravenously at various dietary intakes of Met. Methionine oxidation was evaluated both in the fed and post absorptive state, blood and expired air samples were collected to determine isotope enrichment. The rate at which the men oxidized the labeled tracer was calculated of labelled CO_2 in exhaled breath, the rate at which the men produced CO_2 and the net amount of CO_2 produced during oxidation.

The limitation of the indicator amino acid method is that it involves consumption of synthetic or semi-synthetic diets that are unpalatable. Therefore, in case of a need for an adaptation period like for the direct AA oxidation method, termination of participation by some subjects would be expected. Also, it is proposed that the method could overestimate the AA requirement as a result of tracer dilution with increase in the dietary intake of the test AA (Zello

et al., 1995). It is also not ethically acceptable to feed deficient diets to vulnerable groups like children.

2.3.6 Conclusion

In conclusion, all methods of determining AA requirements have some benefits and limitations thus the choice of the method would depend on the number of experimental animals, availability of time, animal age, and the cost of the study. However, the above methods would not be efficient in determining AA requirement if the health status of the animal is compromised. The use of metabolites or genes involved in production of these metabolites would be beneficial in determining AA requirements in situations where performance response is biased by other factors like illness. However, this is a new field and not much work has been to establish the key metabolites whose changes would be used to ascertain requirement. Below is a discussion on how AA are used during an immune challenge.

2.3 Roles of amino acids in immunity

During a disease condition AA are used for energy and in support of body defense mechanisms therefore creating changes in AA metabolism. Moreover, the proportions and type of AA required for synthesis of proteins needed for support of body's defense mechanism are not similar to muscle protein synthesis. Research has shown that metabolism and requirement for EAA like Thr, Met, Trp, change in monogastric animals during an immune challenge (Remond et al., 2009; Maroufyan et al., 2010; Le' Floc'h et al., 2010). It is evident that disease state changes the normal AA metabolism so that most of them are partitioned towards body

maintenance. Therefore, the recommendations given for healthy animals may not be adequate during disease state. Amino acids especially Met, Thr and Trp are critical in support of innate immunity through production of antioxidants, phagocytes, cytokines and acute phase proteins (**APP**). Thus, if in a healthy situation the portal drained viscera utilize 20 to 35% of the total AA intake (Stoll and Burrin, 2006), during an immune challenge there is increased demand for nutrients to supply energy and synthesis of new cells and proteins. Amino acids like Ala and Glu are highly mobilized for gluconeogenesis to provide energy for leukocytes and macrophages (Kurpad, 2006), while Ser, Thr, Gly, Met and Cys are utilized for APP synthesis (Grimble, 1998) and constitute up to 56% of APP structure. Below is a table showing a summary on the role of AA in pig's immunity.

Table 2.4 Role of amino acids in immune response

Amino acid	**Functions**
Alanine	Inhibition of apoptosis, stimulation of lymphocyte proliferation, and enhancement of Ab production probably through cellular signaling mechanism.
Arginine	Production of NO necessary as signaling molecule, killing of pathogens, regulation of cytokine production and mediator of autoimmune diseases.
Glutamine	A major fuel for cells of the immune system, regulation of T-lymphocyte proliferation, protein synthesis, as well as cytokine and Ab production, and activation of macrophage function; inhibition of apoptosis.
Glutamate	The metabolite GABA[1] is a neurotransmitter, inhibition of T-cell

	response and inflammation
Histidine	Production of histamine necessary in allergic reaction, vasodilator, and central acetylcholine secretion. Produces urocanic acid for modulation of the immune response in skin.
Isoleucine, leucine and valine	Regulation of protein synthesis and activation of cytokine and Ab production through cellular mTOR[2] signaling. Regulation of immune responses through production of HMB[3].
Lysine	Regulation of NO synthesis.
Methionine	Activity is though metabolites: - Homocysteine is an oxidant, inhibitor of NO synthesis - Betaine for methylation of Hcy to Met, one carbon unit metabolism Choline for synthesis of betaine, actylinecholine and phosphatidylcholine - DCSAM[4] for methylation of proteins and DNA, polyamine synthesis, gene expression - Taurine is an anti-inflammatory - Cysteine together with Gly and Glu synthesize GSH an antioxidant and it regulate cellular redox state; Cys metabolism also yields taurine

Phenylalanine	Regulation of tetrahydrobiopterin (a cofactor for NO synthesis), synthesis of tyrosine
Proline	Produced peroxides for killing pathogens, intestinal integrity, a signaling molecule, immunity. Metabolite $P5C^{5}$ maintains cellular redox state, DNA synthesis, lymphocyte proliferation, ornithine and polyamine formation, gene expression.
Threonine	Synthesis of the mucin protein that is required for maintaining intestinal immune function, Thr also inhibits apoptosis, stimulates lymphocyte proliferation, and enhances of Ab production.
Tryptophan	Has the following metabolites: - Serotonin a neurotransmitter, it inhibits the production of inflammatory cytokines and superoxide - NAS^{6} an inhibitor of tetrahydobiopterin synthesis, antioxidant, inhibition of the production of inflammatory cytokines and peroxides. - Production of melatonin an antioxidant it inhibits the production of inflammatory cytokines and superoxide. - ANS^{7} inhibits production of Th-1 cytokines and prevents autoimmune neuroinflammation.

Tyrosine	Produces dopamine a neurotransmitter and regulates of immune response, also produced neurotransmitters EPN[8] and NEPN[9].

Table adapted with permission from Li et al., 2007.
[1]GABA = γ-amino butyris acid; [2]mTOR = the mammalian target of rapamycin; [3]HMB = β-hydroxy-β-methylbutyrate; [4] DCSAM = decarboxylated S-adenosylmethionine; [5]P5C = pyrroline-5-carboxylate.[6]NAS = N-acetylserotonin; [7]ANS = anthranilic acid; [8]EPN = epinephrine; [9] NEPN= norepinephrine.

2.4 Piglet Immunity

The piglet has both innate and adaptive immunity in protection against pathogenic invasion (Calder, 2007). Innate immunity is the first line of defense, therefore occurring in the first days of invasion, it is not specific and has no memory (Gruys et al., 2005). Cells involved in the non-specific response include natural killer cells, monocytes and polymorphonucler neutrophils. Natural killer cells induce and control inflammation as well as stimulate the production of cytokines like IFN-γ and TNF-α (Vivier et al., 2008). Neutrophils, macrophages and monocytes are phagocytes to bacterial antigen where there is production of toxic substances such as superoxide radicals and hydrogen peroxide (Kohchi et al., 2009). Monocytes are antigen presenting cells hence they stimulate the adaptive immune system. Also, they have a role in immunomodulation through stimulating production of chemokines and cytokines like IL-1, IL-5, IFN-γ and TNF-α (Dale et al., 2007).

The adaptive immune response is expressed in vertebrates can be humoral immunity or cell mediated. The humoral mediated immunity involves B lymphocytes produced from the bone marrow or bursa of fabricus in birds and is characterized by its ability to produce antibodies which are specific for an individual antigen. Immunoglobulin G, (IgG) is a kind of antibody that

works efficiently to coat microbes, speeding their uptake by other cells in the immune system. Immunoglobulin M acts against bacterial infection, IgA concentrates in body fluids; tears, saliva, and the secretions of the respiratory and digestive tracts hence guarding the entrances to the body. Immunoglobulin E, whose natural job probably is to protect against parasitic infections, is responsible for the symptoms of allergy. Immunoglobulin D remains attached to B cells and plays a key role in initiating early B cell responses.

The cell mediated immunity involves the T lymphocytes produced in the thymus. T lymphocytes consists of T helper (**Th**), cytotoxic (**Tc**) and regulatory (**Treg**) cells. Th promotes the activation of B lymphocytes as well as cytotoxic T-cells. Naive CD4+ T cells can differentiate into Th1, Th2 or Th17. Th1 acts against intracellular pathogens, by producing IL-2, IL-3, IFN-γ and TNF-β hence supports cellular immunity (Romagnani, 1991). Th 2 act against extracellular pathogens, including helminthes, through production of IL-4, IL-5, IL-6, IL-10, IL-13, and IL-25 hence supports humoral immunity (Romagnani, 1991; Fietta and Delsante, 2009). Th17 acts against gram-negative bacteria, fungi, and some protozoa, through production of IL-17, IL-21, and IL-22, with strong pro-inflammatory effects (Fietta and Delsante, 2009). Cytotoxic T cell functions by killing the target cell through antibody-dependent cell-mediated cytotoxicity or through perforin formation. Regulatory T cell functions by inhibiting, or helping to stop an immune response by releasing signals to other immune cells. For example, the Tregs are able to suppress the proliferation of antigen-stimulated naive T cells (Sakaguchi et al., 2009). The Th responses are tightly controlled to avoid self-antigen reactivity or excessive reactions to non-self-antigens. The T cells do not recognize an antigen on their own, hence work in collaboration with antigen presenting cells. The antigen presenting cells have proteins on their

surface called major histocompatibility complex (MHC) that help T-cells distinguish between self and non-self cells.

Inflammation is characterized by increased production of pro-inflammatory cytokines and acute phase proteins (APP) (Gimbles, 1998) as part of the host defense mechanism. The pro-inflammatory cytokines include interleukin-1, 6 (IL-1 β, IL-6) and tumor necrosis factor-α (TNF-α), while the APP are haptoglobin, serum amyloid P, α-1-acid glycoprotein and C-reactive protein. Pro-inflammatory cytokines released activates the inflammatory cells to produce more cytokines. The effects of IL-1β are fever, anorexia, sleep, induction of hypothalamic-pituitary adrenal axis for production of glucocorticoids, growth and differentiation of T and B cells (Akira et al., 1990, Santangelo, 2002). Tumor necrosis factor-α stimulates increased muscle catabolism for provision of AA for gluconeogenesis, and synthesis of APP, macrophages differentiation and T cell activation and differentiation (Santangelo, 2002, Gruys et al., 2005). Interleukin 6 induces synthesis of APP in the liver and antibody production (Valen et al., 2001). The cytokines are pleotropic for example; IL-1β and TNF-α are potent inducers of IL-6, whereas IL-6 regulates TNF-α expression (Akira et al., 1990). Production of IL-1, IL-4 and IL-10 suppresses the activity of pro-inflammatory cytokines. The function of APP is to opsonize microorganisms, scavenge cellular remnants and free radicals and neutralize proteolytic enzymes while others activate complement system (Gruys et al., 2005). The production of APP increases by more than 50% during an inflammation.

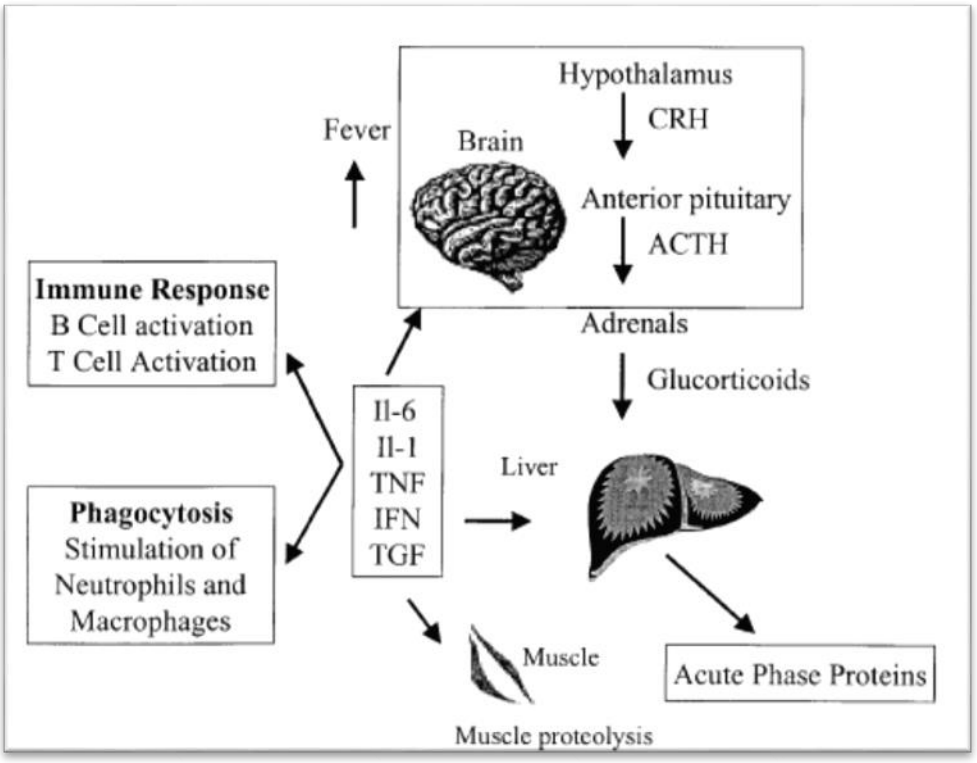

Figure 2.1 Multifunctional properties of cytokines. Used with permission as published by Santangelo (2002).
CRH = corticotropic hormone; ACTH = adenocorticotropic hormone; IL = interleukin; TNF = tumor necrosis factor; IFN = interferon; TGF = transforming growth factor.

2.5 Types of immune challenge models

The pig's immune challenge models can be used to evaluate the interaction of disease with nutrition, in situations where dietary interventions are applied to control an immune challenge. The models used include systemic activation of the immune challenge, which is done through injecting an antigen such as, *Escherichia coli* lipopolysaccharide, turpentine, conjugated Freund's adjuvant and live bacteria in order to create sepsis. The enteric and respiratory infections studies involves introduction of a live pathogen to the site of infection. Some of the

indexes to show the effectiveness of the immune challenge are through measuring cytokines, the antigens, antigen presenting cells, temperature, observing the lymph nodes, intestinal histomorphology, incidence of a diarrhea, and use Ussing chamber to measure electrical resistance and paracellular permeability.

The intensity of the immune stimulation varies between models and the time from infection to inflammation is shorter for the systemic models. Hence, the systemic models are good in measuring acute responses, further, repeated injections over time can allow evaluation of chronic immune stimulation. Rakhshandeh and de Lange (2011) compared chronic immune stimulation in pigs repeatedly injected with *Escherichia coli* lipopolysaccharide or turpentine or use of feed grains that naturally contaminated with mycotoxins. Based on internal organs weight, eye temperature, growth, feed intake, cytokines and acute phase protein production, mycotoxins had the least effect on immune system activation while turpentine and *Escherichia coli* lipopolysaccharide gave the highest indices. However, turpentine was disregarded because the model caused severe damage to the pigs with symptoms such as skin ulcerations. A meta-analysis by Pastorelli et al (2012) showed that, although seven immune challenge models had negative on performance, the enteric infection models caused the highest drop in feed intake while the greatest weight loss was caused by *Escherichia coli* lipopolysaccharide injection model.

The enteric live infection model shows the changes that would occur to an animal when the gut microbiota balance is tipped. The pathogenic bacteria needs to first adapt to the intestinal environment, then compete with the commensal bacteria before they are able to attach to the intestinal wall and produce toxins. Thus the model mimics an incidence of bacterial infection in a pig's gut. The pathogenic bacterial has to pass thorough a barrier, the gut associated lymphoid

tissue (GALT) that forms the major part of the innate immunity. The tissue is made of the villi comprised of intestinal epithelial cells, intraepithelial lymphocytes surrounding the lamina propria that contains lymphocytes, dendritic cells and lymphatic sinuses. At the base of the villi is a crypt that contains intestinal epithelial cells, intraepithelial lymphocytes, mucous secreting globule cells and paneth cells that secrete antimicrobial peptides. On the intestinal epithelium are the Peyer's patches that contains intestinal epithelial cells, B cells, dendritic cells, and M cells on the follicle associated epithelium layer. The main role of M cells is antigen sampling and presentation to the antigens to the immune cells. If presented to a naïve B cells it will recognize an antigen and undergoes maturation to be antibodies secreting cells, antigen presenting cells and memory cells. However, an antigen presenting cell like the dendritic cells take up a foreign particle from either M and present them to naïve T cells (Cook et al., 2000). It is also known that the dendritic cells influence an immune response through induction of B cell production of IgA, promotion of T cell production of cytokines associated with Th2 and production of IL-10 (Ruth and Field 2013; Cook et al. 2000). Once presented with antigens' peptides the naïve T cells migrate via mesenteric lymph node to the systemic circulation and differentiates into Th, Treg, Tc cells.

The enteric infection with live bacteria have been criticized that it requires high protection from biohazard for the workers, it causes loss of appetite and high losses of body weight that lead to mortalities of animals (Deitch, 1998) because of the *Escherichia coli* multiplication. A comparison between live *Escherichia coli* and the *Escherichia coli* lipopolysaccharide was made through injection of the antigens to rats' brain (Schwartz and Bilbo, 2011). It was found that the *Escherichia coli* lipopolysaccharide upregulated genes for interferons, TNF, IL-1 and IL-6 while the live *Escherichia coli* activated IL-1 genes. Further, the

inflammatory response to live *Escherichia coli* lipopolysaccharide was greater than live *Escherichia coli.* Meaning that the host defense system and the gut microbiota are able to antagonize live *Escherichia coli* effects.

Below is a discussion on the gut microbiota and their importance to the host.

2.6 Gut microbial ecology

Bacteria are the predominant microbes of the gut microbiota and have influence on the nutritional, physiological and immunological processes in the host animal (Zootendal et al., 2004). During birth the pigs are sterile but will immediately be exposed to environmental microorganism from some of which will start colonization of the gut. All neonates have indigenous microbiota who are the first colonizers and these are mainly *Lactobacilli, Streptococcus* and *Enterococcus* sp. (Gomez, 2006). Thus, the predominant bacterial group in the proximal GIT of neonates is the Lactobacilli and Streptococci, while towards the distal end of the gut there are the *Bacteroides, Clostridium, Enterobacteria* sp. (Simpson et al., 1999). However, following weaning some gram negative bacteria like *Escherichia coli* start colonizing the gut (Jensen, 1998).

The microbiota population found in the pig gut are different depending on the section of the gut, age of the animal, health status, diet and individual variations. Leser et al., (2002) working on feces from the 12 -18 week pigs was able to detect diverse bacterial phylotypes these were: *Eubacterium* and relatives, *Clostridium* and relatives, *Bacillus-Lactobacillus-Streptococcus* Subdivision, *Flexibacter-Cytophaga- Bacteroides* group, Proteobacteria, *Sporomusa* and relatives, *Mycoplasma* and relatives, High-G+C bacteria, *Spirochetes* and relatives, *Clostridium purinolyticum* group, *Planctomyces* and relatives, *Flexistipes sinusarabici*

assemblage and *Anaerobaculum thermoterrenum.* Whitehead and Coma (2001) determined that over 90% of the viable anaerobes were gram-positive with *Streptococcus* sp., *Clostridium* sp., *Propionibacterium* sp. being the majority, whereas for the gram negative bacteria, *Eubacterium* sp. were among the most numerous observed.

The gut microbes trigger development of the innate immune system, as it has been shown that under germ free conditions mice have poorly developed gut related lymphoid tissue (Round and Mazmanian, 2009). The bacterial colonization presents a first-line mechanism of host defense against infection or intoxication by enteric pathogens or inappropriate overgrowth of potentially pathogenic indigenous opportunists (Kraatz, 2010). Subsequent colonization by pathogens occurs when there is an event causing imbalanced bacterial species, however they need to attach to the gut wall for them to be effective. The gram positive bacteria like streptococci and staphylococci are able to colonize because they have adhesins to establish contact with the host tissue. Their adhesins are lipoteichoic acid or peptidoglycan (Nitsche-Schimdtz et al., 2007). The gram negative have two main types of protein adhesins, (i) the fimbrial adhesins with pili composed of heteropolymers of several subunits and (ii) non-fimbrial adhesins consisting of a single protein or homotrimers (Gerlach and Hensel, 2007).

The animal's diet can be manipulated to influence microbiota population through provision of substrates that favor commensals and reduction of substrates necessary for pathogenic proliferation. The *Bacteroides* group is linked to dietary polysaccharide degradation. They can also utilize host glycans and are thus associated with the mucosa where this energy source is found (Thompsons and Holmes, 2008). *Clostridium leptum* subgroup are abundant within intestinal lumen where they produce butyrate from fermenting substrates such as starch, while the *Enterobacteria* sp. who are considered to be early colonizers of the gut are associated

with the mucus layer. Wellock et al. (2008) investigating effect of fiber found that inulin addition stimulated lactic and butyric acid production in the small and large intestine leading to reduced counts of coliform bacteria. Hermes et al. (2009) observed that feeding weanlings diets containing 7% neutral detergent fiber lead to a decrease in *Escherichia coli* counts (7.77 vs. 6.86 log of cfu/g of feces, $P \leq 0.05$), and an increase in the ratio of *Lactobacilli*:*Enterobacteria* (0.76 vs. 1.37, $P \leq 0.05$) compared to controls. Specific dietary components like Thr, Cys may have a potential to modulate the attachment of bacteria since they have mucosal barrier function as they are a component of mucin (Stoll, 2006). Dietary manipulation through reduction the CP content results in less nutrients available after digestion which can be utilized by pathogenic microbes like *Escherichia coli.* Interestingly, reduction of CP from 23 to 13% increased the population of commensal *Lactobacilli* in the colon (Wellock et al., 2006). Thus the reduction in bacterial population through dietary manipulation may not be for only pathogenic bacteria.

Besides dietary manipulation, subtheraupetic levels of antibiotic have been used to manipulate the host animal gut microbiota, through minimizing the gut microbiota population and eliminating the pathogenic bacteria and subsequently reducing the chances of infection. The antibiotic added to animal feed include, tylosin, bacitracin, virginiamycin, cabardox, ionophore, monensin, oxytetracyline, linomycin, streptomycin, and salinomycin. One of the output from antibiotic use is increase nutrient availability to the host leading to improved growth rates. In the absence of the antibiotics, more nutrients will be utilized by the gut microbiota as well as the host's immune system, hence an increase in the maintenance requirement.

2.7 Lysine and sulfur amino acids requirement in response to use of AGP and immune challenge in piglet

The health status of weaned pigs is especially delicate due to dietary and environmental changes that result in depressed feed intake and growth. The weaning period also predisposes piglet to diarrhea commonly referred to as post weaning diarrhea (**PWD**) caused by opportunistic *Escherichia coli* (Pluske et al., 1997). In such times, there has been use of AGP that reduce gut microbial population leading to reduced nutrient competition between host and gut microflora and reduced production of toxic metabolites (Dibner and Richards, 2005) and thus boost the growth of piglets. A ban in the use of AGP is likely to cause changes in AA requirement because the incidences of diseases like the PWD has been on the rise (Dibner and Richards, 2005; Vigre et al., 2008). Thus, the requirement of all AA involved in health and production in piglets is therefore likely to increase. Currently, the AA requirements given by NRC (2012) are from a collection of studies that included AGP in their diets. It is possible that the AA requirement for piglets under AGP-free feeding regime to be different from current recommendations. This discussion will give specific importance to Lys and SAA requirement as influenced by incidence of disease and dietary AGP.

2.7.1 Lysine

Lysine is required mainly for protein accretion and a small portion is directed towards obligatory oxidation (Klasing, 2009). The requirement for Lys hardly changes under conditions of immune stimulation due to its low involvement in animal's immunity. For example, different immune stressors like *Escherichia coli* LPS, environmental stressors, and circovirus did not increase Lys requirement in pigs (Lindermann et al., 1993; Williams et al., 1997; Shelton et al., 2011). Therefore, the additional Lys in pigs' diets was not able to neutralize the depressing effect

of immune challenge on growth. Nemechesk et al. (2012) reported that piglets raised under experimental and commercial settings had similar Lys requirement even though the growth rate were lower under commercial settings. Similar findings were reported by Webel et al. (1998) where *Escherichia coli* LPS challenge did not decrease efficiency of Lys utilization for protein accretion in chickens. Therefore, Lys requirements are similar between healthy and immune-challenged animals when expressed in g/kg gain or as a percent of the diet. Due to the higher growth rate of healthy pigs Lys requirement would be higher when expressed as intake per day even though the efficiency of Lys utilization would be similar between immune-challenged and non-challenged pigs. Thus, the changes in growth rates as a result of immune challenge does not change Lys requirement.

In a factorial study with 40 to 110 kg pigs fed graded Lys diets with or without AGP and all pigs under healthy conditions, the AGP fed pigs had higher growth and feed efficiency as compared to those on diets without AGP (Bikker et al., 2006) that resulted in a 6% increase in Lys requirement. Thus it was possible that some of the dietary Lys is utilized by gut microbes whose population is reduced by use of AGP, hence creating an increased maintenance requirement under AGP-free feeding regime.

2.7.2 Sulfur amino acids

Sulfur AA (methionine and cysteine) are necessary for protein accretion, production of cytokines, glutathione (**GSH**) and APP (Gimble, 2006). About 30% of the dietary SAA is utilized by the gut for epithelial cell differential, mucosal growth and production of GSH (Bauchart-Theveret et al., 2009). Dietary Met deficiency induces small intestinal villus atrophy,

lower goblet cell number and tissue GSH, decreases immune response through under development of lymphoid organs, reduced antibodies and T cell proliferation, (Jahoor et al., 1995; Bauchart-Thevret et al., 2009; Maroufyan et al., 2010).

Methionine has less activity in the piglet's immune system, however, its metabolites have pro- and anti-inflammatory properties for example, homocysteine (**Hcy**) is pro-inflammatory while GSH and taurine are anti-inflammatory. Methionine has three main metabolic pathways and these are, protein synthesis, transmethylation and transsulfuration (Brosnan and Brosnan, 2006). Methionine transmethylation leads to formation of Hcy via S-adenosylmethionine (**SAM**) by the action of Methionine adenosyltransferase (**MAT**). Methionine adenosyltransferase is the product of two different genes, MAT1A and MAT2A (Stipanuk, 2004). Whereas MAT1A is expressed only in the adult hepatocyte, MAT2A shows a wider distribution in extra hepatic cells such that is can be expressed in all tissues (Stipanuk, 2004). S-adenosylmethionine has to be hydrolyzed by methyltransferase to yield S-adenosylhomocysteine (**SAH**) and methyl that can be donated towards DNA, RNA synthesis. S-adenosylhomocysteine hydrolase, hydrolyzes SAH to homocysteine Adenosine.

Transmethylation is reversible therefore Hcy can be remethylated to Met and is catalyzed by the enzyme methionine synthase where folic acid and vitamin B-12 serve as co-factors. Homocysteine can also undergo transsulfuration thereby donating its sulfur group to serine leading to the formation of Cys. Transsulfuration is catalyzed by the enzyme cystathionine β synthase with vitamin B-6 serving as a co-factor. Cysteine is used for synthesis of mainly proteins and GSH. Cysteine its catabolism yields Tau and inorganic sulfur. Stipanuk et al. (1992) reported that under condition of low Cys availability most of it was directed towards GSH production whereas high Cys levels promoted Tau and sulfates production. In healthy adult men

transsulfuration accounts for 62% of the Hcy usage while the rest is remethylated (Di Buono et al., 2001). The study also showed that supplying dietary Cys can have up to 64% Met sparing effect hence, increasing its availability for protein synthesis. Therefore, higher Cys content in the diet increases remethylation (Di Buono et al., 2003). When the intracellular concentrations of methionine are low, remethylation, via Hcy, will be favored over transsulfuration, and with increasing dietary intake of methionine there will be substrate for transsulfaration pathway (Gimble, 2006).

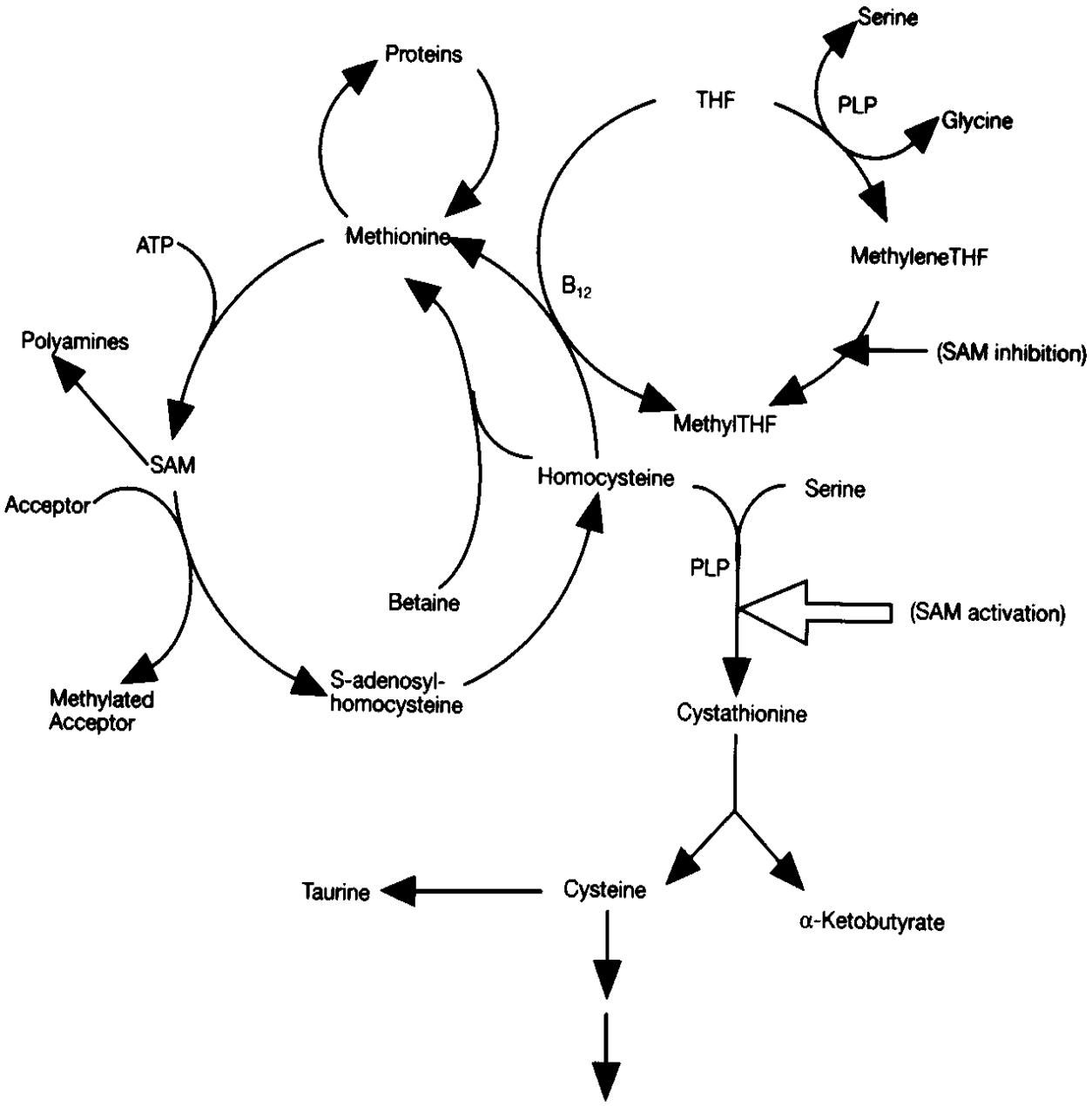

Figure 2.2 Methionine metabolism. Used with permission as published in Selhub (1999).

THF = tetrahydrofolate; PLP = pyridoxal-50-phosphate; SAM = S-adenosylmethionine; ATP = Adenosine triphosphate.

Glutathione is chemically referred to as L-γ-glutamyl-L-cysteinyl-glycine. Glutamate and Cys are covalently joined, a reaction is catalyzed by enzyme glutamate cysteine synthetase (**GSC**) forming γ-glutamylcysteine. Glutathione synthetase will catalyze GSH biosynthesis from γ- glutamylcysteine. The synthesis of GSH is regulated by GSC, Cys availability, and GSH feedback inhibition. The cytosolic GSC is inactive because 80% of it is bound to GSH (Rahman, 2005). Most of the GSH is found in the liver and gastrointestinal tract (van de Poll et al., 2006) but it is present in all tissues. Glutathione is an antioxidant protecting body tissues from free radical injury. Inflammation increases the amount of reactive oxygen species, consequently reducing GSH levels. A decrease in GSH triggers the release of the GSH bound to GSC which in turn results in increased levels of active GSC, and enhances synthesis of GSH (Rahman, 2005). The redox ratio of reduced to oxidized GSH (GSH:GSSG) in cells is greater than 100:1, the ratio is maintained by the enzyme GSSG reductase, which uses the reducing power of NADPH to convert GSSG to GSH. The regulation of GSH synthesis is by a feedback mechanism (Tateishi et al., 1981), thus conversion of Cys to GSH is dependent on the rate of GSH utilization (Gimble, 2006). Glutathione is a store for Cys, therefore it may be hydrolyzed to generate Cys when needed (Stipanuk, 2004).

2.7.2.1 Sulfur amino acid supplementation on immune response

Research indicates that SAA are of importance in the innate immune system and stimulating humoral and cell mediated immune responses. Changes on macrophages, monocytes, granulocytes resulting from differing dietary SAA content have been observed. The SAA involvement in innate immunity was shown by Hunter and Grimble (1994) who injected TNF-α or saline to pair fed rats receiving Cys, Met or Ala. The rats on supplemental SAA had higher levels of polymorphonuclear cells compared to Ala. Therefore, the SAA activated cells required

for body defense while moderating the action of TNF-α which influences muscle catabolism during inflammation. The deficiency of SAA effects on adaptive immunity is reflected by low T-cell proliferation, cytokines, decreased antibodies level and increased levels of reactive oxygen species in poultry and rats (Takahashi et al., 1997; Ronchi et al., 2010).

Sulphur AA also serves to modulate the intestinal function and health (Bauchart-Thevret et al., 2009) through being components of the protective mucin barrier. The mucin barrier quality can be compromised by diseases like infectious bowel disease (Einerhand et al., 2002). However, the mucin barrier damage can be amended by increased provision of the SAA. For example, Faure et al. (2006) reported that rats with damage colon mucosa experienced a restoration of mucin production and epithelial healing after being supplemented with a combination of AA that includes Cys.

Taurine, a sulfur AA is one of the end products of Cys metabolism and mainly functions as a regulator of transmembrane calcium transport (Chesney, 1985). It also reacts with halides to form TauBr or TauCl which are antioxidants. Taurine bromide has the ability to inactivate hydrogen peroxide. In a study by Marcinkiewicz (2010) TauBr had antibacterial activity against *Propionibacterium acnes* and reduced inflammation caused by the bacteria. Taurine chloride anti-inflammatory properties were shown in humans through inhibiting the activity of IL-1β and IL-6 (Chorazy-Massalska et al., 2004), and inhibition of TNF-α and inducible nitric oxide synthase (iNOS) levels in rats (Miao et al. 2011). This response is mediated by suppressing TL-2, 4 and nuclear factor kappa-B (NFκB) signaling pathway (Miao et al., 2011). Taurine at doses of 40, 80, 160 mg/kg resulted in higher than the control lung tumor inhibition rate as well as spleen and thymus index, lymphocyte proliferation and the phagocytic activity of peritoneal

macrophage, peripheral blood neutrophilic granulocyte and monocyte in Lewis lung carcinoma-bearing mice (Wang et al., 2009).

Glutathione an antioxidant, plays an important role in immunomodulation by inhibiting prostaglandins production thereby having anti-inflammatory effects (Nimni et al., 2007). Additionally, NFkB a transcription gene for pro-inflammatory cytokines is activated by oxidants and its activity is decreased by high levels of glutathione and other sulfur containing compounds (Staal et al., 1990). Glutathione depletion triggers the production of TNF-α and its administration reduces the activity of TNF-α, having a modulatory effect. For example, serum TNF-α was 300 fold higher in Met deficient rats challenged with LPS (Chawla et al., 1998), however, administration of exogenous SAM reversed the condition. Since SAM is a precursor of GSH, its administration increased the concentration of GSH in the blood. In murine cells GSH affected the growth and replication of cytotoxic T cells through growth stimulating cytokines such as IL-2 and IL-4 (Liang et al., 1992). Hence GSH was of advantage because IL-4 is responsible in stimulating B-cells for production of antibodies. Similarly, GSH or NAC addition to incubated human cells prevented apoptosis caused by Fas antigen which is a member of TNF-α receptor (Chiba et al., 2005).

It is thus evident that SAA requirement recommended for growth are not enough during an immune challenge. There was reduced urinary sulfur excretion in pigs fed graded SAA content following a 7 day injection *Escherichia coli* LPS, indicating that sulfur-containing amino acids were preferentially conserved or repartitioned thus, suggesting a higher requirement during immune activation (Rakhshandeh et al., 2010). The SAA requirement expressed as SAA:Lys ratio and based on protein deposition and plasma urea nitrogen, was increased from 0.55 in healthy to 0.75 in grower pigs injected twice a week for 6 weeks with *Escherichia coli* LPS (Kim

et al., 2012). It was also noted in the same study that increasing SAA:Lys ratio increased protein deposition in g/day in the LPS injected pigs. The response to Newcastle virus and bovine serum albumin injection in Met supplemented poultry resulted in significant dose dependent increase in total antibodies. The optimal response was at 0.6% dietary Met level which was 120% higher than the recommended requirement for growth (Zhang and Guo, 2008). Thus higher levels of methionine may be required for the synthesis of antibodies. Injection of phytohemaglutitnin on the foot web of quails receiving supplemental Met linearly improved cellular immune response as reflected by the increase in the foot web thickness (Parvin et al., 2009). In addition, Parvin et al. (2009) showed that the Met requirement for immune response was 10% greater than optimal growth.

CHAPTER THREE

HYPOTHESES AND OBJECTIVES

The studies tested the following hypotheses:

1. Amino acid requirement for piglets under AGP-free regime would be higher than those on AGP diets.
2. The requirements for the AA involved in the immune system maintenance are higher when piglets are subjected to an immunological challenge.

The overall objective was to quantify the additional requirements for sulfur amino acids for piglets subjected to sub-optimal health conditions and fed AGP-free diets.

The specific objectives were:

1. To determine the Lys requirement of weaned pigs when fed AGP- free diets subjected to unsanitary conditions.

   Note: Lysine had to be determined first because it is the AA to which the rest of essential AA are expressed as a percentage of, as according to the ideal protein concept.
2. To determine the optimal SAA:Lys for weaned pigs when fed AGP- free diets.
3. To determine the optimal SAA:Lys for weaned pigs when subjected to unsanitary conditions.
4. To quantify the additional SAA needs for weaned piglets consuming diets with no AGP and subjected to an enterotoxigenic *Escherichia coli* challenge.

CHAPTER FOUR

Effect of dietary lysine content and sanitation conditions on performance of weaned pigs fed antibiotic-free diets

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Kahindi R. K.: the graduate student made project proposal in conjunction with Drs. Htoo and Nyachoti. She did the research work, data analyses and wrote the manuscript.

Htoo J. K.: works for Evonik Industries who funded the project. He also assisted in data analyses as well the nutrient composition of the ingredients and diets. He also gave an approval of the version to be published.

Nyachoti C. M.: the supervising professor assisted in providing guidance throughout the research work and in the revision of the research paper.

4.1 ABSTRACT

One hundred and ninety two piglets (7.2 ± 0.50 kg BW) were fed corn-wheat-soybean meal based-diets to determine dietary Lys requirement in a 2 x 4 factorial setting [2 sanitation conditions (**SC**); clean (**CSC**) and unclean (**USC**), and 4 dietary lysine contents (**DLC**); 0.94, 1.09, 1.25 and 1.43%]. The ADG and G:F linearly increased ($P < 0.05$) with DLC, but were lower ($P < 0.0001$) under USC from d 0-7 but not from d 8-21. Overall, DLC did not affect ADFI, but USC reduced ($P < 0.01$) ADFI; however, ADG was increased by both DLC and SC ($P < 0.001$), whereas only DLC affected G:F.

Key words: growth performance, lysine, piglet, sanitation

4.2 INTRODUCTION

Lysine is the first limiting AA in typical diets fed to pigs and therefore, the most studied AA in swine nutrition (NRC, 2012). Unlike other AA, the primary use for Lys is body protein synthesis with the amount supplied in excess of the capacity for protein synthesis degraded (Klasing, 2009). Because of the increased lean gain potential for modern high lean pig genetics, the Lys requirement of pigs across different BW ranges is approximately 20 to 30% higher than NRC (1998) recommendations (Gaines et al., 2003; NRC, 2012). Majority of the studies used to derive the current Lys requirement estimates given by NRC (2012), had pigs fed experimental diets supplemented with antimicrobial growth promoters (**AGP**). The AGP have been used in pig starter diets to overcome post weaning diarrhea and enhance growth performance (Dibner and Richards, 2005). Thus, piglets fed diets containing AGP have better growth compared to those fed AGP-free diets (Bikker et al., 2006).

However, there is mounting pressure to reduce or eliminate the use of AGP in livestock diets as the practice has been linked to the potential problem of increasing transferable bacteria resistance to antimicrobial drugs and therefore, jeopardizing human health (Dibner and Richards, 2005). Feeding weaned pigs antibiotic-free diets under poor sanitary conditions may pose immunological challenges that will impact growth performance leading to changes in the efficiency of nutrient utilization for lean tissue gain (Williams et al., 1997; Le Floc'h et al., 2006). For example, it has been observed that under unclean housing conditions, pigs are often exposed to different kinds of stressors that can lead to the stimulation of the immune system thus, affecting animal performance without any clinical signs (Le Floc'h et al., 2006). Indeed, it has been reported that immunologically challenged pigs have higher requirements for certain AA and especially those that are involved in the immune system (Bikker et al., 2006). Although Lys is primarily used for body protein synthesis and not in the immune system, it is the first limiting AA in most swine diets and is the reference AA in defining the ideal protein ratios for the optimal dietary supply of the other essential AA. However, studies on the Lys requirements of piglets fed AGP-free diets and subjected to an immunological challenge are scarce. Therefore, the objective of this study was to determine the dietary Lys requirement for weaned pigs fed antibiotic-free diets and subjected to clean or unclean housing conditions.

4.3 MATERIALS AND METHODS

4.3.1 Experimental Animals and Design

One hundred and ninety two [Duroc x (Yorkshire x Landrace)] male and female piglets, weaned at 21 ± 1 day and with an initial average body weight (BW) of 7.2 ± 0.50 kg were used in a 6-week study. The protocol for this study was reviewed and approved by the Animal Care

Committee of the University of Manitoba and pigs were cared for in accordance with the guidelines of the Canadian Council on Animal Care (2009). Piglets were allocated to treatments based on BW, gender and litter of origin in a 2 x 4 factorial arrangement with the main factors being dietary Lys content (4 levels) and sanitation condition (clean and unclean). From week 1 to 3, the first batch of 96 pigs was housed (4 pigs/pen) in a clean room that had been disinfected prior to the start of the experiment and was cleaned once a week to determine dietary Lys requirement under clean conditions. From week 4 to 6, the second batch of 96 pigs was housed (4 pigs/pen) in the room occupied by the first batch of pigs without the room being cleaned at the end of week 3 to allow for manure build up. Also, manure slurry from a sow herd (5 kg/pen) was spread on the expanded metal floor of 1.2 x 1.8 m before piglets were introduced into the room to further enhance unclean conditions. The spread of manure slurry (5 kg/pen) from the same source was repeated one week after the introduction of the piglets and the room was not cleaned during the rest of the experimental period.

4.3.2 Experimental diets and animal performance assessment

For each batch, 4 diets with graded levels of Lys were randomly assigned to pens, resulting in six pens per treatment. A corn-soybean meal-wheat-based antibiotic-free diet was formulated to provide 0.83% standardized ileal digestible (**SID**) Lys which is equivalent to 70% of the Lys requirement for 5 to 10 kg pigs recommended by (NRC, 1998). Three additional diets containing 85, 100 and 115% of the NRC (1998) recommendation were constituted by replacing cornstarch in the basal diet with graded levels of crystalline L-Lysine.HCl (Evonik Industries, Hanau-Wolfang, Germany). The analyzed SID Lys contents in the 4 diets were 0.94, 1.09, 1.25 and 1.43% (Table 1). Other AA were balanced to meet or exceed NRC (1998) recommendations. Feed was offered for *ad-libitum* intake and pigs had free access to water through low-pressure

drinking nipples. Pigs were weighed weekly to determine average daily gain (**ADG**). Feed intake was measured weekly to calculate average daily feed intake (**ADFI**) and feed efficiency (**G:F**) was calculated as a ratio of ADG to ADFI; these response criteria were calculated on per pen basis.

4.3.3 Feed and ingredients analyses

Feed ingredient and diet samples were ground in a sample mill (1093 Cyclotec sample mill; Foss Tecator, AB), to pass through a 1 mm sieve and thoroughly mixed prior to DM, CP, and AA analysis. Dry matter was determined according to AOAC (1990 method no. 925.09) and CP (N × 6.25) was determined using a N analyzer (model CNS-2000; Leco Corp., St. Joseph, MI). Amino acid content in feed ingredients and diets were determined by Evonik Industries (Hanau-Wolfang, Germany) using the method described by Llames and Fontaine (1994).

4.3.4 Data analyses

Data was subjected to analysis of variance using the MIXED procedures of SAS 9.2 (SAS Inst., Inc., Cary, NC). The statistical model included effects of sanitation condition and dietary Lys content and their interactions. Linear and quadratic contrasts were made for Lys and significance was set at $P < 0.05$.

Table 4.1 Composition of experimental diets (as is basis)

Dietary SID lysine content, %	0.94	1.09	1.25	1.43
Ingredients, %				
Soybean meal, 46% CP	28.00	28.00	28.00	28.00
Wheat	20.00	20.00	20.00	20.00
Corn	29.00	29.00	29.00	29.00
Corn gluten meal	3.00	3.00	3.00	3.00
Corn starch	3.00	2.80	2.50	2.30
Lactose	8.00	8.00	8.00	8.00
Limestone	1.00	1.00	1.00	1.00
Dicalcium phosphate	1.50	1.50	1.50	1.50
Vegetable oil	4.00	4.00	4.00	4.00
Salt	0.36	0.36	0.36	0.36
Vitamin & mineral premix[1]	1.00	1.00	1.00	1.00
L-Lysine.HCl	0.00	0.23	0.46	0.69
DL-Methionine	0.33	0.33	0.33	0.33
L-Threonine	0.35	0.35	0.35	0.35
L-Tryptophan	0.08	0.08	0.08	0.08
L-Isoleucine	0.12	0.12	0.12	0.12
L-Valine	0.26	0.26	0.26	0.26
Total	100.00	100.00	100.00	100.00
Calculated nutrient content in the diet, %				
ME, kcal/kg	3462	3461	3458	3456
NE, kcal/kg	2290	2290	2289	2288
Calcium	0.87	0.87	0.87	0.87
Available phosphorus	0.41	0.41	0.41	0.41
Total phosphorus	0.73	0.73	0.73	0.73
Analyzed CP & AA content, %				
CP	22.48	22.36	22.80	22.40
Lys	1.07	1.22	1.38	1.56
Met	0.64	0.67	0.65	0.65
Met+Cys	0.99	1.02	0.99	1.00
Thr	1.06	1.08	1.08	1.07
Ile	1.01	0.99	0.98	0.99
Val	1.24	1.22	1.20	1.22
Trp	0.32	0.32	0.31	0.32

[1]Supplied the following per kilogram of diet: 8,250 IU of vitamin A, 835 IU of vitamin D3, 40 IU of vitamin E, 25 µg of vitamin B12, 4 mg of vitamin K, 25 mg of niacin, 600 mg of choline, 12 mg of riboflavin, 200 µg of biotin, 4.5 mg of pyridoxine, 4 mg of folic acid; 2 mg of thiamin, 50 mg of Mn , 150 mg of Zn, 120 mg of Fe, 25 mg of Cu, 0.35 mg of Se, 0.4 mg of I.

4.3 RESULTS

There were no signs of diarrhea or any other illness observed for piglets raised under clean conditions. However, a mild diarrhea characterized by loose feces was observed for piglets raised under unclean conditions from day 3 to 10 after introduction into the pens irrespective of dietary treatment. There were no interaction effects of dietary Lys content and sanitation conditions ($P > 0.10$) on any of the response criteria measured (Table 4.2).

During d 0-7, the ADG and G:F were linearly affected ($P < 0.05$) by the dietary SID Lys content. The ADG and G:F were lower ($P < 0.0001$) for pigs raised under unclean conditions compared with their clean counterparts during d 0-7 (Table 4.2). During d 8-14 as well as from d 15-21, the dietary SID Lys linearly increased ($P < 0.001$) the ADG and G:F. Moreover, sanitary conditions had significant effect on ADG and G:F except during d 15-21 for the ADG. The ADFI was not affected by the dietary Lys level but pigs raised under unclean conditions had a lower ADFI ($P < 0.01$) throughout the experimental period.

During the overall 21 d period, the ADG was affected by the dietary Lys (linear; $P < 0.001$) and the sanitary conditions ($P < 0.001$). The G:F linearly increased ($P < 0.001$) by graded level of dietary Lys, but was not affected by the sanitary conditions during d 0-21 (Table 4.2).

4.5 DISCUSSION

The present study was conducted to provide baseline Lys requirement for weaned piglets fed antibiotic-free diets and raised under different sanitation conditions. Increasing the dietary Lys content linearly increased ADG, which was consistent with results of others (Gaines et al., 2003). There was also a lack of a quadratic response in ADG though some diets had higher Lys content than NRC (1998) recommendation. Various studies have shown that the 1.19% SID Lys recommendation by NRC (1998) for 5 to10 kg pigs does not support adequate growth of the

modern lean pig genotype. For example, Gaines et al. (2003) reported a value of 1.42% SID Lys, to be optimum for growth of weaned pigs. Feed intake was similar at all dietary Lys content, indicating that Lys content had no effect on feed intake and that growth differences across Lys contents was not caused by reduced appetite. This finding is consistent with results of Shelton et al. (2011) showing no differences in feed intake of piglets fed diets with different dietary Lys contents. However, the increase in feed efficiency observed in the present study with increasing dietary Lys content indicates that nutrient utilization for protein accretion was enhanced (Williams et al., 1997). The linear response in ADG and G:F to increasing dietary Lys suggested that the SID Lys requirement for the weaned pigs used in the present study could be greater than the highest level (i.e. 1.43%) used in the current study.

The sanitation model of immune challenge used in the present study confirms work by others showing that subjecting piglets to poor sanitation conditions results in ill health and reduced feed intake, consequently, leading to depressed growth (Lee et al., 2005; Le Floc'h et al., 2006). Indeed in week one, the effect of unclean sanitary condition was so severe that piglets had a mild diarrhea and grew 50% less than those raised under clean conditions. This response could also be associated with the immaturity of the immune and digestive systems of piglets at this stage (Pluske et al., 1997), which compromised the efficiency of nutrient uptake and utilization. During week two, there was accelerated growth indicating that the ability of piglets to deal with an immunological stress improves with age and that the piglets were able to adapt to their environment with time. However, the overall ADG was still lower for pigs raised under unclean conditions compared with those raised under clean conditions, indicating that piglets were unable to fully overcome the effects of being subjected to unclean housing conditions.

Table 4.2 Effects of dietary Lys content and immune challenge on growth, feed intake and feed efficiency in weaned pigs[1]

	Clean				Unclean					*P* values[2]		
Dietary SID Lys content, %	0.94	1.09	1.25	1.43	0.94	1.09	1.25	1.43	SEM	LysL	LysQ	Sanitation
BW initial, kg	7.2	7.2	7.2	7.2	7.2	7.3	7.2	7.3	0.16			
BW final, kg	12.7	13.5	14.3	14.6	12.0	12.8	12.7	13.8	0.29	<0.0001	0.924	0.003
ADG, g												
d 0 – 7	168	146	151	190	71	83	83	123	13.96	0.005	0.161	<0.0001
d 7 – 14	245	290	342	339	208	272	273	313	24.66	0.0001	0.478	0.039
d 14 – 21	380	471	523	529	406	434	440	497	27.02	<0.0001	0.455	0.112
d 0 – 21	264	302	339	352	228	263	266	311	14.13	<0.0001	0.960	<0.0001
ADFI, g												
d 0 – 7	257	258	255	290	227	232	209	230	15.35	0.408	0.249	0.001
d 7 – 14	505	480	500	496	401	436	403	418	25.90	0.961	0.995	<0.0001
d 14 - 21	662	677	687	742	637	635	606	604	32.57	0.553	0.667	0.004
d 0 – 21	475	472	481	509	422	435	406	417	19.47	0.567	0.579	<0.0001
G:F, g/kg												
d 0 – 7	654	554	603	658	300	435	412	547	51.89	0.027	0.452	<0.0001
d 7 – 14	486	623	679	677	520	616	679	751	38.19	<0.0001	0.323	0.384
d 14 -21	570	699	761	725	725	687	728	777	34.34	<0.0001	0.282	0.461
d 0 – 21	570	620	681	687	541	608	656	702	20.49	<0.0001	0.694	0.249

[1]N=6, 4 pigs per pen.

[2]LysL = Lys linear effect; LysQ = Lys quadratic effect; the interaction of Lys x Sanitation was not significant for all response criteria.

Despite the differences seen in ADG and ADFI between piglets in the two sanitation conditions, feed efficiency was similar, which concurs with results of Lee et al. (2005) showing that although feed intake for 3 to 8 weeks old pigs was lower under unclean conditions, feed efficiency was comparable to that of pigs raised under clean conditions. On the contrary, Williams et al. (1997) found a significant trend towards reduced G:F for 10 kg pigs that were subjected to high compared to those subjected to low level of environmental pathogen exposure. In their study, the high level of environmental pathogen exposure was created by not vaccinating the sows, farrowing sows in a nonsanitized room as well as piglets not receiving antibiotic treatment, whereas low level of environmental pathogen exposure was created using medicated early weaning. These contradicting observations could be explained by the differences in the immune challenge model used in the present study and above-mentioned study. Similarity in the feed efficiency of pigs raised under different sanitation conditions imply that although there were changes in metabolic processes that affected piglet performance, the efficiency of Lys utilization for protein accretion was unaffected presumably because Lys is assumed not to be involved in the immune function. It could also imply that feed and quantity of Lys intake were the main factors affecting the growth of piglets.

Although in the present study, the dietary Lys requirement for piglets raised under both clean and unclean sanitary conditions could not be determined by regression analysis, due to lack of plateau in ADG and G:F, the results show that the lysine requirement for piglets fed antibiotic free-diets is higher than NRC (1998) recommendation and could be similar to NRC (2012) recommendation. Unclean sanitary condition resulted in reduced growth rate and feed intake, but had no effect on feed efficiency.

CHAPTER FIVE

Dietary lysine requirement for 7 to 16 kg pigs fed antibiotic-free wheat-corn-soybean meal-based diets

Kahindi, R. K., Htoo, J. K., and C. M. Nyachoti

Kahindi R. K.: the graduate student made project proposal in conjunction with Drs. Htoo and Nyachoti. She did the research work, data analyses and wrote the manuscript.

Htoo J. K.: works for Evonik Industries who funded the project. He also assisted in data analyses as well the nutrient composition of the ingredients and diets.

Nyachoti C. M.: the supervising professor assisted in providing guidance throughout the research work and in the revision of the research paper.

5.1 ABSTRACT

Two experiments were conducted to determine the lysine requirement of weaned pigs [Duroc x (Yorkshire x Landrace)] with an average initial BW of 7 kg and fed antibiotic-free wheat-corn-soybean meal-based diets. The experiments were conducted for 21 d during which piglets had free access to diets and water. Average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain to feed ratio (**G:F**) were determined on d 7, 14, and 21. Blood samples were collected via jugular vein-puncture on d 0 and 14 to determine plasma urea nitrogen (**PUN**) concentration. In Exp. 1, 96 weaned pigs were housed 4 per pen and allocated to 4 dietary treatments with 6 replicates per treatment. The diets contained calculated standardized ileal digestible (**SID**) Lys content of 0.88, 1.10, 1.33, and 1.54%, respectively. The rest of the AA were provided to meet the ideal AA ratio for protein accretion. Increasing dietary Lys content linearly increased ($P < 0.05$) ADG and G:F. In Exp. 2, 90 piglets were housed 3 per pen and allocated to 5 dietary treatments with 6 replicates per treatment. The 5 diets contained calculated SID Lys content of 1.1 to 1.2, 1.3, 1.4, and 1.5%, respectively. Increasing dietary Lys content linearly increased ($P < 0.05$) ADG and G:F, linearly decreased ($P < 0.05$) d-14 PUN, and quadratically ($P < 0.05$) increased ADFI. The ADG data from Exp. 2 was subjected to 2 straight-line and quadratic broken-line regression analyses and the SID Lys requirement was determined to be 1.29 and 1.34%, respectively. On average, optimal dietary SID Lys content for optimal growth of 7 to 16 kg weaned piglets fed wheat-corn-SBM-based diets was estimated to be 1.32%, at this level the ADG and ADFI were 444 and 560 g, respectively. Thus representing an SID Lys requirement, expressed on daily intake basis as, 7.4 g/d or 16.76 mg/g gain.

Key words: lysine, pig, growth, antibiotic-free diets

5.2 INTRODUCTION

Amino acids are required by the body for protein accretion, immune response and regulation of metabolic pathways (Wu, 2010). The need for a specific AA depends on the physiological status, breed, and the health of the animals. Lysine is an essential AA and first limiting in cereal-based diets for pigs. Lysine is mainly used for body protein synthesis which accounts for about 80% in young animals (Klasing, 2009). Weaner pigs (5 to 20 kg) in the 90's had an average daily gain (**ADG**) of 350 g; however current research shows that the piglets of similar body weight have a potential of gaining up to 500 g/day which is as a result of progress in breeding for fast growing, lean pigs (NRC, 1998; Dean et al., 2007). Consequently, Lys requirement of the modern pig would have to change to match its growth demand. The increased Lys requirement has been reported in recent studies with pigs of different body weight (Gaines et al., 2003; Dean et al., 2007; Shelton et al., 2011; Nemeschek et al., 2012; NRC, 2012).

Post-weaning diarrhea reduces piglet growth performance but this effect has been counteracted by addition of antimicrobial growth promoters (**AGP**) to piglet diets. However, long-term use of AGP has been linked to the potential problem of increasing transferable resistance of bacteria to antimicrobial drugs in humans. Within the last decade, the European Union banned use of in-feed AGP in pork production and North America is facing voluntary withdrawals. The use of AGP reduces the microbial load and helps to maintain gut health. Pigs fed without AGP will have reduced performance (Cromwell, 2002) and the requirement for specific AA which are involved in immune response might be modified. However, research on AA requirement for piglets fed antibiotic-free diets is rather limited. Therefore, the objective of this study was to determine the Lys requirements to optimize growth performance of 7 to 16 kg weaned piglets fed antibiotic-free diets.

5.3 MATERIALS AND METHODS

5.3.1 General

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

The experiments were conducted for 21 d with mixed-sex piglets [Duroc x (Yorkshire x Landrace)] sourced from the University of Manitoba's Glenlea Swine Research Unit. A common starter diet containing 1.2 % standardized ileal digestible (**SID**) Lys and 18 % CP was fed during the adaptation period of 4 and 5 d, in Exp. 1 and 2, respectively. Pigs were housed in temperature-controlled room with the initially temperature set at 30^{o}C that was reduced by 1^{o}C per wk.

5.3.2 Experiment 1

Ninety six piglets (initial BW 7.13 ± 0.43 kg) were randomly allocated to 4 dietary treatments in a completely randomized design. Wheat-corn-soybean meal-based diets free of AGP contained 4 increasing levels of dietary SID Lys (0.88, 1.10, 1.33 and 1.54%) that were obtained through increasing dietary soybean meal and crystalline Lys contents. The essential AA in the diets were balanced to meet the ideal protein ratio (Chung and Baker, 1992). Piglets were housed 4 per pen to yield 6 replicates in a room with 1.2 x 1.8 m expanded metal floor pens. Diets were offered *ad libitum* and water was accessed via low pressure drinking nipple. Animals and feeders were weighed weekly to determine average daily gain (**ADG**), average feed intake (**ADFI**), and gain to feed ratio (**G:F**). The G:F was calculated on per pen basis by dividing ADG by ADFI.

5.3.3 Experiment 2

Ninety piglets (initial BW of 6.9 ± 0.5 kg) were randomly assigned to 5 dietary treatments in a completely randomized design. Wheat-corn-soybean meal-based diets free of AGP contained 5 increasing levels of dietary SID Lys (1.1, 1.2, 1.3, 1.4, and 1.5%). Initially 2 diets were made to contain 1.1 and 1.5% SID Lys content, respectively, and the other 3 diets were made from blending the low and high Lys diets in the ratios of 3:1, 1:1, and 1:3. The essential AA were balanced to meet the ideal AA ratio for protein accretion (Chung and Baker, 1992). Diets were offered *ad libitum* and water was accessed via low pressure drinking nipple. The piglets were housed 3 pigs per pen yielding 6 replicates per treatment, in a room with 1.2 x 1.8 m expanded metal floor pens. The ADG, ADFI and G:F were measured as described in Exp. 1. Blood samples were collected via jugular vein-puncture on d 0, 7, and 14 into 10 ml heparinized vacutainers tubes (BD Vacutainer, Franklin Lakes, NJ). The blood samples were centrifuged at 3, 000 rpm for 15 min at 4^{o}C to harvest plasma for determination of plasma urea nitrogen (**PUN**) using blood urea colorimetric slides (VITROS®, Rochester, NY).

5.3.4 Diet and ingredients analyses

For Exp. 1 the AA analysis for diets and ingredients was done using near infra-red spectrophotometry (**NIRS**). For Exp. 2, Dietary AA concentrations in ingredients and diet samples were determined by ion-exchange chromatography with postcolumn derivatization with ninhydrin. Amino acids were oxidized with performic acid, which was neutralized with Na metabisulfite (Llames and Fontaine, 1994; Commission Directive, 1998). Amino acids were liberated from the protein by hydrolysis with 6 *N* HCL for 24 h at 110°C and quantified with the

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

5.3.5 Data analysis

Data were subjected to analysis of variance using Proc mixed procedures of SAS 9.2 (SAS Inst. Inc., Cary, NC). The data were analyzed as a completely randomized design and pen was considered the experimental unit. Initial BW was used as a covariate for performance responses in Exp. 2 but not in Exp. 1 because it did not affect any response. For PUN, d 0 values were used as covariates while analysing d 7 and 14 responses. Effect of dietary Lys content was also analysed using linear and quadratic polynomial contrasts. The ADG, G:F and PUN data was fitted to linear and quadratic broken-lines (Vedenov and Pesti, 2008) to estimate the optimum SID Lys content. Statistical significance level was claimed at $P < 0{\cdot}05$ and a trend at $P > 0.05 < 0.10$.

Table 5.1 Ingredient composition of Exp. 1 diets

	Dietary SID Lys content, %			
Item	1.08	1.22	1.33	1.65
Ingredient, %				
Soybean meal, 46.5% CP	19.00	19.00	19.00	18.50
Wheat	28.00	28.00	27.00	26.00
Corn	28.50	28.00	28.00	28.00
Corn gluten meal	3.00	3.00	4.00	5.00
Corn starch	4.61	4.41	4.08	3.75
Lactose	8.00	8.00	8.00	8.00
Limestone	1.02	1.02	1.02	1.01
Dicalcium phosphate	1.55	1.55	1.55	1.55
Vegetable oil	4.50	4.50	4.00	4.00
Salt	0.50	0.50	0.50	0.50
Vitamin premix[1]	0.50	0.50	0.50	0.50
Mineral premix[2]	0.50	0.50	0.50	0.50
L-Lysine	0.29	0.58	0.86	1.14
DL-Methionine	0.00	0.12	0.23	0.34
L-Threonine	0.03	0.17	0.30	0.44
L-Tryptophan	0.00	0.04	0.08	0.12
L-Isoleucine	0.00	0.00	0.13	0.25
L-Valine	0.00	0.11	0.25	0.40
Total	100.00	100.00	100.00	100.00

[1]Supplied the following per kilogram of diet: 8,250 IU of vitamin A, 835 IU of vitamin D3, 40 IU of vitamin E, 25 µg of vitamin B12, 4 mg of vitamin K, 25 mg of niacin, 600 mg of choline, 12 mg of riboflavin, 200 µg of biotin, 4.5 mg of pyridoxine, 4 mg of folic acid; 2 mg of thiamin.

[2]Supplied the following per kilogram diet: 50 mg of Mn , 150 mg of Zn, 120 mg of Fe, 25 mg of Cu, 0.35 mg of Se, 0.4 mg of I.

Table 5.2 Ingredient composition of Exp. 2 diets

	Dietary SID lysine content, %				
	1.03	1.25	1.31	1.36	1.51
Ingredients, %					
Soybean meal, 46% CP	28.00	28.65	29.30	29.95	30.60
Wheat	15.00	15.00	15.00	15.00	15.00
Corn	37.40	36.41	35.42	34.42	33.43
Corn gluten meal	3.00	3.00	3.00	3.00	3.00
Corn starch	0.00	0.00	0.00	0.00	0.00
Lactose	8.00	8.00	8.00	8.00	8.00
Limestone	0.73	0.75	0.77	0.78	0.80
Dicalcium phosphate	1.95	1.95	1.95	1.94	1.94
Vegetable oil	4.00	4.04	4.08	4.12	4.16
Iodized salt	0.35	0.35	0.35	0.35	0.35
Vitamin premix[1]	0.50	0.50	0.50	0.50	0.50
Mineral premix[2]	0.50	0.50	0.50	0.50	0.50
L-Lysine	0.32	0.43	0.54	0.65	0.76
DL-Methionine	0.12	0.16	0.21	0.26	0.31
L-Threonine	0.10	0.15	0.20	0.24	0.29
L-Tryptophan	0.04	0.05	0.07	0.09	0.11
L-Isoleucine	0.00	0.02	0.04	0.06	0.07
L-Valine	0.00	0.05	0.09	0.14	0.19
Total	100.00	100.00	100.00	100.00	100.00

[1]Supplied the following per kilogram of diet: 8,250 IU of vitamin A, 835 IU of vitamin D3, 40 IU of vitamin E, 25 µg of vitamin B12, 4 mg of vitamin K, 25 mg of niacin, 600 mg of choline, 12 mg of riboflavin, 200 µg of biotin, 4.5 mg of pyridoxine, 4 mg of folic acid; 2 mg of thiamin.

[2]Supplied the following per kilogram diet: 50 mg of Mn , 150 mg of Zn, 120 mg of Fe, 25 mg of Cu, 0.35 mg of Se, 0.4 mg of I.

5.4 RESULTS

5.4.1 Experiment 1

Piglets were healthy throughout the study. The SID Lys contents corrected from the analyzed Lys values were 1.01, 1.22, 1.33 and 1.65% in diets 1, 2, 3, and 4, respectively, (Table 5.3). Response data on ADG, ADFI and G:F ratio are given in Table 5. Final BW linearly increased ($P < 0.0001$) with increasing dietary SID Lys content. Increasing dietary SID Lys linearly increased ($P < 0.05$) ADG in wk 2 and 3, and quadratically increased ($P < 0.05$) ADG in wk 3. Similarly, increasing dietary SID Lys content linearly and quadratically increased ($P < 0.05$) ADFI in wk 3. In contrast, ADFI was similar ($P > 0.05$) across the dietary treatments for wk 1 and 2, respectively. However, the overall ADFI was not affected by increasing dietary SID Lys content. Increasing dietary Lys content linearly increased ($P < 0.0001$) G:F throughout the trial. Due to lack of a plateau in the G:F the broken line analysis was fitted only for ADG.

Table 5.3 Calculated and analyzed nutrient composition of Exp. 1 diets (as fed basis)

	Dietary SID Lys content, %			
Item	0.99	1.23	1.51	1.81
Calculated nutrient content				
ME, MJ/kg	14.56	14.55	14.45	14.46
NE, MJ/kg	10.49	10.47	10.40	10.40
CP, %	19.30	19.25	19.75	19.99
Total Lys, %	0.98	1.20	1.42	1.63
SID Lys, %	0.87	1.09	1.31	1.51
SID Met, %	0.26	0.37	0.49	0.61
SID Met+Cys, %	0.52	0.64	0.76	0.89
SID Thr %	0.55	0.69	0.83	0.97
SID Ile %	0.61	0.61	0.75	0.88
SID Trp %	0.17	0.21	0.25	0.29
SID Val %	0.68	0.79	0.94	1.10
Analyzed nutrient content %				
CP	18.60	18.39	19.63	20.55
Total Lys	1.11	1.35	1.64	1.94
SID Lys	0.99	1.23	1.51	1.81
Total Met	0.29	0.40	0.52	0.64
Total Met+Cys	0.61	0.72	0.85	0.96
Total Thr	0.68	0.81	0.95	1.11
Total Trp	0.20	0.24	0.27	0.31
Total Val	0.83	0.93	1.03	1.28

5.4.2 Experiment 2

Piglets used in this experiment were received from a barn with streptococcus outbreak therefore; all piglets were injected once with a long acting antibiotic (Duplocillin® LA, Wellington, NZ) at the beginning of the study. However, the piglets were healthy throughout the study and maintained normal behaviour.

The calculated and analyzed nutrient compositions of the diets are given in Table 5.4. Formulated dietary SID Lys contents (1.1, 1.2, 1.3, 1.4, and 1.5%) were close to the values calculated from analyzed Lys contents (1.03, 1.25, 1.31, 1.36, and 1.51%). In addition, the analyzed values for other AA were similar to calculated values.

Effects of dietary Lys content on final BW, ADG, ADFI, G:F, and PUN contents are given in Table 5.6. Increasing dietary Lys content linearly increased ($P < 0.05$) final BW and overall ADG, quadratically increased ($P < 0.05$) ADG and tended to increase ($P = 0.06$) final BW. In contrast, there was no linear ($P > 0.10$) but a quadratic ($P < 0.05$) effect on the overall ADFI with increasing dietary Lys content. Increasing dietary Lys content lineally increased ($P < 0.05$) G:F ratio in wk 2 but not wk 1 and 3. Thus, the overall G:F had only a linear ($P < 0.01$) response to increasing dietary Lys content. Increasing dietary Lys content linearly ($P < 0.01$) but not quadratically reduced PUN content both on d 7 and 14. The decline in PUN content was steady up to 1.36% SID Lys and the decrease was slower thereafter.

The broken line analyses showed that the optimum SID Lys contents to be 1.30 and 1.34%, for ADG and 1.25 and 1.56% for G:F using linear and quadratic broken-lines, respectively, (Table 5.7). Thus, based on ADG and G:F averages the corresponding optimal SID Lys content were 1.32 and 1.41%, respectively.

Table 5.4. Calculated and analyzed nutrient composition of Exp. 2 diets (as fed basis).

	Dietary SID Lys content, %				
	1.03	1.25	1.31	1.36	1.51
Calculated nutrient content					
ME, MJ/kg	14.45	14.45	14.45	14.45	14.46
NE	10.40	10.40	10.40	10.40	10.40
CP, %	19.86	20.08	20.31	20.54	20.76
Total Lys, %	1.23	1.33	1.43	1.53	1.64
SID Lys, %	1.10	1.20	1.30	1.40	1.50
SID Met	0.40	0.45	0.50	0.55	0.60
SID Met+cys	0.70	0.75	0.80	0.85	0.91
SID Thr %	0.72	0.78	0.83	0.89	0.94
SID Ile %	0.71	0.74	0.77	0.80	0.83
SID Val %	0.78	0.84	0.90	0.95	1.01
SID Trp %	0.24	0.26	0.28	0.30	0.32
Analyzed nutrient content, %					
CP	18.68	21.34	20.55	19.98	20.70
Total Lys	1.15	1.38	1.44	1.48	1.65
SID Lys	1.03	1.25	1.31	1.36	1.51
Total Met	0.44	0.45	0.52	0.66	0.66
Total Met+Cys	0.75	0.79	0.85	0.98	0.98
Total Thr	0.80	0.89	0.92	1.00	1.03
Total Ile	0.76	0.88	0.88	0.85	0.90
Total Val	0.85	1.00	1.02	1.05	1.12
Total Trp	0.26	0.30	0.31	0.33	0.34

Table 5.5 Effect of dietary Lys on growth, feed intake and feed efficiency of weaned pigs fed antibiotic free diets. Exp. 1[1]

	Dietary SID Lys content, %					*P*-value	
Item	0.99	1.23	1.51	1.81	SEM	Linear	Quadratic
Body weight, kg							
Initial	7.13	7.13	7.13	7.13	0.19	-	-
Final	13.31	13.97	15.04	15.31	0.28	0.001	0.649
Average daily gain g/d							
wk 1	137	120	174	142	11.2	0.193	0.522
wk 2	359	364	444	467	27.6	0.004	0.744
wk 3	325	462	515	549	18.9	0.001	0.015
wk 1 to 3	291	325	379	386	13.9	0.001	0.333
Average daily feed intake, g/d							
wk 1	250	217	246	250	12.8	0.518	0.576
wk 2	569	511	567	570	26.2	0.619	0.259
wk 3	745	842	892	856	25.9	0.005	0.020
wk 1 to 3	535	524	563	551	14.1	0.189	0.993
Gain to feed ratio, g/kg							
wk 1	562	550	711	631	39.5	0.062	0.405
wk 2	626	714	806	815	32.3	0.001	0.024
wk 3	440	552	578	641	26.6	0.001	0.383
wk 1 to 3	548	619	644	704	12.9	0.001	0.237

[1]N=6, 4 pigs per pen.

Table 5.6 Effect of dietary Lys on growth, feed intake and feed efficiency of weaned pigs fed antibiotic-free diets. Exp. 2[1]

	Dietary SID Lys level						*P*-value	
	1.03	1.25	1.31	1.36	1.51	SEM	Linear	Quadratic
Body weight, kg								
Initial	6.86	6.85	6.85	7.09	6.9	0.27	-	-
Final	13.91	15.44	15.75	15.74	15.98	0.39	0.003	0.063
Average daily gain, g								
wk 1	198	238	240	223	239	26.1	0.443	0.515
wk 2	359	454	476	503	544	26.2	0.001	0.218
wk 3	463	566	561	535	541	41.0	0.381	0.177
wk 1 to 3	339	421	441	429	443	17.7	0.001	0.019
Average daily feed intake, g								
wk 1	235	265	309	239	283	29.2	0.484	0.455
wk 2	538	600	643	552	588	29.7	0.598	0.107
wk 3	769	819	853	814	770	56.2	0.984	0.234
wk 1 to 3	501	550	560	543	545	22.3	0.335	0.034
Gain to feed, g/kg								
wk 1	766	901	779	928	811	38.7	0.541	0.084
wk 2	677	776	752	909	909	46.6	0.001	0.931
wk 3	595	701	658	673	708	45.5	0.213	0.648
wk 1 to 3	670	749	740	802	813	31.8	0.004	0.617
Plasma urea nitrogen[2], mmol/L								
d 0	3.55	2.97	4.03	2.58	3.83	0.42	0.899	0.507
d 7	3.59	2.93	2.96	1.42	1.21	0.53	0.001	0.733
d 14	2.72	2.45	1.41	1.63	1.00	0.32	0.001	0.502

[1]N=6, 3 pigs per pen.

[2]The d 0 values were used as covariates in analysis of values from subsequent days.

Table 5.7. The standardized ileal digestible Lys estimates from Exp. 2.

	Linear broken-line					Quadratic broken-line				
			95% confidence limit					95%confidence limit		
	Estimate	SE	Lower	Upper	R^2	Estimate	SE	Lower	Upper	R^2
Exp. 2										
ADG	1.29	0.03	1.18	1.41	0.98	1.34	0.01	1.29	1.38	0.97
G:F	1.25		Failed to converge			1.56		Failed to converge		

5.5 DISCUSSION

The optimal Lys requirements for weaned pigs may differ among the response criteria and due to factors such as the breed, diet, and environmental conditions. It has also been shown that pigs fed diets containing AGP have faster growth rates and higher feed efficiencies compared to those without in feed AGP (Bikker et al., 2006). Thus, differences in piglet performance create a possibility that Lys requirement between piglets on in-feed antibiotic and antibiotic-free feeding regimens would differ. The two experiments were conducted to establish Lys requirement for weaned pigs fed antibiotic-free diets using growth, feed intake, feed efficiency and PUN as response criteria. For Exp. 1 the AA analysis for diets and ingredients was done using near infra-red spectrophotometry (**NIRS**). The analyzed AA content in the diets were very different from calculated values from wet chemistry. Thus there was a wide gap between 2 diets containing the highest SID Lys content (1.51 and 1.81%) and NIRS was found not a suitable method for diet analysis. A second experiment was conducted to verify these findings and all the ingredients and diets were analyzed using wet chemistry. Hence, only the results from Exp. 2 shall be discussed. The composition of experimental diets was optimized since the diets were formulated according to ideal protein concept (Chung and Baker, 1992). Also, the ME content was within NRC (2012) recommendations allowing for SID Lys:ME ratios that would maximize piglet performance (Roth et al.,1999; Oresanya et. al., 2007).

The ADG was not affected by dietary Lys content in the first wk; such performance could be attributed to a low feed intake normally observed in piglets immediately after weaning (Dong and Pluske, 2007). However, the linear increase in dietary SID Lys content resulted in 31% increase in the higher overall ADG for piglets fed the highest SID Lys diet compared to the lowest SID Lys diet. A positive correlation between ADG and dietary Lys content has also been

observed in growing pigs of different BW (Coma et al., 1995; Kendall et al., 2008; Moore et al., 2013).

The optimum SID Lys estimates based on linear and quadratic broken-lines were 1.30 and 1.34% for ADG and 1.25 and 1.56% for G:F. Due to lack of a plateau the quadratic broken-line the estimated G:F optimum was beyond dietary SID Lys content used in the present diets. Using averages of the broken-line values it was noted that G:F gave higher estimates of SID Lys requirement compared to ADG (i.e. 1.41 vs. 1.32%). Similar observations where G:F resulted in higher optimum Lys requirement than ADG have been reported (Friesen et al., 1994; Gaines et al., 2003; Yi et al., 2006; Nemechek et al., 2012). For instance, Gaines et al. (2003) reported SID Lys of 1.42 and 1.52% to be optimal for ADG and G:F, respectively, whereas Nemechek et al. (2012) had optimal SID Lys for ADG at 1.30% and 1.37% for G:F in 7 to 14 kg pigs. Similarly, findings of Yi et al. (2006) reported that SID Lys values of 1.3 and 1.4% gave maximum ADG and G:F, respectively, for 11 to 26 kg pigs. Hence, the results from the present study agree with results of studies showing that SID Lys requirement would differ depending on the response criteria. The ADG response criterion determines the overall body weight increase that is, bones, muscles and visceral organs. Therefore, if the body weight increase is more towards visceral organ then more nutrients will be partitioned towards maintenance requirement. However, the G:F ratio indicates how much a nutrient can be used for a production function, hence the greater the ratio the higher the nutrient partitioning towards a production function. Therefore, the Lys requirement for G:F will be higher than ADG. The differences have been attributed to the need for AA for purposes other than growth, hence even as maximal growth is attained there could still be a metabolic demand for Lys (Ball et al., 2007).

Plasma urea N is an indicator of protein utilization and change in PUN concentration reflects use of AA for either anabolic or catabolic purposes (Coma et al., 1995). The PUN concentration should decrease as the dietary content of a limiting AA increases and plateau as the optimum requirement is attained. In this study, the dietary AA were offered on an ideal ratio for protein accretion but PUN values could not be used to estimate the optimum SID Lys requirement. The PUN content linearly decreased with increasing dietary Lys content and although there was a point of inflection, showing that there was reduced response with increasing input, this response was not strong enough to cause plateau. Therefore, the PUN could not be used to estimate the optimum SID Lys requirement in this study.

The optimum SID Lys estimate for 7 to 16 kg (1.32%) based on ADG is higher than NRC (1998) recommendation (1.19%) for 5 to 10 kg pigs, but similar to 1.35% recommendation by NRC (2012) for 7 to 11 kg pigs. Pigs are constantly selected for leanness and fast growth rates and as a consequence, their Lys requirement for protein accretion is higher and is as suggested by NRC (2012). The optimum SID Lys requirement determined in the present study is in close agreement with values reported by Oresanya et al. (2007; 1.33%) for 7.5 to 13 kg pigs and Nemechek et al. (2012; 1.30 to 1.37%) for 7 to 14 kg pigs. However, Gaines et al. (2003) and Dean et al. (2007) reported slightly higher values of 1.42 and 1.40% SID Lys, for 7 to 14 and 6 to 12 kg PIC pigs, respectively. These variations in Lys requirement could be due to differences in the diets used as well as the pig's genotype. For example, Cameron et al. (2003) predicted 30% greater Lys requirement for pigs with high than low lean growth rates.

The overall ADFI also showed a quadratic response to increasing dietary Lys content, a response that was similar to ADG. The lower feed intake by the piglets receiving diet with lowest Lys content could be attributed to less preference to AA-deficient diets (Henry, 1993;

Ettle and Roth, 2009). Also, piglets on Lys deficient diet gained less BW and thus they were the lightest. A study by Bruininx et al. (2001) demonstrated that piglet eats according to BW pattern and their feed consumption increases with BW.

At 1.32% SID Lys, the ADG and ADFI were 444 and 560 g, respectively. Hence, the SID Lys requirement expressed on a g/day and mg/g gained basis, was 7.4 g Lys/d and 16.76 mg Lys/g gain, respectively. A study by Nemechek et al. (2012) reported Lys requirement values of 17 to 19 mg Lys/g gain at SID Lys 1.30 to 1.32% and Gaines et al. (2003) reported values of 17.08 to 18.5 mg Lys/ g gain. Some of the mechanisms by which AGP improves performance are thought to be through reduction of gut microbiota that competes with host for nutrients and through minimizing proliferation of pathogenic microbes (Bikker et al., 2006). Thus, it was expected that piglets on diets without in-feed AGP would have lower growth performance and hence slightly lower Lys requirement compared to those on AGP diets. However, the estimated SID Lys requirement when expressed on mg/g gain in the present study compared with some studies where AGP were used (Oxytetracyline, 22 g/kg; Dean et al., 2007), (Linomycin, 22 g/kg and streptomycin, 22 g/kg; Oresanya et al., 2007), and (Carbodox, 27.5 mg/kg; Nemechek et al., 2012), thus suggesting Lys requirement was not changed due to antibiotic-free feeding regime. However, the high sanitation standards maintained could have contributed to the pigs' well-being, through prevention of increased AA requirement caused by illness (Le, Floc'h, 2006).

In conclusion the SID Lys requirement for 7 to 16 kg pigs fed antibiotic-free wheat-corn-soybean meal-based diets ranged between 1.29 and 1.34% for ADG, with an average of 1.32%. The ADG and ADFI corresponding to 1.32% SID Lys were 444 and 560 g, respectively. This represents a SID Lys requirement, expressed on daily intake basis to be, 7.4 g/d.

CHAPTER SIX

Optimal sulfur amino acids to lysine ratio for weaned pigs fed antibiotic-free wheat-corn-soybean meal-based diets and raised under clean and unclean conditions

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Kahindi R. K.: the graduate student made project proposal in conjunction with Drs. Htoo and Nyachoti. She did the research work, data analyses and wrote the manuscript.

Htoo J. K.: works for Evonik Industries who funded the project. He also assisted in data analyses as well the nutrient composition of the ingredients and diets.

Nyachoti C. M.: the supervising professor assisted in providing guidance throughout the research work and in the revision of the research paper.

6.1 ABSTRACT

Unsanitary production conditions can stimulate an immune response leading to increased sulfur amino acids (**SAA**) maintenance needs and consequently increasing the SAA to lysine ratio (**SAA:Lys**), especially under antibiotic-free feeding regimen. Two 14-d experiments were conducted to determine the optimum SAA:Lys ratio in piglets when reared under clean or unclean condition and fed antibiotic-free diets. For each experiment, 90 mixed-sex pigs (Duroc x [Yorkshire x Landrace]; initial average BW of 7.3 ± 0.6 kg) weaned at 21 ± 1 d with 6 replicates of 3 pigs per pen were used. The diets were corn-wheat-soybean meal-based (1.18% standardized ileal digestible (**SID)** Lys; 51% SID SAA:Lys). Diets 2 to 5 were the basal diet supplemented with 4 graded levels of DL-Met (55, 60, 64 and 68% SID SAA:Lys). Piglets were allowed free access to feed and water. In Exp. 1, piglets were raised for 13 days in a clean room that was previously disinfected and washed weekly, whereas in Exp. 2, piglets were introduced into a room previously occupied by other pigs and was not disinfected. In addition, manure slurry from a sow herd was spread (5 kg per pen) on d 0 and 7 of the study and the room was not cleaned throughout the study. Blood was collected on d 0 and 13 or 14 for Exp. 1 and 2, respectively, for measurement of plasma urea nitrogen (**PUN**). At the end of the experiment, one pig per pen was slaughtered to collect jejunal tissue for measurement of villus height (**VH**), crypt depth (**CD**), and VH:CD. In both experiments, quadratic broken-line model was used to estimate optimum SAA:Lys ratio. The highest average daily gain was at SAA:Lys ratio of 60% in Exp. 1 and 64% in Exp. 2. Increasing SAA:Lys ratio linearly increased ($P < 0.05$) VH and VH:CD in Exp. 2 and both linearly and quadratically in Exp. 1. In Exp. 2, increasing SAA:Lys ratio linearly reduced ($P < 0.05$) average daily feed intake and linearly and quadratically decreased PUN. Based on response criteria, the optimum SAA:Lys was 58 and 61 for piglets raised under clean and unclean conditions, respectively, using ADG. However, using VH the optimum SAA:Lys

was 60 and 66 under clean and unclean conditions, respectively. Hence SAA:Lys for gut health was higher under unclean conditions.

Key words: Pigs, Sanitary condition, Sulfur amino acids

6.2 INTRODUCTION

Methionine requirement varies depending on body weight of the pig, sex, and health status. Cysteine and Met form the dietary sulfur amino acids (**SAA**) that are used for protein synthesis. Methionine is metabolized to Cys and 50% of the required dietary SAA can be provided by Cys (Chung and Baker, 1992a). In piglets, 30% of the total dietary Met is used by the splanchnic tissue, as a source of energy, for synthesis of mucoproteins and mucin, antioxidant and maintenance of redox potential (Stipanuk, 2004; Stoll and Burrin, 2006). Some of the SAA is utilized by the gut commensal bacteria thereby preventing attachment to the intestinal wall by pathogenic microbes (Dahiya et al., 2007).

Piglets fed diets containing antimicrobial growth promoters (**AGP**) have less intestinal bacterial mass, thinner intestinal wall and less mucin production compared to those without in-feed AGP (Dibner and Richards, 2005). Since feeding pigs AGP-free diets increases mucin production and intestinal thickness, there would be a great allocation of SAA towards maintenance. Thus, to maintain similar growth to AGP-fed counterpart the pig would either increase feed intake or require increased dietary SAA content. Increasing SAA requirement eventually increases the standardized ileal digestible (**SID**) SAA:Lys ratio. The current SID SAA:Lys ratio for 5 to 12 kg piglets ranges from 50 to 60 (Chung and Baker, 1992b; Chung and Baker, 1992c; Gaines et al., 2005; Dean et al., 2007; NRC, 2012).

Piglets raised under unsanitary conditions had lower protein retention; consequently their Lys requirement was (g/day) was lower than the sanitary group (Williams et al., 1997). Unsanitary housing conditions cause moderate immune system activation in piglets (Le Floc'h et al., 2006) thus partitioning amino acids towards an immune response rather than protein accretion. It was hypothesized that pigs in AGP-free feeding regimen and when raised under unclean housing conditions will have increased SAA requirement, consequently a high SID SAA:Lys ratio. Thus, the objective of the study was to determine optimum SID SAA:Lys ratio for piglets fed AGP-free diets and raised under clean or unclean housing conditions.

6.3 MATERIALS AND METHODS

6.3.1 General

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

6.3.2 Experiment 1 and 2

The experiments were conducted for 13 or 14 days at the University of Manitoba's T. K. Cheung Center for Animal Research with male and female piglets sourced from the University of Manitoba Glenlea Swine Research farm. For each experiment, 90 piglets (Duroc x [Yorkshire x Landrace]; initial average BW of 7.3 ± 0.63 kg) weaned at 21 ± 1 d with 6 replicates of 3 pigs per pen (1.2 x 1.8 m) were used. A common starter diet containing 1.2 % SID Lys and 18 % CP was fed for 5 days before the start of experiments. Five experimental diets offered in a completely randomized design were wheat- corn-soybean meal-based (1.18% SID Lys and 51,

55, 60, 64, and 68 SID SAA:Lys; Table 6.1). The 1.18% SID Lys was marginally limiting according to work by Kahindi et al. (2014a) who reported that the SID Lys requirement for 7 to 16 kg piglet fed AGP-free diets was 1.32%. The graded levels of SID SAA:Lys were attained through replacing cornstarch with crystalline Met and the contents of essential AA were similar for all diets and balanced to meet the ideal AA ratio for protein accretion (Chung and Baker, 1992c). However, the analyzed lysine content for diets 3, 4 and 5 was higher (1.24% SID lysine) than calculated. Piglets were allowed free access to feed and water. The pigs were housed in temperature-controlled room with an initially temperature of 30^{o}C that was reduced by 1^{o}C per week.

Piglets and feeders were weighed on day 6 and 13 for Exp. 1 and weekly in Exp. 2 to determine average daily gain (**ADG**), average feed intake (**ADFI**), and gain to feed ratio (**G:F**). The G:F was calculated on per pen basis by dividing ADG by ADFI. Blood samples were collected via jugular vein-puncture on d 0 and 13 or 14 into 10 ml heparinized vacutainers tubes (BD Vacutainer, Franklin Lakes, NJ). The blood samples were centrifuged at 3,000 rpm for 15 min at 4^{o}C to harvest plasma for determination of urea nitrogen (**PUN**) using blood urea colorimetric slides (VITROS®, Rochester, NY). The piglets were monitored for the incidences and severity of diarrhea was assessed using the fecal consistency score on day 6 and 13 for Exp. 1 and weekly in Exp. 2 using method of Marquardt et al. (1999). Fecal consistency scoring was (1 = normal; 2 = soft feces; 3 = mild diarrhea and 4 = severe diarrhea). Air quality status in the rooms was analyzed thrice each week. The air samples taken were hydrogen sulphide on a JEROME 631-X machine (Arizona Instrument Corporation, Phoenix, AZ) and ammonia measured using detector tube (RAE Systems, San Jose, CA).

One pig per pen was slaughtered on d 13 and 14 for Exp. 1 and 2, respectively, to determine jejunal morphology (villus height, crypt depth and villus heght:crypt depth ratio). A 1-cm section of the mid ileum was collected, rinsed with cold phosphate buffed solution and stored in 10% buffered formalin to fix the villi and the crypts. The sections were processed for histological examination using the standard hematoxylin and eosin method. Villus height (**VH**; the tip of the villous to the crypt-villous junction) and crypt depth (**CD**; the crypt-villous junction to the base) were measured on 10 intact, well-oriented villi per specimen using a compound light microscope equipped with a video camera.

In Exp. 1, piglets were kept in a room maintained as per standard operating procedures of the University of Manitoba's T. K. Cheung Center for Animal Research. In Exp. 2, the piglets were introduced into a room previously occupied by the Exp. 1 pigs without the room being cleaned at the end of week 3 to allow for manure build up. Also, manure slurry from a sow herd (5 kg per pen) was spread on the expanded metal floor before piglets were introduced into the room to further enhance unclean conditions. The spread of manure slurry (5 kg per pen) from the same source was repeated 1 week after the introduction of the piglets and the room was not cleaned during the rest of the experimental period.

6.3.3 Diet and ingredients analyses

Dietary AA concentrations in ingredients and diet samples were determined by ion-exchange chromatography with postcolumn derivatization with ninhydrin. Amino acids were oxidized with performic acid, which was neutralized with Na metabisulfite (Llames and Fontaine, 1994; Commission Directive, 1998). Amino acids were liberated from the protein by

hydrolysis with 6 *N* HCL for 24 h at 110°C and quantified with the internal standard by measuring the absorption of reaction products with ninhydrin at 570 nm. Tryptophan was determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm), after alkaline hydrolysis with barium hydroxide octahydrate for 20 h at 110°C (Commission Directive, 2000). Tyrosine was not determined.

6.3.4 Data analyses

Data were analyzed using Proc mixed procedures of SAS 9.2 (SAS Inst. Inc., Cary, NC). The data were analyzed as a completely randomized design and pen was considered the experimental unit for performance responses and fecal scores, while piglet was experimental unit for PUN and histomorphological data. Initial BW was used as a covariate for performance responses. Whereas, d 0 PUN values were used as covariates while analysing d 13 or 14 responses. Effects of increasing SAA:Lys ratios were also analyzed using linear and quadratic polynomial contrasts. The ADG, G:F, VH, CD, VH:CD, and PUN data were fitted broken-line regression (Vedenov and Pesti, 2008) to estimate the optimum SAA:Lys ratio. Though all the response criteria were fitted to the broken-line some failed to converge for example ADG and VH:CD from the unclean sanitary group. Polynomial regression was used for unclean group's ADG to obtain the requirement estimate. Statistical significance level was claimed at $P < 0{\cdot}05$ and a trend at $0.05 > P < 0.10$.

6.4 RESULTS

In Exp. 1, increasing dietary SID SAA:Lys ratio quadratically increased ($P < 0.05$) ADG in wk 2 (Table 6.2) but showed no effect ($P > 0.10$) on the overall ADG. Increasing SID SAA:Lys ratio linearly increased wk 2 and overall ADFI, also tended to linearly increase ($P < 0.10$) wk 2 G:F. The VH and VH:CD ratio were linearly and quadratically increased ($P < 0.05$) with increasing SID SAA:Lys, however, the CD did not show any significant effects (Table 6.4). Increasing SID SAA:Lys ratio resulted in a trend towards a linear decrease in PUN. Ammonia and hydrogen sulphide concentrations ranged between 25 to 32 and 0.06 to 0.07 ppm (Table 6.5). For all the response criteria apart from ADG the optimum SAA:Lys ratio was 60 (Table 6.6). Using ADG the optimum SAA:Lys ratio was 61.

In Exp. 2, increasing SID SAA:Lys ratio linearly reduced ($P < 0.05$) ADFI but had no effect on the G:F (Table 6.3). Increasing SAA:Lys ratio linearly and quadratically decreased ($P < 0.05$) PUN and linearly increased ($P < 0.05$) VH and VH:CD (Table 6.4). Table 6.5 shows the ammonia and hydrogen sulphide concentration in the rooms over time. Ammonia concentrations ranged between 32 to 38 ppm, whereas the hydrogen sulphide concentration was between 0.14 to 0.20 ppm. The optimum SAA:Lys ratio were 66, 58, and 63 when using VH, G:F, and PUN as response criteria.

Table 6.1 Ingredient composition of experimental diets

	SID SAA:Lys ratio %				
Ingredients, g/kg	52	56	60	64	68
Wheat	602.3	602.3	602.3	602.3	602.3
Corn	100.0	100.0	100.0	100.0	100.0
Soybean meal, 46% CP	208.2	208.2	208.2	208.2	208.2
Vegetable oil	36.6	36.6	36.6	36.6	36.6
Corn starch	5.0	4.5	4.0	3.6	3.1
Monocalcium phosphate	13.9	13.9	13.9	13.9	13.9
Limestone	11.8	11.8	11.8	11.8	11.8
Salt	3.2	3.2	3.2	3.2	3.2
Mineral-vitamin premix[1]	10.0	10.0	10.0	10.0	10.0
L-Lysine-HCl	4.9	4.9	4.9	4.9	4.9
L-Threonine	1.8	1.8	1.8	1.8	1.8
L-Trptophan	0.2	0.2	0.2	0.2	0.2
L-Valine	2.1	2.1	2.1	2.1	2.1
DL-Methionine	0.0	0.5	1.0	1.4	1.9
Calculated nutrient composition, g/kg or as specified					
NE (MJ/kg)	10.40	10.40	10.40	10.40	10.40
CP	213.9	213.9	213.9	213.9	21.39
SID Lys	11.8	11.8	11.8	11.8	11.8
SID Met	2.8	3.3	3.8	4.2	4.7
SID Cys	3.3	3.3	3.3	3.3	3.3
SID Met + Cys	6.1	6.6	7.1	7.5	8.0
SID Thr	7.7	7.7	7.7	7.7	7.7
SID Trp	2.6	2.6	2.6	2.6	2.6
SID Ile	7.1	7.1	7.1	7.1	7.1
SID Val	8.3	8.3	8.3	8.3	8.3
SID Leu	13.1	13.1	13.1	13.1	13.1
SID Arg	11.6	11.6	11.6	11.6	11.6
SID His	4.6	4.6	4.6	4.6	4.6
SID Phe	9.0	9.0	9.0	9.0	9.0
SID SAA:Lys	5.2	5.6	6.0	6.4	6.8
Total Ca	8.0	8.0	8.0	8.0	8.0
Available P	4.5	4.5	4.5	4.5	4.5
Analyzed nutrient composition, g/kg					
Total Lys	13.1	13.2	13.7	13.7	13.7
Total Met+Cys	6.9	7.5	8.1	8.4	8.8

[1]Supplied the following per kilogram of diet: 8,250 IU of vitamin A, 835 IU of vitamin D3, 40 IU of vitamin E, 25 µg of vitamin B12, 4 mg of vitamin K, 25 mg of niacin, 600 mg of choline, 12 mg of riboflavin, 200 µg of biotin, 4.5 mg of pyridoxine, 4 mg of folic acid; 2 mg of thiamin. [2]Supplied the following per kilogram diet: 50 mg of Mn , 150 mg of Zn, 120 mg of Fe, 25 mg of Cu, 0.35 mg of Se, 0.4 mg of I.

Table 6.2 Effect of SID SAA:Lys ratio on body weight, average daily gain, average feed intake, and gain to feed ratio of weaned piglets raised under clean conditions (Exp. 1)

	SID SAA:Lys ratio, %						*P*-value	
	52	56	60	64	68	[1]SEM	Linear	Quadratic
BW d 0, kg	7.45	7.48	7.47	7.45	7.47	0.25	-	-
BW d 14, kg	10.62	10.85	11.06	10.92	10.96	0.16	0.209	0.291
Average daily gain, g								
d 1 to 7	139	141	174	148	163	17.2	0.307	0.620
d 8 to 14	342	368	381	403	364	14.2	0.096	0.032
d 1 to 14	248	263	279	278	271	11.9	0.116	0.177
Average daily feed intake, g								
d 1 to 7	258	241	292	256	256	13.3	0.776	0.299
d 8 to 14	479	513	536	566	560	21.9	0.005	0.378
d 1 to 14	368	378	426	405	409	14.7	0.028	0.151
Gain to feed ratio, g/kg								
d 1 to 7	545	590	594	572	640	57.0	0.358	0.922
d 8 to 14	714	717	712	713	649	23.0	0.091	0.152
d 1 to 14	674	700	694	686	665	20.0	0.631	0.227

N = 6

[1]SEM = pooled standard error of mean for all treatment means.

Table 6.3 Effect of SID SAA:Lys ratio on body weight, average daily gain, average feed intake, and gain to feed ratio of weaned piglets raised under unclean conditions (Exp. 2)

	SAA:Lys ratio						*P*-value	
Item	52	56	60	64	68	[1]SEM	Linear	Quadratic
BW d 0	7.24	7.14	7.13	7.13	7.19	0.10	-	-
BW d 14	11.12	11.09	11.45	11.41	10.62	0.31	0.583	0.117
Average daily gain, g								
d 1 to 7	194	208	228	236	184	23.3	0.901	0.118
d 8 to 14	359	357	368	374	306	31.6	0.384	0.268
d 1 to 14	277	282	298	305	245	22.7	0.586	0.119
Average daily feed intake, g								
d 1 to 7	346	327	318	356	278	18.1	0.079	0.290
d 8 to 14	555	531	559	531	457	31.8	0.070	0.203
d 1 to 14	450	429	438	444	367	22.2	0.046	0.182
Gain to feed ratio, g/kg								
d 1 to 7	558	633	722	658	659	54.0	0.200	0.151
d 8 to 14	644	672	658	703	666	32.0	0.488	0.566
d 1 to 14	612	660	682	682	662	27.0	0.172	0.142

N = 6

[1]SEM = pooled standard error of mean for all treatment means.

Table 6.4. Effect of SID SAA:Lys ratios on plasma urea nitrogen and jejunal VH, CD and VH:CD of weaned piglets raised under clean and unclean conditions

	SID SAA:Lys ratio, %						*P*-value	
Item	52	56	60	64	68	[2]SEM	Linear	Quadratic
Plasma urea nitrogen, mmol/L								
Clean								
d 0	3.51	3.68	3.53	2.74	3.82	0.47	0.820	0.510
d 14	4.34	3.87	4.73	4.53	4.71	0.20	0.089	0.814
Unclean								
d 0	3.66	3.55	3.36	3.48	3.4	0.40	0.648	0.809
d14	4.61	4.22	3.66	3.36	3.92	0.26	0.014	0.041
Histomorphology[1]								
Clean								
VH, µm	475	538	622	553	571	26.1	0.015	0.021
CD, µm	216	220	213	209	218	4.78	0.536	0.548
VH:CD	2.2	2.42	2.93	2.64	2.63	0.14	0.020	0.031
Unclean								
VH, µm	458	537	531	534	560	22.7	0.027	0.451
CD, µm	222	225	221	210	207	5.95	0.030	0.400
VH:CD	2.19	2.37	2.41	2.71	2.71	0.14	0.006	0.828

[1]VH = villus height; CD = crypt depth; VH:CD = villus height to crypt depth ratio.

[2]SEM = pooled standard error of mean for all treatment means.

N = 6

Table 6.5 Concentration of ammonia and hydrogen sulphide (ppm) for clean and unclean conditions

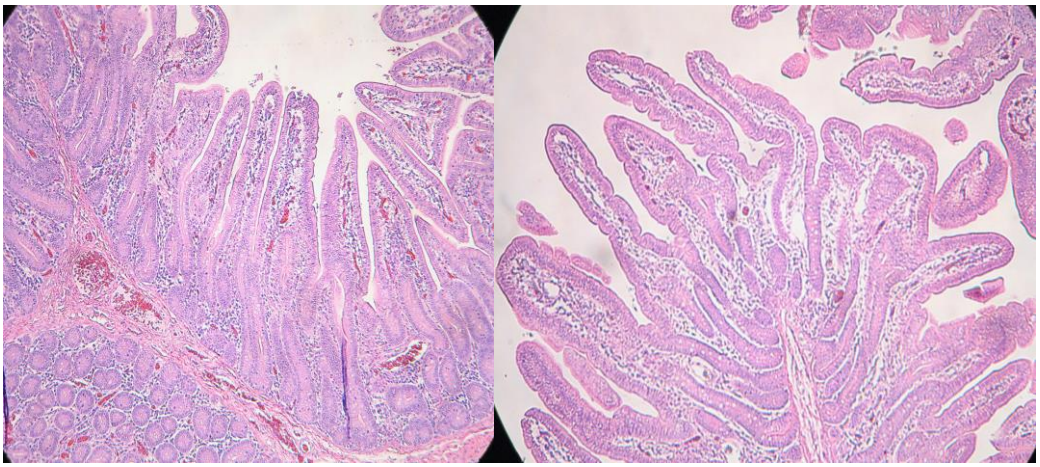
	Hydrogen sulphide				Ammonia			
	Clean	SE	Unclean	SE	Clean	SE	Unclean	SE
d 0	0.06	-	ND[1]	-	< 5	-	ND	-
wk1	0.06	0.011	0.14	0.003	32	1.667	32	2.778
wk2	0.07	0.006	0.20	0.004	25	3.632	38	1.667

[1]ND = Not determined.

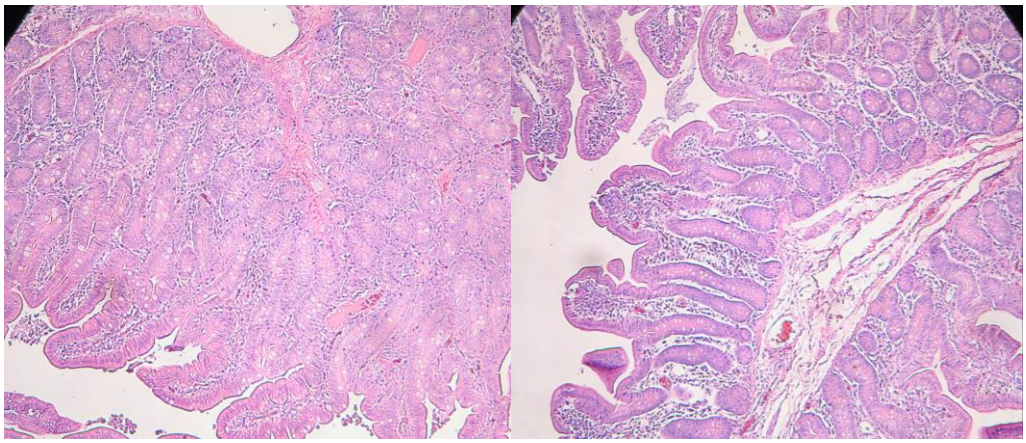
Table 6.6 The estimated SAA:Lys ratios from clean and unclean experiments[1]

	Estimates at 95% confidence level			
	Estimate	Model	SEM	R^2
Clean broken-lines				
ADG	61.1	Y = 46.33+3.88*ADG- 5.63*(ADG>61.09)*(ADG-61.09)	0.16	99.9
VH	60	Y = -476+18.22*VH- 24.45*(VH > 60)*(VH-60)	5.93	80.4
VH:CD	60	Y = -2.33+03086*VH:CD- 0.1186*(VH:CD > 60)*(VH:CD-60)	3.22	91.6
PUN	60	Y = 1.76+0.045*PUN- 0.021*(PUN > 60)*(PUN-60)	-	40.3
Unclean broken-lines				
ADG	64.5	Y = 369.6-1.53*ADG- 3.36*(ADG > 64.5)*(ADG-64.5)	-	9.9
VH	65.9	Y = 317.3+3.53*VH + 1.46*(VH > 65.9)*(VH-65.9)	3.26	72.4
G:F	58.4	Y = -12+12*G:F- 14.5*(G:F > 58.4)*(G:F-58.4)	1.16	97.9
VH:CD	Failed to converge			
PUN	63.4	Y = 10.81-0.118*PUN+0.26*(PUN> 63.4)*(PUN-63.4)	0.49	99.5
Unclean polynomial regression				
ADG	54.2	Y = 0.8516*SAA:LYS 2+96.26*SAA:LYS – 2424.7	-	72.3

[1]ADG = Average daily gain; VH = villus height; CD = crypt depth; VH:CD = villus height to crypt depth ratio; PUN = plasma urea nitrogen; G:F = gain to feed ratio. N = 6



A: without diarrhea



B: with diarrhea

Figure 6.1 Hematoxylin and eosin stained jejunal segments from piglets with and without diarrhea.

The stressors under unsanitary condition were expected to cause diarrhea and reduce piglet growth as reported by Kahindi et al. (2014b). However, there were no observable changes in piglets' health or incidences of diarrhea. The lack of illness could be due to piglets' predisposal to maternal feces and that the feces could have had low levels of pathogenic microbes. Addition of manure from sow herd did not only make the room filthy but also increased NH_3 and H_2S concentration in the unclean room. Toxic levels of NH_3 and H_2S would cause piglet' growth retardation through reduction in feed intake and discomfort to the respiratory system (Ni et al., 2000). The H_2S concentration throughout the study period was below the 10 ppm level that affects pig growth (Kim et al., 2008). Although the recommended maximum NH_3 concentration in a swine barn is 20 ppm (Donham, 2000), pigs can withstand higher concentration of up to 40 ppm (Jones et al., 1997). Hence it is possible that the piglets in the unclean room got acclimatized to the increasing NH_3 concentrations.

Diet formulation using ideal AA ratio for protein accretion is adopted because it minimizes feed inefficiencies due to dietary AA excesses or inadequacy. In healthy 5 to 11 kg pigs, the SAA:Lys ratio for protein accretion recommendations by NRC (1998) and (2012) are 58 and 55, respectively. Studies however, reported that the 55 ratio may not be sufficient for optimal performance of growing pigs at different body weight. Gaines et al. (2005) estimated based on ADG SAA:Lys of 59 for 8 to 19 kg pigs and 60 for 8 to 26 kg pigs. The SAA:Lys ratio of 58 was reported optimum for growth and feed efficiency of 6 to 12 kg pigs (Dean et al., 2007) and 11 to 26 kg pigs (Yi et al., 2006). Earlier work by Chung and Baker (1992c) on 10 kg piglets reported ideal SAA:Lys ratio for protein accretion to be 60, however, Chung and Baker (1992b) had reported 50 as optimal SAA:Lys ratio for 5 to 10 kg pig. In the current study, the optimal

SID SAA:Lys was 61 for clean based on ADG and 58 for unclean conditions based on G:F. The SID SAA:Lys ratio for clean group compare with findings of Chung and Baker (1992c), whereas the unclean group estimate is similar to values reported by Gaines et al. (2005) and Dean et al. (2007) for piglets.

Increasing SAA:Lys ratio linearly increased ADFI of piglets raised under clean conditions. On the contrary, under unclean conditions ADFI was similar for all treatments except one with the highest SID SAA:Lys ratio where there was a depression in ADFI. Excess Met beyond an optimal SID SAA:Lys ratio can lead to a reduction in piglets' feed intake. The depressed feed intake is consistent with work others (Harper et al., 1970; Pearson and Carr, 1979; Iyayi et al., 2014). And has been attributed to AA imbalances caused by excess dietary Met. It is clear that the response in ADFI to increasing SID SAA:Lys conflicted between the clean and unclean conditions. As noted the feed intake for piglets under unclean condition was higher than clean condition, thus it is possible that there was a tendency to consume excess Met. The diet with the highest SID SAA:Lys ratio depressed ADFI under unclean condition, consequently a reduction in ADG. As a result the effect of increasing SAA:Lys was not significant on the G:F from d 1 to 14 in both experiments. However, although dietary Met was not efficiently utilized for growth it was directed towards other functions as indicated by the PUN and VH:CD responses. The gut sequesters about 30% of the dietary Met (Stoll and Burrin, 2006) for production of fuel or is metabolized to Cys for production of antioxidants, mucin, enterocytes cell proliferation and maintenance of tissue redox state (Stipanuk, 2004). The VH and VH:CD were increased by 20 and 22% from the lowest to the highest SAA:Lys ratio under clean and unclean conditions, respectively. Similarly, Kaewtapee et al. (2010) and Bauchart-Theveret et al. (2009) reported increased VH as a result of increasing Met content in pigs' diets.

Therefore, dietary Met content affects intestinal growth and that deficiency in SAA will reduce enterocytes production by the crypt leading to shorter villi.

The study objective was to establish the ideal SID SAA:Lys ratio for piglets under two sanitary conditions. The SAA:Lys ratio was expected to differ due to increased maintenance requirement under unclean housing condition (Le Floc'h et al., 2006). However, due to minimal changes in piglets' performance or incidences of diarrhea under unclean conditions the optimum SID SAA:Lys ratio for under unclean condition and based on G:F was lower that is, 58 than the 61 for piglets raised under clean condition. When VH and PUN were used as response criteria the optimum SID SAA:Lys ratio for piglets under unclean condition was higher compared clean (63 and 66 vs. 60). The high SAA:Lys ratio indicate that though the SAA requirement for ADG was not affected by sanitary conditions, the gut maintenance requirement increased in piglets under unclean condition. Dahiya et al. (2007) reported that increasing dietary Met content significantly reduced growth of *Clostridium perfringens* and coliforms in birds that had been inoculated with *Clostridium perfringens*. In the same study, increasing dietary Met content beyond growth requirement increased the population of lactobacillus, the beneficial bacteria. Exposure of piglets to foreign particles, pathogenic or not, through introduced feces could have increased maintenance requirement of SAA for gut barrier function. The SID SAA requirements based on performance parameters were 10.89 and 10.43 mg SAA/gain for piglets under clean and unclean conditions, respectively. The level is higher than 8.7 mg SAA/g gain reported for 5 to 10 kg pigs (Chung and Baker, 1992b) and 10.2 mg SAA/g gain for 6 to 12 kg pigs (Dean et al., 2007) but similar to 10.87 mg SAA/g gain reported by NRC (2012). When the SAA estimate is based on VH, the requirement for piglets under unclean condition was 11.50 mg SAA/g gain.

In conclusion, the SID SAA:Lys requirement for 7 to 12 kg pigs raised under clean or unclean conditions did not differ. However, when the requirement is based on histomorphology and PUN the piglets under unclean condition required higher SID SAA compared to the clean. Therefore, in situations where sanitary condition or gut health is compromised it is prudent to increase the dietary SAA content beyond the growth requirement for piglets.

CHAPTER SEVEN

Sulfur amino acid to lysine ratio for piglets challenged with enterotoxigenic *Escherichia coli*

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Kahindi R. K.: the graduate student made project proposal in conjunction with Drs. Htoo and Nyachoti. She did the research work, data analyses and wrote the manuscript.

Regassa A.H.: a post-doctoral fellow assisted in laboratory work and analysis of the gene expression data.

Htoo J. K.: works for Evonik Industries who funded the project. He also assisted in data analyses as well the nutrient composition of the ingredients and diets.

Nyachoti C. M.: the supervising professor assisted in providing guidance throughout the research work and in the revision of the research paper.

7.1 ABSTRACT

The sulfur AA (**SAA**; methionine and cysteine):Lys ratio for protein accretion is 55 for 7 to 11kg pigs (NRC, 2012), however the use of SAA to support the immune system as opposed to accretion of lean tissue may exert additional requirements in immune compromised pigs. Moreover, due to negative whole body protein balance resulting from of muscle wasting during an immune challenge, performance may not be a suitable response criterion for determining SAA requirement. Thus a study was conducted to determine the standardized ileal digestible (**SID**) SAA:Lys ratio of weaned pig under an enterotoxigenic *Escherichia coli* (**ETEC**) challenge by measuring the mRNA levels of key enzymes genes in the Met metabolic pathway as response criteria. Thirty five [Duroc x (Yorkshire x Landrace)] male and female piglets, with an initial average BW of 7 kg were assigned to 5 dietary treatments in a completely randomized design in a 12 d study. The corn-wheat-soybean-meal based AGP-free diets had graded SID SAA:Lys ratios (48, 54, 60, 66, and 72) and 1.18% SID Lys. Feed was offered at 4% BW to reduce within treatment differences in the amount of SAA intake and fed twice a day at 0800 and 1600 h. The piglets had a 6 d diet adaptation period and were all orally challenged with 6 and 15 mL of ciprofloxacin-resistant ETEC $K88^+$ (5×10^9cfu/mL) on d 7 and 10, respectively. Blood samples were collected via jugular venipuncture in serum tubes at 0, 6 and 24 h after oral inoculation. Rectal temperatures were measured at daily from d 6 and 12 h after oral inoculation. On d 13 all pigs were killed to collect liver and ileal tissues to determine the mRNA levels for methionine adenosyltransferase 1 and 2-α (**MAT1A** and **MAT2A**), 5-methyltetrahydrofolate-homocysteine methyltransferase (**MTR**), and cystathionine γ-lyase (**CTH**) using real-time PCR. The two ETEC inoculations resulted in increased rectal temperature ($P < 0.05$) that was similar across all treatment. Serum TNF-α concentration after 6 h of inoculation was higher ($P < 0.05$) than before challenge and increasing SAA:Lys ratio linearly increased the TNF-α concentration. Increasing

SAA:Lys ratio linearly decreased ($P < 0.01$) hepatic MTR gene expression, while linearly quadratically increasing ($P < 0.01$) CTH and MAT1A genes expression. Ileal expression of MTR and MAT2A were linearly increased ($P < 0.05$) whereas that of CTH and MAT2A were quadratically increased ($P < 0.05$) by increasing SAA:Lys ratio. The results indicate that dietary SAA:Lys ratio of 60 was enough to immune challenge support piglets.

Key word: pig, *E.coli* K88, MAT1A and 2A, CTH and MTR genes, SAA:Lys ratio

7.2 INTRODUCTION

Sulfur AA (**SAA**; methionine and cysteine) are essential not only for protein accretion but also involved in the production of cytokines, glutathione (**GSH**), and acute phase proteins (**APP**) (Grimble, 2006). The dietary SAA requirement expressed as SAA:Lys is 55 for 7 to 11 kg pigs (NRC, 2012), a ratio considered optimum for muscle protein accretion. Our study showed SAA: Lys of 60 as an optimum ratio for growth of piglets fed antibiotic-free diets (Kahindi et al., 2014c). In case of immune challenge, there can be negative whole body protein balance resulting from muscle wasting in order to release AA for energy and synthesis of cytokines and APP (Grimble, 1998; Le F'loch et al., 2004, 2007; Melchior et al., 2005). The SAA are some of the AA utilized for APP synthesis (Grimble, 1998) and constitute up to 56% of their structure. Moreover, a dietary Met deficiency decreases immune response through under-development of lymphoid organs, reduced antibodies and T cell proliferation, in pigs, rats and poultry (Jahoor et al., 1995; Maroufyan et al., 2010; Kim et al., 2012). Thus, increased SAA provision can minimize the negative effects of an immune challenge thereby sustaining health and reducing muscle tissue loss (Rakhshandeh et al., 2013).

The need for SAA to support the immune system as opposed to accretion of lean tissue calls for a response criteria besides performance to determine its requirements in immune compromised pigs. Methionine is metabolized in the liver and small intestine through 3 major pathways; transmethylation, remethylation and transsulfuration. From the small intestine, the ileum would be a site of choice for immune compromised piglets because besides its function in AA absorption it contains gut-associated lymphoid tissue, hence plays an active role in gut health. The transmethylation yields methionine adenosyl transferase an enzyme that regulates remethylation and trassulfuration depending on the need for Cys and dietary SAA content (Stipanuk, 2004). For example, there was an upregulation of the transsulfuration pathway demonstrating increased need for Cys in septic in rats (Malmezat et al., 2000). Using enzymes of Met metabolism pathways (transmethylation, remethylation and transulfuration) would show the favored pathway with increasing SAA:Lys to a point of maximum positive change in piglets exposed to an immune challenge.

Hence, the objective of this study was to determine SID SAA:Lys requirement for piglets subjected to an enterotoxigenic *Escherichia coli* (**ETEC**) immunological challenge by measuring the mRNA levels of key enzymes genes in the Met metabolic pathway as response criteria.

7.3 MATERIALS AND METHODS

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

7.3.1 Experimental Diets and Feeding Regimen

Five corn-wheat-soybean-meal based AGP-free diets with graded SID SAA:Lys ratios (48, 54, 60, 66, and 72) and 1.18% SID Lys were used in the current study (Table 7.1). The 1.18% SID Lys was second limiting for the piglets used according to work by Kahindi et al. (2014b) who reported that the SID Lys requirement for 7 to 16 kg piglet fed AGP-free diets was 1.32%. The Lys was kept marginally limiting to avoid underestimation of the SAA:Lys ratio. The graded levels of SID SAA:Lys were attained through replacing corn starch with of DL-Met and the contents of essential AA were similar for all diets and balanced to meet the ideal AA ratio for protein accretion (Table 7.2; NRC, 1998). The diets were offered at 4% body weight in order to ensure consistency in the content of SAA intake and fed twice a day at 0800 and 1600 h. Feed refusal was weighed daily to determine average feed intake (**ADFI**), Piglets had unlimited access to water via a low-pressure water drinking nipple.

7.3.2 Animals and Housing

Thirty five [Duroc x (Yorkshire x Landrace)] male and female piglets, weaned at 21 ± 1 d and with an initial average BW of 6.9 ± 0.5 kg were assigned to dietary treatments in a completely randomized design for a 13 d study. The experiment was conducted in University of Manitoba's T. K. Cheung Centre for Animal Science Research and the piglets were sourced from Glenlea Swine Research Unit, University of Manitoba (Winnipeg, MB). Piglets were kept in individual pens in a temperature-controlled room. Piglets were weighed on d 0, 6, and 12

determine average daily gain (**ADG**). The gain to feed ratio (**G:F**) was calculated on per pen basis by dividing ADG by ADFI.

7.3.3 Bacterial challenge

The piglets had a 6 d diet adaptation period, on d 7 (primary challenge) and 10 (secondary challenge) all the piglets were orally challenged with 6 and 15 mL, respectively, of ciprofloxacin-resistant ETEC $K88^+$ (5×10^9 cfu/mL) as described by Opapeju et al. (2009). The ETEC strain was confirmed positive for $K88^+$ fimbriae by PCR genotyping using published primers (Setia et al., 2009). Also, samples of the inoculums was serially diluted and plated on Eosin Blue Agar (Becton, Dickson and Company, Sparks, MD) containing ciprofloxacin to confirm the ETEC concentration.

7.3.4 Fecal Consistency Scoring and Rectal Temperature

Fecal consistency scores and incidence of diarrhea were determined before and after ETEC inoculation. The fecal consistency was scored according to the method of Marquardt et al. (1999), where, 1 = normal; 2 = soft feces; 3 = mild diarrhea; 4 = severe diarrhea. Rectal temperature was measured a day before and every day after the ETEC inoculation.

7.3.5 Sample collection

Blood samples were collected via jugular venipuncture into serum tubes (Becton Dickinson and company, Rutherford, NJ) at 0, 6 and 24 h after oral inoculation. The serum tubes containing blood were left to stand at room temperature for 3 hours and then centrifuged at 3,000 rpm for 15 min at 4^{o}C and serum stored at -80^{o}C until analyses. Tumor necrosis factor alpha (TNF-α) was analyzed using quantikine® colorimetric sandwich ELISA kits (R & D Systems Inc. Minneapolis, MN).

Fecal swabs were collected on from each pig before challenge, 3 and 5 d after challenge for detection of ciprofloxacin-resistant ETEC $K88^+$ shedding through plating using Eosin Methyl Blue agar (Becton Dickinson and company, Rutherford, NJ) with ciprofloxacin. A gram of feces was serially diluted using sterile PBS (pH 6.8) before plating.

7.3.6 Tissue Collection, RNA Isolation and qRT-PCR

On d 13, all the piglets were anesthetized by an intramuscular injection of ketamine:xylazine (20:2 mg/kg; Bimeda-MTC Animal Health Inc., Cambridge Ontario, Canada). This was followed by euthanization through intravenous administration of sodium pentobarbital (50 mg/kg of BW; Bimeda-MTC Animal Health Inc.) in order to collect liver and ileum samples for RNA isolation. The samples were immediately snap frozen in liquid nitrogen and stored at -80^{o}C. Using real-time PCR the mRNA expression of the following genes was done for the liver and ileum mucosa: methionine adenosyltransferase 1 and 2-α (MAT1 and 2 A), 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) and cystathionine γ-lyase (CTH). The genes with exception of CTH are responsible for synthesis of rate limiting enzymes for the Met metabolic pathways. Absence of complete pig cystathionine β-synthase sequence in the National Center for Biotechnology Information (NCBI) gene bank lead to a substitution with CTH as a gene representative of the transulfuration pathway. Total RNA was extracted from liver or ileum mucosal scrapping using TRIzol reagent (Invitrogen, Canada) according to the manufacturer's instruction. The purity of the extracted RNA was assessed by measuring the 260/280 absorbance ratio with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA), where the 260/280 ratio was ranged from 1.95-2.00, hence the extracted samples were pure. Electropherogram was done for all samples to determine RNA integrity using Agilent 2100 bioanalyzer (Agilent Technologies Canada Inc., Mississauga, ON,

Canada) that gave values ranging from 5 to 9. Thus, some samples were partially degraded but most had intact RNA. The isolated total RNA was reverse transcribed using high capacity reverse transcription kit (Applied Biosystems, Burlington, ON). Pairs of primers for each gene were designed using the NCBI database (Table 7.3). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in duplicate reactions including nuclease free water, the forward and reverse primers of each gene, cDNA and SYBR Green as a detector using CFX Connect ™ Real-Time PCR Detection System (Life Science Research, Bio-Rad).). One µl of complementary DNA from each sample were pooled per treatment and used for qRT-PCR followed by agarose gel electrophoresis of the qRT-PCR products to determine reliability of glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) as a housekeeping gene. Data were generated using ΔΔCt method by normalizing the expression of the target gene to a housekeeping gene, GAPDH. GAPDH was chosen as a housekeeping gene because it has been for normalization of gene expression levels from tissues and cells harvested from *Escherichia coli* infected treatments without affecting the mRNA expression (Wehkamp et al., 2004; Shifflett et al., 2005; Theivanthiran et al., 2012). The values were reported as a fold change of the expression of the target genes in the 54 SID SAA:Lys treatment as compared with the rest.

7.3.7 Data analyses

Data were subjected to analysis of variance using the MIXED procedures of SAS 9.2 (SAS Inst., Inc., Cary, NC). Each pig was an experimental unit. Orthogonal polynomial contrasts were performed to determine linear and quadratic effects of dietary SAA:Lys ratio. The statistical significance level was claimed at $P < 0{\cdot}05$ and a trend $P > 0.05 < 0.10$.

7.4 RESULTS

The piglets were healthy in the pre-challenge period and although their feed intake differed, the fecal consistency score was or close to 1, meaning that they had normal feces (Table 7.4). The fecal score slightly changed in the post-challenge period due to increased incidence of diarrhea of 17% (data not shown), thus mean fecal consistency score was 1.33. Increasing SAA:Lys had no significant effect on fecal consistence during the post-challenge period. Four pigs died each from SAA:Lys treatments of 48, 60, 66, and 72. Although the diets were offered at 4% BW to ensure consistency in the amount of SAA intakes, some piglets did not eat all the feed allocated to them. Despite this, increasing SAA:Lys ratio quadratically increased ADG, ADFI and G:F in the pre-challenge period (Table 7.5). The diet with a SAA:Lys ratio of 72 had the lowest ADG, ADFI and G:F in the pre-challenge period. On the contrary, in the post-challenge period, increasing SAA:Lys resulted in similar ADG, ADFI and G:F.

The total coliforms population was similar across the dietary treatments. Increasing SAA:Lys linearly increased and tended to quadratically decrease ciproflaxin ETEC population on d 3 and 5, respectively, after the primary challenge with ETEC. The two ETEC K88 inoculations resulted in increased rectal temperature (Figure 7.3) and the increase in rectal temperature was similar across all dietary treatments (Table 7.4). Serum TNF-α concentration at 6 h after primary inoculation was significantly higher ($P < 0.05$) than before challenge concentration (Figure 7.4). However, serum TNF-α concentration 24 h after the secondary inoculation was similar ($P > 1.0$) to before challenge concentration. Increasing SAA:Lys quadratically increased the serum TNF-α concentration after the secondary inoculation. The diet with SAA:Lys ratio of 48 had the lowest TNF-α concentration after both inoculations.

The housekeeping gene had no influence the mRNA expression across the treatments as shown by similar bands across treatments from the agarose gel electrophoresis (Figure 7.1). Increasing SAA:Lys ratio linearly decreased ($P < 0.01$) MTR gene expression and both linearly and quadratically increased ($P < 0.01$) CTH and MAT1A expression in the liver (Figure 7.2). In the ileum, increasing SAA:Lys ratio linearly increased ($P < 0.05$) MTR and MAT2A expression and quadratically increased CTH and MAT2A expression (Figure 7.3). There was a tendency ($P < 1.0$) towards a quadratic increase in MTR gene expression with increasing SAA:Lys ratio.

Table 7.1 Ingredient composition (in %) of diets

	SID SAA:Lys ratio				
Item	48	54	60	66	72
Soybean meal, 46% CP	23.69	23.69	23.69	23.69	23.69
Wheat	45.62	45.62	45.62	45.62	45.62
Corn	12.34	12.34	12.34	12.34	12.34
Corn starch	0.50	0.43	0.36	0.27	0.21
Lactose	10.00	10.00	10.00	10.00	10.00
Limestone	1.14	1.14	1.14	1.14	1.14
Monocalcium Phosphate	1.45	1.45	1.45	1.45	1.45
Vegetable oil	3.19	3.19	3.19	3.19	3.19
Iodized salt	0.31	0.31	0.31	0.31	0.31
Vitamin premix	0.50	0.50	0.50	0.50	0.50
Mineral premix	0.50	0.50	0.50	0.50	0.50
L-Lysine	0.51	0.51	0.51	0.51	0.51
DL-Methionine	0.00	0.07	0.14	0.23	0.29
L-Threonine	0.18	0.18	0.18	0.18	0.18
L-Tryptophan	0.05	0.05	0.05	0.05	0.05
L-Valine	0.03	0.03	0.03	0.03	0.03
Total	100.00	100.00	100.00	100.00	100.00

Table 7.2 Calculated and analyzed nutrient composition of experimental diets

	SID SAA:Lys ratio				
Item	48	54	60	66	72
Calculated nutrient content					
ME, MJ/kg	13.94	13.94	13.94	13.94	13.93
NE, MJ/kg	10.25	10.25	10.25	10.25	10.25
CP, %	19.52	19.52	19.52	19.52	19.52
Total Lys, %	1.29	1.29	1.29	1.29	1.29
SID Lys, %	1.18	1.18	1.18	1.18	1.18
SID Met, %	0.26	0.33	0.40	0.49	0.54
SID Cys, %	0.30	0.30	0.30	0.30	0.30
SID Met+cys, %	0.56	0.63	0.70	0.79	0.84
SID Thr %	0.75	0.75	0.75	0.75	0.75
SID Trp %	0.28	0.28	0.28	0.28	0.28
SID Arg, %	1.11	1.11	1.11	1.11	1.11
SID Ile %	0.71	0.71	0.71	0.71	0.71
SID Val %	0.80	0.80	0.80	0.80	0.80
SID Leu, %	1.33	1.33	1.33	1.33	1.33
SID His, %	0.43	0.43	0.43	0.43	0.43
SID Phe, %	0.86	0.86	0.86	0.86	0.86
SID SAA:Lys ratio	**0.48**	**0.54**	**0.60**	**0.66**	**0.72**
Analyzed nutrient content, %					
CP	19.43	20.85	20.56	21.23	20.53
Total Met	0.27	0.39	0.43	0.48	0.60
Total Cys	0.35	0.36	0.35	0.38	0.36
Total Met+Cys	0.62	0.75	0.79	0.86	0.96
SID Met+Cys	0.54	0.66	0.71	0.78	0.87
Total Lys	1.41	1.32	1.35	1.35	1.34
SID Lys	1.28	1.20	1.23	1.23	1.22
Total Thr	0.85	0.84	0.84	0.86	0.85
Total Trp	0.29	0.29	0.29	0.30	0.29
Total Arg	1.17	1.25	1.23	1.27	1.21
Total Ile	0.76	0.84	0.82	0.84	0.80
Total Leu	1.37	1.49	1.45	1.52	1.44
Total Val	0.89	0.96	0.95	0.96	0.91
Total His	0.47	0.50	0.49	0.51	0.49
Total Phe	0.93	1.01	0.98	1.03	0.98
SID SAA:Lys ratio	**0.42**	**0.55**	**0.58**	**0.63**	**0.71**

Table 7.3 Primer sequences used for real time PCR

Gene[1]	Primer sequences 5'- 3'	Gene bank Access	Annealing Temperature, ^{o}C	Product length, bp
MTR	F: GTCTGTGCTTGATGCTCCCT	XM_001927058.2	58	146
	R CAAGCTTCTGGCAAACGTCC		58	137
MAT1A	F: GGCTTGTGCGACCATTCTCT	NM_001243187.1	60	143
	R: AAGTCAAAGCCACAGGCCAC		60	130
MAT2A	F: GCTCGTTGGGTGGCAAAATC	NM_001167650.1	60	147
	R: GCTGTCCCTACCAAAGTGGC		60	170
CTH	F: TATCCTGGGTTGCCCTCTCA	NM_001044586.1	58	159
	R: AATGGGTCATGATTGCCGGA		58	142
GAPDH	F: GGTGAAGGTCGGAGTGAACG	NM_001206359.1	58	238
	R: GGGATCTCGCTCCTGGAAGA			

[1]MAT1A = methionine adenosyltransferase 1 alpha, MAT2A = methionine adenosyltransferase 2 alpha, MTR = 5-methyltetrahydrofolate-homocysteine methyltransferase, CTH = cystathionine gamma-lyase, GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Table 7.4 Fecal consistence score, rectal temperature and serum TNF-α concentration of ETEC challenged pigs fed diets with graded SAA:Lys levels

	SAA:Lys ratio						*P*-value		
	48	54	60	66	72	SEM[2]	Model	linear	Quadratic
Diarrhoea score									
Pre-challenge	1.00	1.00	1.03	1.06	1.14	0.04	0.167	0.023	0.316
Post-challenge	1.30	1.43	1.38	1.30	1.23	0.27	0.986	0.760	0.673
Rectal temperature[1]									
Before	38.79	38.74	39.09	39.12	38.53	0.26	0.482	0.865	0.158
24 h after	39.04	39.11	39.43	39.24	39.14	0.18	0.628	0.578	0.232
Microbial[2] CFU									
Coliforms	6.19	6.42	5.65	5.60	5.73	0.48	0.674	0.255	0.765
ETEC, d 3	2.35	2.67	2.67	4.41	5.46	0.95	0.123	0.014	0.382
ETEC, d 6	4.85	2.86	3.23	2.77	5.28	1.12	0.378	0.834	0.065
TNF-α[3]									
Before	56.15	50.21	49.69	59.04	59.86	14.94	0.980	0.741	0.685
6 h after	30.04	82.04	109.85	239.59	38.11	42.26	0.008	0.112	0.011
24 h after	35.84b	73.89a	46.63b	71.56a	42.81b	9.69	0.026	0.707	0.042

[1]Rectal temperature: before challenge and 24 h after primary challenge; [2]Microbial: coliforms before challenge, Ciproflaxin resistant ETEC 3 and 6 days after challenge; [3]TNF-α before challenge, 6 h after primary challenge and 24 h after secondary challenge.

[2]SEM = pooled standard error of mean for all treatment means. N = 6

Table 7.5 Performance of pigs fed diets with graded SAA:Lys levels before and after ETEC challenge

	SAA:Lys ratio						*P*-value		
	48	54	60	66	72	SEM[2]	Model	linear	Quadratic
Before challenge									
ADG, g	73c	126ab	109b	157a	60c	22.02	0.031	0.945	0.009
ADFI, g	150b	150b	172ab	205a	110c	17.67	0.019	0.655	0.012
G:F, g/kg	484	689	613	764	482	87.92	0.113	0.798	0.031
SAA intake, g/d	0.81	0.99	1.22	1.60	0.52	-	-	-	-
After challenge									
ADG, g	178	175	195	182	157	31.68	0.945	0.737	0.525
ADFI, g	269	250	289	278	248	21.17	0.573	0.835	0.375
G:F, g/kg	640	713	642	633	553	80.80	0.754	0.336	0.435
SAA intake, g/d	1.45	1.65	2.05	2.17	2.16	-	-	-	-

[1]ND = Not determined; [2]SEM = pooled standard error of mean for all treatment means. N = 6

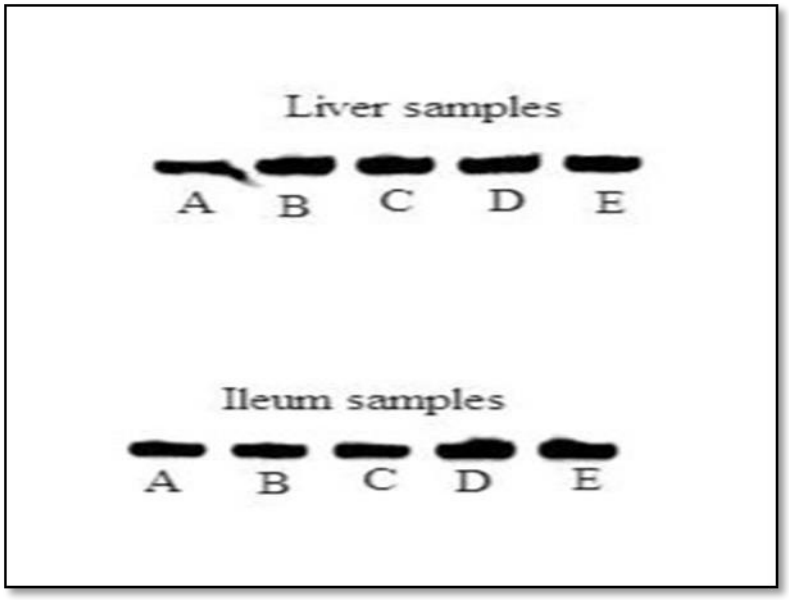


Figure 7.1 Agarose gel of the treatments' PCR products for validation of GAPDH as a housekeeping gene. The SAA:Lys treatment diets of 48, 54, 60, 66 and 72 are represented by letters A, B, C, D, and E, respectively.

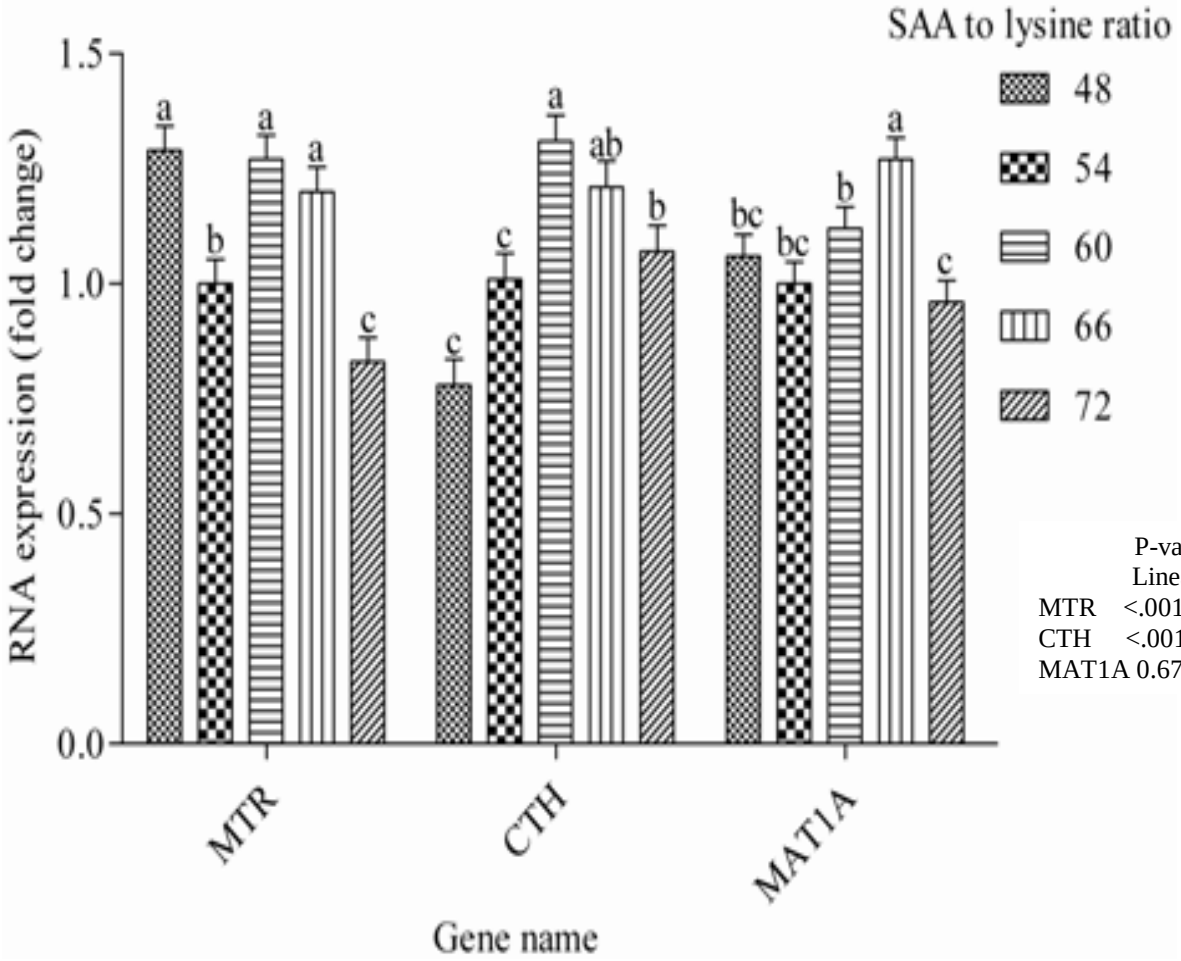
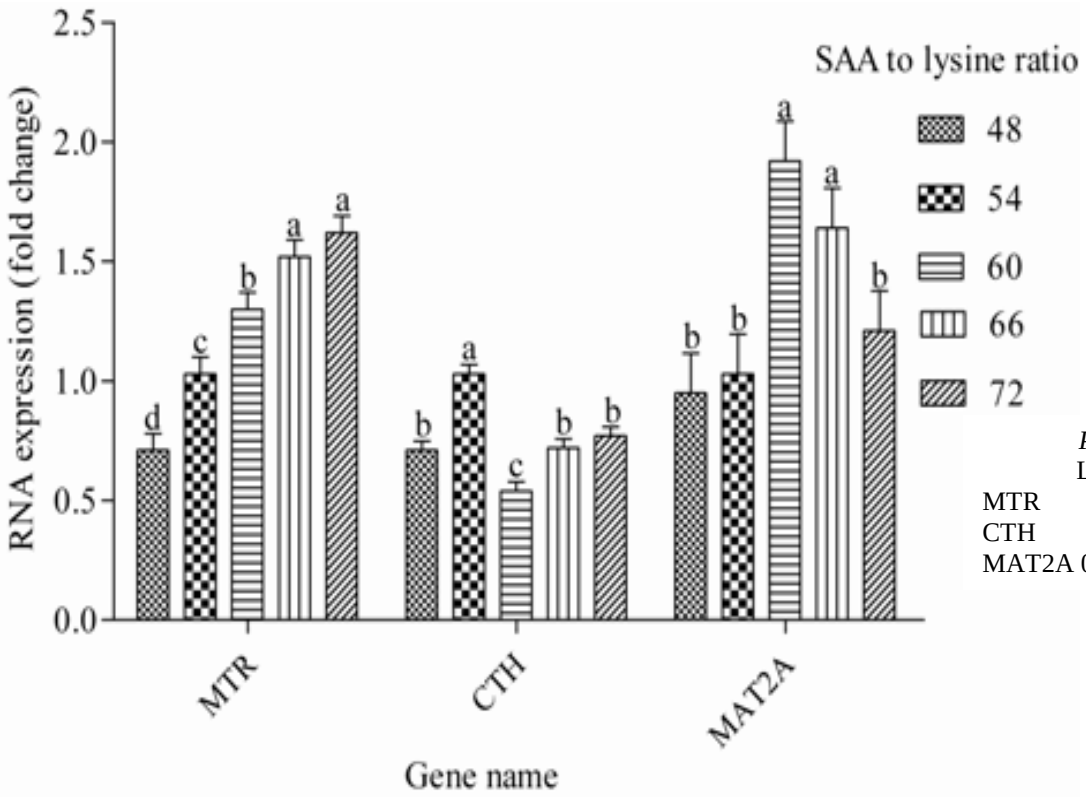


Figure 7.2 The mRNA expression of 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), methionine adenosyltransferase 1 alpha (MAT1A), and cystathionine gamma-lyase (CTH) in the liver of piglets fed diets with graded SAA:Lys. Bars (Mean ± SD) marked with different letter are significantly different ($P < 0.05$). N = 6



	P-value; Linear	Quadratic
MTR	<.001	0.079
CTH	0.102	0.038
MAT2A	0.040	0.002

Figure 7.3 The mRNA expression of 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), methionine adenosyltransferase 2 alpha (MAT2A), and cystathionine gamma-lyase (CTH) in the ileum of piglets fed diets with graded SAA:Lys. Bars (Mean ± SD) marked with different letter are significantly different ($P < 0.05$). N = 6

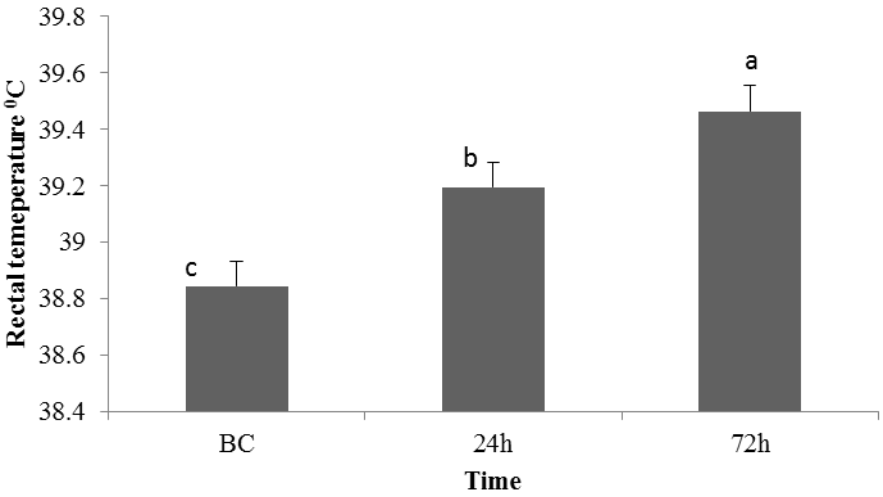


Figure 7.4 Rectal temperature of *E.coli* challenged piglets; before challenge (BC) and 24 and 72 h after primary challenge. Bars (Mean ± SEM) marked with different letter are significantly different ($P < 0.05$). N = 6

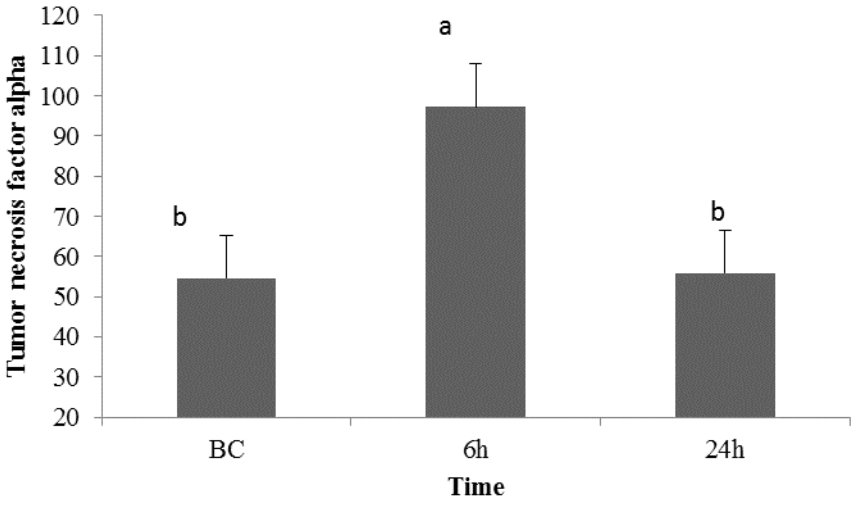


Figure 7.5 Tumor necrosis factor alpha of E.coli challenged piglets; before challenge (BC) and 6 and 24 h after primary challenge. Bars (Mean ± SEM) marked with different letter are significantly different ($P < 0.05$). N = 6

7.5 DISCUSSION

There was a presence of ETEC in the piglets' intestine as shown by ETEC shedding on d 3 and 5 after inoculation. However, the fecal score was low (< 1.5) for all treatments therefore the ETEC inoculation mainly caused soft feces. There were mortalities from 4 treatments that were caused by low feed intake and diarrhea caused by ETEC infection that led to loss of body condition and eventually death. Inoculation with ETEC triggered an immune response in the piglets as reflected by the increase in rectal temperature both at primary and secondary inoculations. Further, the concentration of TNF-α, an inflammatory marker, 6 h after the primary inoculation was elevated compared to before challenge. The TNF-α is produced by macrophages during an inflammation, it stimulates the hypothalamus causing fever and triggers the liver to produce acute phase proteins (Gruys et al., 2005). The production of TNF-α in the current study was dependent on the dietary SAA:Lys ratio. Hence, the higher SAA:Lys ratio the more TNF-α concentration in the blood with exception of the diet with a 72 SAA:Lys ratio. This confirms the need for SAA during an immune response for both pro- and anti-inflammatory purposes and that, high dietary SAA contents exerts strong inflammatory responses. Since the synthesis of APP is triggered by cytokines like TNF-α, IL-1β and IL-6, the quantity of these cytokines will positively influence APP concentration in the blood. Interestingly, exposing piglets to a secondary immune challenge caused a minor change to the serum TNF-α as compared to the concentrations before challenge. The response could be due to the fact that secondary immune response is rapid and less severe since the body would have already synthesized the necessary antibodies and memory cells as a result of the primary response (Abbas et al., 2012).

In the pre-challenge period, the highest ADG and ADFI was at SAA:Lys ratio of 66. Feeding SAA:Lys ratio of 48 and 72 dropped ADG by 54 and 62% and ADFI by 27 and 46%, respectively, compared to SAA:Lys ratio of 66. Hence the drop in growth for piglets fed diets

with SAA:Lys ratio of 48 and 72 was not only because of reduce feed intake but mainly the reduced dietary SAA content. Consequently, the drop in the SAA intake by piglets on the 72 SAA:Lys treatment compromised their immune response as indicated by the low 6 h TNF-α concentration. The post-challenge ADFI and ADG were similar regardless of the dietary treatment, however, the G:F improved with increasing dietary SAA:Lys ratio. These ADFI and ADG responses in the current study are comparable to Capozzalo et al. (2012) who did not observe difference in ADG and ADFI of piglets orally inoculated with *Escherichia coli* and fed diets with graded Trp levels. During an immune challenge, growth is of secondary importance because nutrients are partitioned towards support of an immune response and that the body is undergoing muscle catabolism for release AA required for gluconeogenesis and synthesis of APP. Therefore, although there was increasing dietary SAA content in the current study, the SAA were not preferential utilized for growth but to support the immune system.

We had hypothesized that the need of Cys for GSH, mucin and APP synthesis would increase in case of an enteric infection and that the Met transmethylation, remethylation and transsulfuration pathways should change adjusting to the body's requirement. The point where remethylation pathway is more dominant than transsulfuration pathway would be the optimum SAA requirement for metabolic needs during an ETEC immune challenge. Moreover, the transsulfuration pathway was expected to be dominant because of the ETEC inoculation in piglets creates an immune challenge and hence an increased need for Met metabolites such as Cys and GSH. Beside protein synthesis, Cys is used for mucin and GSH formation thus supporting the innate immunity through maintenance of gut barrier function and redox status. A study by Malmezat et al. (2000) reported 80% increased liver transsulfuration in rats injected with live *Escherichia coli* and consequently a 45% increase in the liver GSH. It was also

expected that the remethylation pathway would be dominant over transsulfuration when the dietary SAA content is low, as explained by Grimble (2006) and that increasing dietary Met intake favors the transsulfuration pathway over remethylation.

The gene expression for CTH was low in the ileum and thus although the MAT2A gene expression was increased with increasing dietary SAA:Lys ratio, the key metabolite (homocysteine), was remethylated to Met as show by the complementary increase in MTR gene expression. A study by Riedijk et al. (2007) proved that there is not only Met transmethylation but also transsulfuration in the pig intestine. Therefore, low CTH gene expression in the ileum does not indicate absence of transsulfuration but that this pathway was not affected by dietary SAA:Lys ratio. Additionally, the expression of CTH mRNA confirms that the bulk of the transsulfuration is done by the liver due to low activity of CTH in the intestine (Stipanuk 2004). Intriguingly, MTR gene expression in the liver dropped as MAT1A and CTH increased with increasing SAA:Lys ratio. Thus, in the liver of ETEC inoculated piglets, transsulfuration pathway was favored over remethylation with increasing SAA:Lys ratio. In healthy neonates, Met deficiency up regulated intestinal SAM, methionine synthase and cystathionine β-synthase and down regulated SAM and up regulated cystathionine β-synthase in the liver (Bauchart-Thevert et al., 2009). Thereby implying that remethylation was dominant in the liver but all pathways were active at equal measure in the intestine of Met deficient neonates. Therefore, the changes in metabolites with varying dietary SAA content in the current study were similar to those seen in the study by Bauchart-Thevert et al. (2009).

The highest expression of CTH mRNA and consequently transsulfuration was at SAA:Lys ratio of 60 and further increase in the SAA content did alter this gene expression. The SAA:Lys ratio of 60 is higher than the 55 recommendation by NRC (1998 and 2012) for protein

accretion. The findings are contrary to study by Kim et al. (2012) who using protein deposition as response criteria on LPS-injected grower pigs reported SAA:Lys ratio of 75 to be the requirement. The differences in the models used could explain variability in the optimum SAA:Lys. Injecting LPS will induce sepsis that stimulates inflammation within 60 min of inoculation (Thorgersen et al., 2010) unlike enteric infection where diarrhea can take a minimum 6 h to set, hence it is possible that piglets would require a lower dietary SAA boost in case of an enteric unlike systemic infection.

In conclusion, data of the current study show that mRNA expression of CTH gene unlike t MAT2A gene expression in the small intestine of piglets was not increased by dietary SAA:Lys hence, most of the Met is remethylated. Met metabolic pathway in the liver indicate that dietary SAA:Lys of 60 as enough to support piglet's immune response and performance during an enteric immune challenge. It was also evident that SAA:Lys ratio of 54 and below depressed both performance and immune response of piglets.

CHAPTER EIGHT

GENERAL DISCUSSION

The current amino acids (**AA**) requirements recommended for growing pigs were compiled from studies where animal performance was the response criteria. Performance as a response criteria is dependable in healthy growing animals, however in a situation when the health is compromised the AA requirement might differ. This is because rather than growth the AA would be utilized to support the immune system hence the maintenance requirement is increased. As published in NRC (1998), the AA requirement expressed in ideal AA ratio differ between maintenance and growth. The weaned pigs are challenged with an abrupt change in the environment, diet type and that their digestive system is immature thus will not digest the new diet. As a result the piglets tend to have post weaning diarrhea (**PWD**) (Pluske, et al., 1997). To overcome PWD and enhance growth performance pig starter diets have often been fortified with antimicrobial growth promoters (**AGP**) such as sub-theraupeutic levels of antibiotics and excess supply of minerals like Zn. However, there is increasing pressure discontinue the use of in-feed antibiotic in livestock production to prevent the development of antibiotic-resistant bacteria and therefore jeopardizing human health (Dibner and Richards, 2005). The mineral overdosing raises environmental concerns through causing ground and water pollution. We hypothesized that AA requirement for weaned pigs fed AGP-free diets would be higher and more so when the piglets are subjected to an immunological. Immune challenge depresses growth performance of piglets leading to changes in the efficiency of nutrient utilization for lean tissue gain (Williams et al., 1997; Le Floc'h et al., 2006). It has been shown that under immune challenge pigs fed diets with higher than recommended AA contents for performance have fast recovery and strong immunity

(Bikker et al., 2006; Li et al., 2007). These AA such Trp, Thr and Met are not only need for piglets' growth but are also involved in maintaining the immune system.

The research work from this thesis is aides in increasing knowledge of dietary manipulation to improve the health and performance of piglets. Therefore, the AA requirement of healthy and immune challenged piglets whose gut health was compromised were estimated. The measuring of the SAA:Lys ratio was done using other indexes, besides the contemporary performance and plasma urea nitrogen. These indexes considered the role of the AA in the body and its metabolism. The major assumption being that the role in question is important enough to affect amino acid requirement. These non-performance indexes are particularly necessary in cases of immune challenge when the piglets naturally reduce their feed intake and growth. Since the SAA are important in the maintenance of gut health and development of innate immunity, it was necessary to know if an enteric immune challenge would lead to their increased requirement.

In this thesis research, AA requirement for weaned piglets were determined under both healthy and immune challenged conditions for comparison purposes while making recommendations. Two immune challenge models were used: 1) Unsanitary condition where there manure from sow herd was spread in each pen. This model represented a general cause of immune challenge where the piglets were exposed to different kind of stressors such as housing condition and microbial load. The model was new our lab group and since the exact causative factor was not established the effect on piglets was variable. For instance, this model resulted in depressed feed and growth when piglets were fed graded Lys levels but this effect was not shown in piglets feed graded SAA:Lys ratios. 2) Oral inoculation with enterotoxigenic *Escherichia coli* (**ETEC**) K88, this model has been established and used to determine piglets' responses to a specific cause of immune challenge (Owusu-Asiedu et al., 2002; Opapeju et al., 2009) and hence

the clinical signs after inoculation are known. Effectiveness of both models depends on the presence of pathogenic microbes and age of piglets because as the piglet grew older, their immune system matured and were less susceptible to the immune challenge. Both models were used to assess AA requirements when the piglet's gut health is compromised.

For years, animals have been used to understand the mechanisms of different types of immune challenges. The animal used depends on availability, costs and personal preferences. The pig has been an animal of interest because of its close relation to humans in that they are both omnivores, monogastrics, and their immune system closely resembles, therefore, pigs could provide information applicable to humans (Philipson et al. 2013). The pig models used are for study of enteric, respiratory and systemic infections. The enteric and respiratory infections studies involves introduction of a live pathogen to the site of infection whereas injection of an antigen is used for systemic infection. For this thesis, two models of causing enteric infection were used one was general while the other one was specific.

The introduction of piglets to unsanitary condition was a general model and is highly variable among research groups because there is no one overall definition of the model. Several researchers have previously used the sanitary model to assess its effects and the interaction with diets on pigs' performance and immunity (Williams et al. 1997; Bassaganya-Riera et al. 2001; Lee et al. 2005; Le Floc'h et al. 2006; Zhao et al. 2007). Bassaganya-Riera et al. (2001) studying effects of conjugated linoleic acid on nursery pigs had an unsanitary condition as a room where older pigs had occupied for a week before starting the experiment, no biosecurity rules were applied to this room, and the piglets did not receive any antibiotic injection from farrowing to the start of the experiment. Le Floc'h et al. (2006) had an unsanitary condition as a non-sanitized room that was not disinfected nor cleaned after previous occupation by pigs from the same herd

and these piglets fed on antibiotic-free diets. In addition, non-experimental pigs were introduced to double the number per pen a week after the start of the experiment. For Williams et al. (1997), the pigs were placed in a non-sanitized nursery facility that had been previously occupied but not washed via high pressure spraying. Also, the facility was co-occupied by 7-wk old non-experimental pigs introduced into the room at 21 d intervals.

In all these studies, unsanitary conditions had detrimental effects on piglets' performance and immunity. The presence of manure from a different pig herd and the lack of cleaning of the pens is meant to increase microbial load to a level intolerable by the piglets. Thus there is a chronic immune system activation and chances of re-infection as the bacterial load increases into the room. The type of bacteria the piglets exposed is not known, however, due to the immature piglets' immune system their susceptibility to infection from opportunistic bacteria is high. Further, there is build-up of odour as shown by Lee et al. (2005) where the ammonia, carbon dioxide and dust level under unsanitary condition doubled the levels in the sanitary condition. The odour from unsanitary rearing is capable of causing respiratory tract infections. The main limitation is that the causative agent to a sickness is not known. The strength of this model is that it imitates a real life situation where unknown and perhaps several causative agents will induce an immune challenge that can be subclinical or have clinical manifestations.

The response criteria that has been used to assess the effects of unsanitary conditions are growth, feed intake, blood cytokines, lymphocytes and acute phase protein content. The feed intake and growth are criteria of choice because immune system activation normally depresses appetite that will in-turn lead to slow growth rates. Whereas, the lymphocytes, cytokines and acute phase proteins give an indication and the extent of an immune challenge.

The gastrointestinal tract is host to many bacteria and many of whom are commensals, the alteration of the bacterial population by introduction of pathogen or foreign particles can lead to an infection. The *Escherichia coli* model is specialized thus the causative agent and clinical symptoms are known. One of the major symptom seen is diarrhea that is caused by the heat labile (LT), heat-stable (STa and STb) toxins produced as the ETEC colonizes the intestine. The LT toxin activates adenylate cyclase receptor, whereas the heat stale toxins activate guanylate cyclase C receptor to increase production of cAMP or cGMP that limit sodium absorption leading to water secretion into the intestinal lumen (Giannella and Mann, 2003; Johnson, et al., 2009). Therefore, when determining the effectiveness of the model diarrhea is one of the response criteria used. The severity of the diarrhea varies depending on the virulence of the *Escherichia coli* and the host resistance to infection. Considering that there is water loss from the body, weight loss is also a common occurrence and if the weight loss is intensive there could be mortalities.

The response criteria used to assess the effects of the interaction between nutrition and *Escherichia coli* infection are blood cytokines, lymphocytes, acute phase proteins content, incidences of diarrhea, animal performance, and indices of intestinal health. Also, the presence of fimbriae enabling attachment to the intestinal mucosa has to be confirmed on the bacteria.

The effects of the enteric pathogen infestation model on the piglets are higher than the unsanitary condition model as shown in a meta-analysis by (Pastorelli, et al., 2012). The unsanitary condition model lead to a 4 and 12% decline in ADFI and ADG, respectively, whereas the enteric pathogen infestation model resulted in in 10 and 30% decline in ADFI and ADG, respectively, (Pastorelli, et al., 2012). The effects of the unsanitary condition model in this thesis were inconsistent as shown by the 13 and 15% decline in ADFI and ADG, respectively,

when used in piglets fed graded Lys levels but there were no observable of changes in piglets feed graded SAA:Lys ratios. The lack of illness was attributed to piglets' predisposal to maternal feces and that the feces could have had low or no levels of pathogenic microbes. Therefore, it could have been beneficial if some feces from pigs used in chapters 4 and 6 had been sampled for microbial population and identification of pathogenic and commensal bacteria. The type of fecal microbes present when the piglets arrived from the sow barns and during the experimental period could be compared to determine the effects of initial bacterial populations and dietary treatments on health of the piglets.

Lysine was the first AA whose requirement was determined because it is the key AA in making recommendations in the ideal protein ratio. Although it is known that most Lys is used for protein accretion and not immune challenge, it was still necessary to establish whether the AGP-free feeding regime would cause major changes. The sulfur amino acids were studied because they play a critical role not only in piglet performance but also in innate immunity and maintenance of intestinal integrity. The SAA comprise of Met, Cys and Tau, however, only Met and Cys are required for protein synthesis. Methionine is an essential AA thus cannot be synthesized by the body. Cysteine can be obtained from dietary source or synthesized from Met and 50% of the required dietary SAA can be provided by Cys (Chung and Baker, 1992). In piglets, 30% of the total dietary Met is used by the splanchnic tissue, as a source of energy, for synthesis of mucoproteins and mucin, antioxidant and maintenance of redox potential (Stipanuk, 2004; Stoll and Burrin, 2006). Additionally, some of the SAA is utilized by the gut commensal bacteria thereby preventing attachment of pathogenic microbes to the intestinal wall (Dahiya et al., 2007).

The Lys requirement for weaned piglets fed antibiotic-free diets and raised under different sanitation conditions was compared. Four graded Lys levels (from SID Lys 1.09 to 1.43%) resulted in linear but no quadratic response in both ADG and G:F. This linear response in ADG and G:F to increasing dietary Lys suggested that the SID Lys requirement for the weaned pigs could be at or greater than the 1.43%. Even though unsanitary conditions caused mild diarrhea, reduced feed intake, and depressed growth in piglets the feed efficiency was comparable to piglets raised under clean housing condition. The findings implied that although there were changes in metabolic processes that affected piglet performance, the impact on feed efficiency there negligible. Thus confirming that Lys requirement would not differ due to an immune challenge because of the minimal role played by Lys in the immunity. However, the dietary Lys requirement for piglets raised under both clean and unclean sanitary conditions could not be determined in this study therefore, chapter 5 discusses more Lys requirement studies. Two studies were conducted the first with 4 and second with 5 dietary treatments. Four dietary treatments had been used initially like in the chapter 4's experiment, however, getting an optimum level was difficult due to lack of more than 1 level above the plateau point. Hence fitting the 4 levels to broken-line models resulted in non-convergence of linear and nonlinear regression functions. Therefore, we opted to use 5 dietary treatments for all the rest of the studies in the thesis. A different challenge observed in all experiments was the accuracy at which the calculated AA values matched analyzed values. Initially it was thought that the method of analysis was the causative factor. In as much as analyzing diets using Near Infra-Red Spectrophotometry could have heavily contributed to the inaccuracy, the problem was not immediately solved with use of wet chemistry for ingredients and diets analyses. Thus in all experiment there were diets with excess AA, especially Lys. The optimum SID Lys estimates

based on linear and quadratic broken-lines were 1.30 and 1.34% based on ADG, thus average of 1.32%. These values were higher than NRC (1998) recommendation (1.19%) for 5 to 10 kg pigs, but similar to 1.35% recommendation by NRC (2012) for 7 to 11 kg pigs. Considering that the success of AGP in improving performance has been due to factors such as, reduction of gut microbiota that competes with host for nutrients (Bikker et al., 2006), we had expected piglets on diets without in-feed AGP to have high Lys requirement. However, the estimated SID Lys requirement compared with some studies where AGP were used therefore, Lys requirement was not changed due to antibiotic-free feeding regime.

In chapters 6 and 7, the SAA:Lys ratio was established under healthy, unsanitary and immune challenged conditions. The Lys requirement had been determined to be 1.32%, however in these chapters the diets were slightly limiting in Lys content (1.18%) to prevent underestimation of the SAA:Lys ratios. Therefore, Lys was the second limiting AA after the SAA in all diets. In these studies non-performance parameters were also used as response criteria. In chapter six, intestinal histomorphology specifically, jejunal villus height (**VH**), crypt depth (**CD**), VH:CD were included besides growth, as response criteria. The intestinal histomorphology has not been used before in determining AA required and is usually a measure of gut health. Normally the AA requirements are based on the function of the AA in the body and in a situation like the SAA where their utilization for growth equates gut health maintenance it raises questions whether the gut health can be used as a response criteria. In chapter 7, the gene expression of key enzymes in the Met pathway was used as response criteria in immune challenged piglets. It has been established that SAA play a key role in innate immunity, therefore it was expected any increase in demand due to immune challenge would be reflected by the genes. Unfortunately, the gene expression data given was not compared to healthy piglets or

those on diets with in-feed antibiotics within the same study. Thus the discussion was one sided and comparisons were made with literature findings, this was a major limitation because the animals and dietary ingredients used vary among the experiments.

We had hypothesized that unsanitary housing conditions and inoculation with enterotoxigenic *Escherichia coli* would stimulate an immune response leading to increased SAA maintenance needs and consequently the SAA to Lys ratio. Interestingly, the optimal SID SAA:Lys were similar when growth was used a response criteria but higher for unclean conditions when intestinal histomorphology was the response criteria. The results imply that SAA should be supplied beyond growth requirement in case foreign particles introduction of into the gut, so as to support the gut barrier function. However, using gene expression data the optimal was 60, a value similar to requirement for growth in chapter 6. Therefore, implying that a SAA:Lys ratio of 60 is enough to support the piglet's performance and immune challenge.

In chapter 6, the unsanitary piglets on the highest SAA:Lys ratio diet dropped their feed intake resulting to reduced growth. Similarly in Chapter 7, the SAA:Lys ratio of 72 depressed growth and feed intake before challenge, however, there was no effect after immune challenge the piglets. As evident that there was AA imbalance from a diet used in chapter 6, the dietary PUN that was linearly decreasing with increasing SAA:Lys ratio and increased at the SAA:Lys ratio of 68. The high SAA:Lys created AA imbalance that affect feed intake and it shows that these effects are more pronounced in case of an immune challenge. According to Harper (1964) the consumption of excess of an AA leads to high plasma concentration of that AA and one of the ways to maintain homeostasis is by reduction in feed intake. The reduction in feed intake consequently leads to growth depression. Amino acid imbalances are likely to occur with the current methodology used to determine requirements where there are graded levels of test AA

but the rest of the AA are kept constant in all diets. Also, there is a possibility of occurrence of a second limiting AA at higher levels of supplementation whose increased inclusion would normalize or reduce the negative effects.

In chapter 7, we used ETEC model of immune challenge to determine the optimum SID SAA:Lys when piglet's gut health is compromised. The gene expression as a response criteria used for immune challenged piglets gave similar SAA:Lys ratio to healthy piglets whose requirement was measured using performance parameters in chapter 6. The similarity could be due to low immune stimulation as shown by mild of diarrhea from *Escherichia coli* infected pigs. Although there was immune stimulation, the effects were not strong enough to cause moderate or severe diarrhea. However, mortalities were recorded, where 4 pigs from different treatments died due the infection, mainly as a result of loss of appetite. Thus the severity of the immune challenge differed among the piglets. In the pre-challenge period the peak ADG and ADFI was at SAA:Lys ratio of 66. Feeding SAA:Lys ratio of 48 and 72 dropped ADG by 54 and 62% and ADFI by 27 and 46%, respectively, compared to SAA:Lys ratio of 66. Hence the drop in growth for piglets fed diets with SAA:Lys ratio of 48 and 72 was not only because of reduce feed intake but mainly the reduced dietary SAA content. Consequently, the drop in the SAA intake by piglets on the 72 SAA:Lys treatment compromised their immune response as indicated by the low 6 h TNF-α concentration.

Literature on use of intestinal gene expression and the intestinal morphology in determining AA requirement in pigs is scarce. Most of the studies show the importance of SAA in maintenance of gut health, where there is a comparison of piglets fed either SAA deficient or normal or excess diets. Similarly, gene expression is done to understand SAA metabolism, which can be in relation to immune challenge. Therefore, this is the only work using gene expressions

and the intestinal morphology as response criteria in determining AA requirement in pigs. Thus in as much as the non-performance response criteria are good in times of an immune challenge, there is need of repetition of this kind of study so as to validate the values.

In conclusion, the Lys and SAA:Lys requirements for piglets fed AGP-free diets were established. The Lys requirement was similar to NRC (2012) recommendations. However, the SID SAA:Lys requirement for 7 to 12 kg pigs was 60 and higher than the 55 ratio from NRC (2012). The SAA:Lys ratio was similar the despite health status or sanitary condition of the piglets. This is because the performance parameters and gene expression gave similar estimate the SAA:Lys ratio for piglets. However, when the requirement is based on histomorphology, the piglets under unclean condition required higher SID SAA compared to the clean. Therefore, in situations where sanitary condition or gut health is compromised it is prudent to increase the dietary SAA content beyond the growth requirement for piglets. This thesis provided knowledge of Lys and SAA requirement in piglets fed AGP-free diets when healthy or when the gut is infected with pathogens. The information is necessary in developing rations for piglets that would maximize production with economic benefits.

CHAPTER NINE

CONCLUSIONS AND FUTURE DIRECTION

CONCLUSIONS

1. The feed efficiency was unaffected by sanitary conditions; therefore Lys requirement under clean or unclean housing conditions was similar.

2. The SID Lys requirement for weaned pigs fed AGP-free wheat-corn-soybean meal-based diets was 1.32% and thus similar to NRC (2012; 1.35%) recommendation.

3. The optimum SAA:Lys based on performance parameters was 58 and 61 under clean and unclean housing conditions, respectively. These ratios are higher than the 55 recommendation by NRC (2012).

4. The SID SAA requirement for gut health was 11.50 mg SAA/g gain for piglets under unclean condition, a level higher than 10.87 mg SAA/g gain by NRC (2012) for healthy piglets. Thus accentuating the increased need for SAA for piglets under unsanitary condition.

5. The SAA:Lys ratio of 60 was optimum for enterotoxigenic *E.coli* challenged piglets. This ratios are higher than the 55 recommendation by NRC (2012) for healthy piglets.

6. The results showed that the SAA:Lys is increased during an immune challenge and when piglets are fed AGP-free diets. The percentage increase in the SAA:Lys ratio when compared to NRC (2012) it between 9 to 16%.

FUTURE DIRECTIONS

1. A potential clearer way of observing met metabolic pathways is by using a stable isotope tracer as done by Riedijk et al. (2007). The quantities of Met used for Cys synthesis, incorporated into mucin, glutathione and muscle protein accretion can be calculated after infusion. These values will give better quantitative changes in Met requirement in piglets when evaluating nutrition and immunity interactions.

2. A long term assessment of health and performance of pigs that had previous AA supplementation at nursery stage is paramount. Therefore, the economies of AA supplementation during an immune challenge would be evaluated.

3. It is necessary to establish whether the AA requirement obtained under AGP-free feeding would suffice or be in excess with use of AGP alternatives such as probiotic, prebiotic, and acidifiers in piglet' diets.

4. Diet ingredients with functional components such as spray dried porcine plasma, egg products, milk products have been added to nursery pig' diets with success on

performance and health. It would be of an advantage to determine AA requirement of piglets when fed diets containing ingredients with functional components in comparison to a corn-soybean meal based diet.

5. The requirement of the rest of the EAA should be determined to establish the complete ideal AA profile under AGP-free feeding in healthy and immune compromised piglets.

CHAPTER 10

LITERATURE CITED

Abbas, A., A. H. Lichtman, and S. Pillai. 2012. Basic Immunology: Functions and disorders of the immune system. 4th ed. Philadelphia, PA.

Akira, S., T. Hirano, T. Taga, and T. Kishmoto. 1990. Biology of multifunctional cytokines: IL 6 and related molecules (IL1 and TNF). FASEB J. 4:2060-2067.

AOAC. 1990. Official Methods of Analysis. 15th ed. Assoc. Off. Anal. Chem., Washington, DC.

Bae, J. Y., I. H. Ok, S. Lee, S. S. O. Hung, T. S. Min, and S. C. Bai. 2011. Re-evaluation of dietary methionine requirement by plasma methionine and ammonia concentrations in surgically modified rainbow trout, *Oncorhynchus mykiss*. Asian-Aust. J. Anim. Sci. 24:974-98.

Baker, D. H. 1986. Problems and pitfalls in animal experiments designed to establish dietary requirements for essential nutrients. J. Nutr. 116:2339-2349.

Bassaganya-Riera, J., R. Hontecillas-Magarzo, K. Bregendahl, M. J. Wannemuehler, and D. R. Zimmerman. 2001. Effects of dietary conjugated linoleic acid in nursery pigs of dirty and clean environments on growth, empty body composition, and immune competence. J. Anim. Sci. 79:714-721.

Bauchart-Thevret C., B. Stoll, S. Chacko, and D. G. Burrin. 2009. Sulfur amino acid deficiency upregulates intestinal methionine cycle activity and suppresses epithelial growth in neonatal pigs. Am. J. Physiol. Endoc. M. 296:E1239-E1250.

Bertolo, R. F., S. Moehn, P. B. Pencharz, and R. O. Ball. 2005. Estimate of the variability of the lysine requirement of growing pigs using the indicator amino acid oxidation technique. J. Anim. Sci. 83:2535-2542.

Bikker, P., J. Fledderus, L. Le Bellego, and M. Rovers. 2006. AGP ban effects on amino acid requirements. Feed Mix 14:22-24.

Bikker, P., M. W. A. Verstegen, R. G. Campbell, and B. Kemp. 1994. Digestible lysine requirement of gilts with high genetic potential for lean gain, in relation to the level of energy intake. J. Anim. Sci. 72:1744–1753.

Brosnan, J. T., and M. E. Brosnan. 2006. The sulfur-containing amino acids: an overview. J. Nutr. 136: 1636S–1640S.

Bruininx, E. M. A. M., C. M. C. van der Peet-Schwering, J. W. Schrama, P. F. G. Vereijken, P. C. Vesseur, H. Everts, L. A. den Hartog, and A. C. Beynen. 2001. Individually measured feed intake characteristics and growth performance of group-housed weanling pigs: Effects of sex, initial body weight, and body weight distribution within groups. J. Anim. Sci. 79:301–308.

Calder, P. C. 2007. Immunological Parameters: What Do They Mean? J. Nutr. 137: 773S–780S.

Callesen, J. 2002. Effects of termination of AGP use on pig welfare and productivity. In Abstracts of the International Invitational Symposium: Beyond Antibiotic Growth Promoters in Food Animal Production, Foulum, Denmark, 2002, p. 6. Danish Veterinary Institute, Copenhagen, and the Danish Institute of Agricultural Science, Tjele, Denmark.

Cameron, N. D., E. McCullough, K. Troup, and J. C. Penman. 2003. Serum urea concentration as a predictor of dietary lysine requirement in selected lines of pigs. J. Anim. Sci. 81:91-100.

Canada Veterinary Drug Directorate 2014. http://healthycanadians.gc.ca/drugs-products-medicaments-produits/antibiotic-resistance-antibiotique/antimicrobial-framework-cadre-antimicrobiens-eng.php

Capozzalo, M. M., J. C. Kim, J. K. Htoo, C. F. M. de Lange, B. P. Mullan, C. F. Hansen, J. W. Resink, P. A. Stumbles, D. J. Hampson, and J. R. Pluske. 2012. An increased ratio of dietary tryptophan to lysine improves feed efficiency and elevates plasma tryptophan and kynurenine in the absence of antimicrobials and regardless of infection with enterotoxigenic *Escherichia coli* in weaned pigs. J. Anim. Sci. 2012.90:191–193.

CCAC. 2009. Guidelines on: The care and use of experimental animals in research, teaching and testing. Canadian Council on Animal Care, Ottawa, ON, Canada.

Chawla, R. K., W. H. Watson, C. E. Eastin, E. Y. Lee, J. Schmidt, and C. J. McClain. 1998. S-adenosylmethionine deficiency and TNF-alpha in lipopolysaccharide-induced hepatic injury. Am. J. Physiol. 275:G125-129.

Chiba, A., S. Kaieda, S. Oki, T. Yamamura, and S. Miyake. 2005. The involvement of Vα14 natural killer T cells in the pathogenesis of arthritis in murine models. Arthritis Rheum. 52:1941-1948.

Chorazy-Massalska M., E. Kontny, A. Kornatka, M. Rell-Bakalarska, J. Marcinkiewicz, and W. Maśliński. 2004. The effect of taurine chloramine on pro-inflammatory cytokine production by peripheral blood mononuclear cells isolated from rheumatoid arthritis and osteoarthritis patients. Clin. Exp. Rheumatol. 22:692-698.

Choudhary, C., C. Kumar, F. Gnad, M. L. Nielsen, M. Rehman, T. C. Walther, J. V. Olsen, and M. Mann. 2009. Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science. 325:834-840.

Chung, T. K., and D. H. Baker. 1992a. Maximal portion of the young pig's sulfur amino acid requirement that can be furnished by cystine. J. Anim. Sci. 70:1182-1187.

Chung, T. K., and D. H. Baker. 1992b. Methionine requirement of pigs between 5 and 20 kilograms of body weight. J. Anim. Sci. 70:1857-1863.

Chung, T. K., and D. H. Baker. 1992c. Ideal amino acid pattern for 10 kg pigs. J. Anim. Sci. 70:3102-3111.

Coma, J., D. R. Zimmerman, and D. Carrion. 1995. Relationship of rate of lean tissue growth and other factors to concentration of urea in plasma of pigs. J. Anim. Sci. 73. 3649-3656.

Coma, J., D. R. Zimmerman, and D. Carrion. 1996. Lysine requirement of the lactating sow determined by using plasma urea nitrogen as a rapid response criterion. J. Anim. Sci. 74:1056-1062.

Commission Directive. 1998. Establishing community methods for the determination of amino acids, crude oils and fats, and olan-quindox in feeding stuff and amending Directive 71/393/EEC, annex part A. Determination of Amino Acids. Offic. J. L257:14-23.

Commission Directive. 2000. Establishing community methods for the determination of vitamin A, vitamin E and tryptophan, annex part C. Determination of Tryptophan. Offic. J. L174:45-50.

Cook, D. N, D. M. Prosser, R. Forster, J. Zhang, N. A. Kuklin, et al. 2000. CCR6 Mediates Dendritic Cell Localization, Lymphocyte Homeostasis, and Immune Responses in Mucosal Tissue. Immunity 12:495-503.

Cromwell, G. L. 2002. Why and how antibiotics are used in swine production. Anim. Biotechnol.13:7-27.

Cromwell, G. L., T R Cline, Crenshaw, J. D., Crenshaw, T. D., Ewan, R. C., Hamilton, C. R., Lewis, A. J., Mahan, D. C., Miller, E. R., and J. E. Pettigrew. 1993. The dietary protein and (or) lysine requirements of barrows and gilts. NCR-42 Committee on Swine Nutrition. J. Anim. Sci. 17:1510-1519.

Dahiya, J. P., D. Hoehler, A. G. Van Kessel, and M. D. Drew. 2007. Effect of different dietary methionine sources on intestinal microbial populations in broiler chickens. Poult. Sci. 86:2358-2366.

Dale, D. C., L. Boxer, and W. C. Liles. 2008. The phagocytes: neutrophils and monocytes. Blood.112:935-945.

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 pigs. J. Anim. Sci. 90:3485-3491.

Dean, D. W., L. L. Southern, B. J. Kerr, and T. D. Bidner. 2007. The lysine and total sulfur amino acid requirements of six- to twelve-kilogram pigs. Prof. Anim. Sci. 23:527-535.

Deitch, E. A. 1998. Animal models of sepsis and shock: a review and lessons learned. Shock. 9:1-11.

Di Buono M., L. J. Wykes, R. O. Ball, and P. B. Pencharz. 2001. Total sulfur amino acid requirement in young men as determined by indicator amino acid oxidation with L-[1–13C] phenylalanine. Am. J. Clin. Nutr.74: 756–60.

Di Buono, M., L. J. Wykes, D. E. C. Cole, R. O. Ball and P. B. Pencharz. 2003. Regulation of sulfur amino acid metabolism in men in response to changes in sulfur amino acid intakes. J. Nutr. 133: 733–739.

Dibner, J. J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture: History and mode of action. Poult. Sci. 84:634-643.

D'Mello F. J. P. 2003. Responses of growing poultry to amino acids. In amino acids in animal nutrition (ed. F. J. P. D'Mello), pp. 237-264. CAB International, Wallingford, UK.

Dong, G. Z., and J. R. Pluske. 2007. The low feed intake in newly-weaned pigs: Problems and possible solutions. Asian-Aust. J. Anim. Sci. 20:440-452.

Donham, K. J., 2000. The concentration of swine production: Effects on swine health, productivity, human health and environment. Vet. Clin. N. Am. Food A. 16:559-597.

Duffy, B., T. Gunn, J. Collinge and P. B. Pencharz. 1981. The effect of varying protein quality and energy intake on the nitrogen metabolism of parenterally fed very low birthweight (<1600 g) infants. Pediatr. Res. 15:1040-1044.

Einerhand, A. W. C., I. B. Renes, M. K. Makkink, M. van der Sluis, H. A. Bu¨ ller, and J. Dekker. Role of mucins in inflammatory bowel disease: important lessons from experimental models. Eur. J. Gastroenterol. Hepatol. 14:757–765.

Elango, R., Goonewardene, L. A., Pencharz, P. B., and R. O. Ball. 2004. Parenteral and enteral routes of feeding in neonatal piglets require different ratios of branched-chain amino acids. J. Nutr.134:72-8.

Elango, R., M. A. Humayun, R. O. Ball, and P. B. Pencharz. 2007. Lysine requirement of healthy school-age children determined by the indicator amino acid oxidation method. Am. J. Clin. Nutr. 86:360-366.

Ettle, T., and F. X. Roth. 2009. Dietary selection for lysine by piglets at differing feeding regimen. Livest. Sci. 122:259-263.

Faure, M., C. Mettraux, D. Moennoz, J. P. Godin, J. Vuichoud, F. Rochat, D. Breuille´, C. Obled, and I. C. Theulaz. 2006. Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium–treated rats. J. Nutr. 136: 1558‑1564.

Friesen K.G., J. L. Nelssen, R. D. Goodband, M. D. Tokach, J. A. Unruh, D. H. Kropf, and B. J. Kerr. 1994. Influence of dietary lysine on growth and carcass composition of high-lean-growths gilts fed from 32 to 72 kilograms. J. Anim. Sci. 72:1761-1770.

Friesen, K. G. J. L. Nelssen, R. D. Goodband, M. D. Tokach, J. A. Unruh, D. H. Kropf, and B. J. Kerr. 1994. Influence of dietary lysine on growth and carcass composition of high-lean-growth gilts fed from 34 to 72 kilograms. J. Anim. Sci. 72:1761-1770.

Fuller, M. F., R. Mc William, T. C. Wang, and L. R. Giles. 1989. The optimum dietary amino acid pattern for growing pigs, 2. Requirements for maintenance and for tissue protein accretion. Br. J. Nutr. 62:255-261.

Gaines A. M., G. F. Yi, B. W. Ratliff, P. Srichana, D. C. Kendall, G. L. Allee, C. D. Knight, and K. R. Perryman. 2005. Estimation of the ideal ratio of true ileal digestible sulfur amino acids:lysine in 8 to 26 kg nursery pigs. J. Anim. Sci. 83:2527-2534.

Gaines, A. M., D. C. Kendall, G. L. Allee, M. D. Tokach, S. S. Dritz, and J. L. Usry. 2003. Evaluation of the true ileal digestible (TID) lysine requirement for 7 to 14 kg pigs. J. Anim. Sci. 81 (Suppl. 1):549. (Abstr.).

Gerlach, R. G. and M. Hensel. 2007. Protein secretion systems and adhesins: The molecular armory of Gram-negative pathogens. Int. J. Med. Microbiol. 297:401-415.

Giannella, R. A. and E. A. Mann. 2003. E. coli heat-stable enterotoxin and guanylyl cyclase C: new functions and unsuspected actions. Trans. Am. Clin. Climatol. Assoc. 114: 67–86.

Gimble, R.F. 2006. The effects of sulfur amino acid intake on immune function in humans. J. Nutr. 136: 1660S–1665S.

Gomez, M. S. C. 2006. Development of gut microbiota in the pig: modulation of bacterial communities by different feeding strategies. Doctoral thesis, University of Barcelona.

Grimble, R F., and G. K. Grimble. 1998. Immunonutrition: Role of sulfur amino acids, related amino acids, and polyamines. Nutr. 14: 605-610.

Gruys, E., M. J. M. Toussaint, T. A. Niewold, and S. J. Koopmans. 2005. Acute phase reaction and acute phase proteins. J. Zhejiang Univ. Sci. 6B:1045-1056.

Hack, V., D. Schmid, R. Breitkreutz, C. Stahlhenning, P. Drings, H. Kinscherf, F. Taut, E. Holm, and W. Droge. 1997. Cystine levels, cystine flux, and protein catabolism in cancer cachexia, HIV/SIV infection, and senescence. FASEB J. 11:84-92.

Harper A. E., N. J. Benevenga, and R. M. Wohlhueter. 1970. Effects of ingestion of disproportionate amounts of amino acids. Physiol. Rev. 50:428-558.

Hauschild, L., C. Pomar, and P. A. Lovatto. 2010. Systematic comparison of the empirical and factorial methods used to estimate the nutrient requirements of growing pigs. Animal 4:714-723.

Henry, Y. 1993. Self-selection of lysine by growing pigs: choice combinations between deficient or sub-optimal and adequate or super-optimal dietary levels according to sex. Reprod. Nutr. Dev. 33: 489-502.

House J. D., P. B. Pencharz, and R. O. Ball. 1997. Tyrosine kinetics and requirements during total parenteral nutrition in the neonatal piglet: the effect of glycyl-L-tyrosine supplementation. Pediatr. Res. 41:575-83.

Hunter, E. A. L., and R. F. Grimble. 1994. Cysteine and methionine supplementation modulate the effect of tumor necrosis factor on protein synthesis, glutathione and zinc concentration of liver and lung in rats fed a low protein diet. J. Nutr. 124: 2319-2328.

Iyayi E. A., F. A. Aderemi, O. O. Ladele, and A. S. Popoola. 2014. Effects of low protein diets supplemented with high amino acids (methionine or lysine) on performance of broilers. Am. J. Exp. Agr. 4:525-531.

Jahoor F., L. J. Wykes, P. J. Reeds, J. F. Henry, M. P. del Rosario, and M. E. Frazer. 1995. Protein-deficient pigs cannot maintain reduced glutathione homeostasis when subjected to the stress of inflammation. J. Nutr. 125:1462–1472.

Jan Wehkamp, J. Harder, K. Wehkamp, B. Wehkamp-von Meissner, M. Schlee, C. Enders, U. Sonnenborn, S. Nuding, S. Bengmark, K. Fellermann, J. M. Schröder, and E. F. Stange. 2004. NF-κB- and AP-1-Mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a Novel Effect of a Probiotic Bacterium. Infect. Immun. 72:5750-5758.

Jensen-Waern, M., L. Melin , R. Lindberg, A. Johannisson, L. Petersson, and P. Wallgren. 1998. Dietary zinc oxide in weaned pigs--effects on performance, tissue concentrations, morphology, neutrophil functions and faecal microflora. Res. Vet. Sci. 64:225-31.

Johnson, A. M., Kaushik, R. S., Francis, D. H., Fleckenstein, J. M., and P. R. Hardwidge. 2009. Heat-labile enterotoxin promotes *Escherichia coli* adherence to intestinal epithelial cells. J. Bacteriol. 191:178-186.

Jones J. B., C. M. Wathes and A. J. F. Webster. 1997. Behavioural responses of pigs to atmospheric ammonia. In: Proceedings of the 5th International Livestock Environment Symposium. 875-882. St. Joseph, MI: ASAE.

Kaewtapee C, N. Krutthai, K. Poosuwan, T. Poeikhampha, S. Koonawootrittriron, and C. Bunchasak. 2010. Effects of adding liquid DL-methionine hydroxy analogue-free acid to drinking water on growth performance and small intestinal morphology of nursery pigs. J.Anim. Physiol.Anim. Nutr. 94:395-404.

Kahindi R. K., J. K. Htoo, and C. M. Nyachoti. 2014c. Optimal sulfur amino acid to lysine ratio for weaned pigs fed antibiotic-free diet and raised under clean and unclean conditions Joint Animal Science Meeting of ASAS/ADSA/CSAS, Kansas City, Missouri. 20-24 July.

Kahindi, R. K., J. K. Htoo, and C. M. Nyachoti. 2014a. The standardised ileal digestible lysine requirement of 7 to 16 kg weaned pigs fed antibiotic-free diet. AVTRW/BSAS/WPSA Conference - Innovation from animal science - Science into Practice University of Nottingham, Jubilee Campus, Wollaton Road, Nottingham, UK 29 - 30 April, 2014.

Kahindi, R. K., J. K. Htoo, and C. M. Nyachoti. 2014b. SHORT COMMUNICATION: Effect of dietary lysine content and sanitation conditions on performance of weaned pigs fed antibiotic-free diets. Can. J. Anim. Sci. 94:115-118.

Kendall, D. C., A. M. Gaines, G. L. Allee, and J. L. Usry. 2008. Commercial validation of the true ileal digestible lysine requirement for eleven- to twenty-seven-kilogram pigs. J. Anim. Sci. 86:324-332.

Kim, J.C., B. P. Mullan, B. Frey, H. G. Payne, and J. R. Pluske. 2012. Whole body protein deposition and plasma amino acid profiles in growing and/or finishing pigs fed increasing levels of sulfur amino acids with and without *Escherichia coli* lipopolysaccharide challenge. J. Anim. Sci. 90:362-365.

Kim, K. Y., H. J. Ko, H. T. Kim, Y. S. Kim, Y. M. Roh, C. M. Lee, and C. N. Kim. 2008. Quantification of ammonia and hydrogen sulfide emitted from pig buildings in Korea. J. Environ. Manage. 88:195-202.

Klasing, K.C. 2009. Minimizing amino acid catabolism decreases amino acid requirements. J. Nutr. 139:11-12.

Kohchi, C., H. Inagawa, T. Nishizawa, and G. I. Soma. 2009. ROS and Innate Immunity. Anticancer Research 29:817-822.

Kraatz M. 2010. Lactobacilli and other lactic acid-related bacteria in the mucosal proximal gastrointestinal tract of pigs: a review of ecology for two derivative approaches for isolation of novel species. Current research, technology and educational topics in applied microbiology and microbial biotechnology (A Mendez- Vilas) (ed). Pages 674 -686.

Kurpad, A. V., and T. Thomas. 2011. Methods to assess amino acid requirements in humans. Curr. Opin. Clin. Nutr. Metab. Care14:434-439.

Kurpad, A. V., M. M. Regan, S. Varalakshmi, J. Gnanou, and V. R. Young. 2004. Daily requirement for total sulphur amino acids of chronically undernourished Indian men. Am. J. Clin. Nutr. 80:95–100.

Kurpad, A. V., M. M. Regan, T. Raj and J. V. Gnanou. 2006. Branched-chain amino acid requirements in healthy adult human subjects. J. Nutr. 136:256S–263S.

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. Sc. 87:37-45.

Le Floc'h, N., and S. Bernard. 2007. Biological roles of tryptophan and its metabolism: Potential implications for pig feeding. Livest. Sci. 112:23-32.

Le Floc'h, N., C. Jondreville, J. J. Matte, and B. Seve. 2006. Importance of sanitary environment for growth performance and plasma nutrient homeostasis during the postweaning period in piglets. Arch. Anim. Nutr. 60:23–34.

Le Floc'h, N., J. J. Matte, D. Melchior, J. van Milgen, B. Sève. 2010. A moderate inflammation caused by the deterioration of housing conditions modifies Trp metabolism but not Trp requirement for growth of post-weaned piglets. Animal 4:1891-1898.

Lee, C. L., R. Giles, W. L. Bryden, J. L. Downing, P. C. Owens, A. C. Kirby, and P. C. Wynn. 2005. Performance and endocrine responses of group housed weaner pigs exposed to the air quality of a commercial environment. Livest. Prod. Sci. 93:255-262.

Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller. 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microbiol. 68:673–690.

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.

Liang, S. M., N. Lee, D. S. Finbloom, and C. M. Liang. 1992. Regulation by glutathione of interleukin-4 activity on cytotoxic T cells. Immunol. 75:435-440.

Litvak, N., J. K. Htoo, and C. F. M. de Lange. 2013. Restricting sulfur amino acid intake in growing pigs challenged with lipopolysaccharides decreases plasma protein and albumin synthesis. Can. J. Anim. Sci. 93: 505-515.

Llames, C. R., and J. Fontaine. 1994. Determination of amino acids in feeds: Collaborative study. J. Assoc. Off. Anal. Chem. 77:1362–1402.

Loughmiller, J. A., J. L. Nelssen, R. D. Goodband, M. D. Tokach, E. C. Titgemeyer, and I. H. Kim. 1998. Influence of dietary total sulfur amino acids and methionine on growth performance and carcass characteristics of finishing gilts. J. Anim. Sci. 76:2129–2137.

Lumpkins, B. S., A. B. Batal, and D. H. Baker. 2007. Variations in the digestible sulfur amino acid requirement of broiler chickens due to sex, growth criteria, rearing environment, and processing yield characteristics. Poult. Sci. 86:325–330.

Malmezat, T., D. Breuille, P. Capitan, P. P. Mirand, and C. Obled. 2000. Methionine transsulfuration is increased during sepsis in rats. Am. J. Physiol. Endocrinol. Metab. 279: E1391-1397.

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

Marcinkiewicz J. 2010. Taurine bromamine (TauBr) - its role in immunity and new perspectives for clinical use. J. Biomed. Sci. 17(Suppl 1):S3.

Maroufyan, E., A. Kasim, S. R. Hashemi, T. C. Loh, M. H. Bejo, and H. Davoodi. 2010. The effect of methionine and threonine supplementations on immune responses of broiler chickens challenged with infectious bursal disease. Am. J. Applied Sci. 7: 44-50.

Marquardt R., L. Z. Jin, J. W. Kim, L. Fang, A. A. Frohlich, and S. K. Baidoo. 1999. Passive protective effect of egg-yolk antibodies against enterotoxigenic Escherichia coli K88+ infection in neonatal and early-weaned piglets. FEMS Immunol. Med. Microbiol. 23:283-288.

McLaughlan, J. M., and W. I. Illman. 1967. Use of free plasma amino acid levels for estimating amino acid requirements of the growing rat. J. Nutr. 93:67-70.

Melchior, D., B. Se`ve, and N. Le Floc'h. 2004. Chronic lung inflammation affects plasma amino acid concentrations in pigs. J. Anim. Sci. 82:1091–1099.

Metges, C. C., El-Khoury, A. E., Henneman, L., Petzke, K. J., Grant, I., Bedri, S., Pereira, P. P., Ajami, A. M., Fuller, M. F., and V. R. Young. 1999. Availability of intestinal microbial lysine for whole body lysine homeostasis in human subjects. Am. J. Physiol. (Endocrinol. Metab. 40) 277: E597-E607.

Miao, J., L. Zheng, J. Zhang, Z. Ma, W. Zhu, and S. Zou. 2011. The effect of taurine on the toll-like receptors/nuclear factor kappa B (TLRs/NF-κB) signaling pathway in Streptococcus uberis-induced mastitis in rats. Int. Immunopharmacol. 11:1740–1746.

Moehn, S., Shoveller, A. K., Rademacher, M. and R. O. Ball. 2008. An estimate of the methionine requirement and its variability in growing pigs using the indicator amino acid oxidation technique. J. Anim. Sci.86:364-369.

Moore, K. L., B. P. Mullan, R. G. Campbell, and J. C. Kim. 2013. The response of entire male and female pigs from 20 to 100 kg liveweight to dietary available lysine. Anim. Prod. Sci. 53:67-74.

Morris, T. M. 2004. Nutrition of chicks and layers. World's Poult. Sci. Assoc. 60:5-12.

Nemechek, J. E., A. M. Gaines, M. D. Tokach, G. L. Allee, R. D. Goodband, J. M. DeRouchey, J. L. Nelssen, J. L. Usry, G. Gourley, and S. S. Dritz. 2012. Evaluation of standardized ileal digestible lysine requirement of nursery pigs from seven to fourteen kilograms. J. Anim. Sci. 90:4380-4390.

Ni, J. Q, A. J. Heber, C. A. Diehl, and T. T. Lim. 2000. Ammonia, hydrogen sulphide and carbon dioxide release from pig manure in under-floor deep pits. J. Agr. Eng. Res. 77, 53-66.

Nimni, M., B. Han, and F. Cordoba. 2007. Are we getting enough sulfur in our diet? Nutr. Metab. 4: 24.

Nitsche-Schmitz, D. P., M. Rohde, and G. S. Chhatwal. 2007. Invasion mechanisms of Gram-positive pathogenic cocci. Thromb. Haemost. 98: 488-496.

NRC, 1998. Nutrient requirements of swine. 10th ed. Natl. Acad.Press, Washington, DC.

NRC, 2012. Nutrient requirements of swine. 11th ed. Natl. Acad.Press, Washington, DC.

Opapeju, F.O., D. O. Krause, R. L. Payne, M. Rademacher, and C. M. Nyachoti. 2009. Effect of dietary protein level on growth performance, indicators of enteric health and gastrointestinal microbial ecology of weaned pigs induced with post-weaning colibacillosis. J. Anim. Sci. 87:2635–2643.

Oresanya, T. F., A. D. Beaulieu, E. Beltranena, and J. F. Patience. 2007. The effect of dietary energy concentration and total lysine/digestible energy ratio on the growth performance of weaned pigs**.** Can. J. Anim. Sci. 87:45-55.

Owusu-Asiedu, A., S. K. Baidoo, C. M. Nyachoti, and R. R. Marquardt. 2002. Response of early-weaned pigs to spray-dried porcine or animal plasma-based diets supplemented with egg-yolk antibodies against enterotoxigenic *Escherichia coli*. J. Anim. Sci. 80:2895-2903.

Parr, T. M., B. J. Kerr and D. H. Baker. 2003. Isoleucine requirement of growing (25 to 45 kg) pigs. J. Anim. Sci. 81:745–752.

Parvin, R., A. B. Mandal, S. M. Singh, and R. Thakur. 2010. Effect of dietary level of methionine on growth performance and immune response in Japanese quails (*Coturnix Japonica*). J. Sci. Food Agric. 90:471-481.

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

Pearson, G., and J. R. Carr. 1979. Methionine supplementation of weaner pig diets containing lupin-seed meal. New Zeal. J. Exp. Agr. 7:99-101.

Pedersen, C., J. E. Lindberg, and S. Boisen 2003. Determination of the optimal dietary threonine:lysine ratio for finishing pigs using three different methods. Livest. Prod. Sci., 82, 233‑243.

Pencharz, P. B., and R. O. Ball. 2003. Different approaches to define individual amino acid requirements. Annual Review of Nutrition 23: 101-116.

Philipson, C.W., J. Bassaganya-Riera, and R. Hontecillas. 2013. Animal models of enteroaggregative *Escherichia coli* infection. Gut Microbes 4:281-91.

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.

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.

Pope, T., L. N. Loupe, P. B. Pillai, and J. L. Emmert. 2004. Growth performance and nitrogen excretion of broilers using a phase-feeding approach from twenty-one to sixty-three days of age. Poult. Sci. 83:676–682.

Quiniou, N. and J. Noblet. 2012. Effect of the dietary net energy concentration on feed intake and performance of growing-finishing pigs housed individually. J. Anim. Sci. 90:4362-4372.

Rahman, I. 2005. Regulation of glutathione in inflammation and chronic lung diseases. Mutation Res. 579:58–80.

Rakhshandeh, A, J. K. Htoo, and C. F. M. de Lange. 2010. Immune system stimulation of growing pigs does not alter apparent ileal amino acid digestibility but reduces the ratio between whole body nitrogen and sulfur retention. Livest. Sci. 134:21-23.

Rakhshandeh, A., and C. F. M. de Lange. 2011. Evaluation of chronic immune system stimulation models in growing pigs. Animal 6:305-310.

Reeds, P. J. 2000. Dispensable and indispensable amino acid for humans. J. Nutr. 130:1835S–1840S.

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.

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.

Richard, L., C. Vachot, A. Surget, V. Rigolet, S. J. Kaushik and I. Geurden. 2011. The effect of choline and cystine on the utilisation of methionine for protein accretion, remethylation and trans-sulfuration in juvenile shrimp *Penaeus monodon*. Bri. J. Nutr. 106:825-835.

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.

Robbins, K. R., H. W. Norton, and D. H. Baker. 1979. Estimation of nutrient requirements from growth data. J. Nutr. 109:1710- 1714.

Romagnani, S. 2006. Regulation of the T cell response. Clin. Exp. Allergy. 36:1357–1366.

Ronchi, V. P., A. M. Giudici, J. R. Mendieta,V. J. Caballero, A. N. Chisari, P. M. Sanllorenti, and R. D. Conde. 2010. Oxidative stress in mouse liver caused by dietary amino acid deprivation: protective effect of methionine. J. Physiol. Biochem. 66:93–103.

Roth, F. X., K. Eder, and M. Kirchgessner. 1999. The effect of energy density and the lysine to energy ratio of diets on the performance of piglets. J. Anim. Physiol. Anim. Nutr. 82:1-7.

Round, J. L., and Mazmanian, S. K. 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nature Rev. Immunol. 9:313-323.

Ruth, M. R., and C. J. Field. 2013. The immune modifying effects of amino acids on gut associated lymphoid tissue. J. Anim. Sci. Biotechnol. 4:27.

Sakaguchi, S. K. Wing, Y. Onishi, P. Prieto-Martin, and T. Yamaguchi. 2009. Regulatory T cells: how do they suppress immune responses? Int. Immunol. 21:1105–1111.

Santangelo, F. 2002. The regulation of sulfurated amino acid junctions: Fact or fiction in the field of inflammation? Amino Acids 23: 359–365.

Schutte, J. B., J. De Jong, W. Smink, and M. Pack. 1997. Replacement value of betaine for DL-methionine in male broiler chicks. Poult. Sci. 76:321–325.

Schwartz, J. M. and S. D. Bilbo. 2011. LPS elicits a much larger and broader inflammatory response that E. coli infection within the hippocampus of neonatal rats. Neurosci. Lett. 497:110-115.

Selhub, J. 1999. Homocysteine metabolism. Annu. Rev. Nutr. 19:217-246.

Setia, A., S. K. Bhandari, J. D. House, C. M. Nyachoti, and D. O. Krause. 2009. Development and in vitro evaluation of an Escherichia coli probiotic able to inhibit the growth of pathogenic E. coli K88. J. Anim. Sci. 87:2005–2012.

Shelton, N. W., M. D. Tokach, S. S. Dritz, R. D. Goodband, J. L. Nelssen, J. M. DeRouchey, and J. L. Usry. 2011. Effects of porcine circovirus type 2 vaccine and increasing standardized ileal digestible lysine:ME ratio on growth performance and carcass composition of growing and finishing pigs. J. Anim. Sci. 89:2440-2451.

Shifflett, D. E., D. R. Clayburgh, A. Koutsouris, J. R. Turner, and G. A. Hecht. 2005. Enteropathogenic E. coli disrupts tight junction barrier function and structure in vivo. Lab. Investi. 85:1308-1324.

Shoveller, A. K., Brunton, J. A., House, J. D., Pencharz, P. B., and R. O. Ball. 2003. Dietary cysteine reduces the methionine requirement by an equal proportion in both parenterally and enterally fed piglets. J. Nutr. 133:4215-24.

Shoveller, A. K., J.A. Brunton, P. B. Pencharz, and R. O. Ball. 2003. The methionine requirement is lower in neonatal piglets fed parenterally than in those fed enterally. J. Nutr.133:1390–1397.

Simpson, J. M., V. J. McCracken, B. A. White, H. R. Gaskins, and R. I. Mackie. 1999. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. J. Microbiol. Methods 36:167-179.

Staal, F. J., M. Roederer, and L. A. Herzenberg. 1990. Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA. 87:9943-9947.

Stein, H. H., B. Seve, 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.

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

Stipanuk, M. H., R. M. Coloso, et al. 1992. Cysteine concentration regulates cysteine metabolism to glutathione, sulfate and taurine in rat hepatocytes. J Nutr. 122: 420-427.

Stoll, B. and D. G. Burrin. 2006. Measuring splanchnic amino acid metabolism in vivo using stable isotopic tracers. J. Anim. Sci. 84(E. Suppl.):E60–E72.

Taylor, A.E., P. Toplis, I.J. Wellock, and H.M. Miller. 2012. The effects of genotype and dietary lysine concentration on the production of weaner pigs. Livest. Sci. 149: 180-184.

Theivanthiran, B., S. Batra, G. Balamayooran, S. Cai, K. Kobayashi, R. A. Flavell and S. Jeyaseelana. 2012. Nod2 signaling contributes to host defense in the lungs against *Escherichia coli* infection. Infect. Immun. 80:2558-2569.

Thompson, C. L. and A. J. Holmes. 2008. A window of environmental dependence is evident in multiple phylogenetically distinct subgroups in the faecal community of piglets. FEMS Microbiol. Lett. 290:91–97.

Thorgersen, E. B., B. C. Hellerud, E. W. Nielsen, A. Barratt-Due, H. Fure, J. K. Lindstad, A. Pharo, E. Fosse, T. I. Tønnessen, H. T. Johansen, A. Castellheim, and T. E. Mollnes.

2010. CD14 inhibition efficiently attenuates early inflammatory and hemostatic responses Escherichia *coli* sepsis in pigs. FASEB J. 24: 712-722.

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* K88. J. Anim. Sci. 87:148–156.

Valen, G. Z., Q. Yan, and G. K. Hansson. 2001. Nuclear factor kappa-B and the heart. J. Am. Coll. Cardiol. 38:307-314.

van de Poll, M. C. G., C. H. C. Dejong, and P. B. Soeters. 2006. Adequate range for sulfur-containing amino acids and biomarkers for their excess: lessons from enteral and parenteral nutrition. J. Nutr. 136: 1694S–1700S.

Vedenov, D., and G. M. Pesti. 2008. A comparison of methods of fitting several models to nutritional response data. J. Anim. Sci. 86:500-507.

Vieira, S. L., A. Lemme, D. B. Goldenberg, and I. Brugalli. 2004. Responses of growing broilers to diets with increased sulfur amino acids to lysine ratios at two dietary protein levels. Poult. Sci. 83:1307–1313.

Vigre, H., P. B. Larsen, M. Andreasen, J. Christensen, and S. E. Jorsal. 2008. The effect of discontinued use of antimicrobial growth promoters on the risk of therapeutic antibiotic treatment in Danish farrow-to-finish pig farms. Epidemiol. Infect. 136:92-107.

Wang, F. R., X. F. Dong, J. M. Tong, X. M. Zhang , Q. Zhang, and Y. Y. Wu. 2009. Effects of dietary taurine supplementation on growth performance and immune status in growing Japanese quail (*Coturnix japonica*). Poult. Sci. 88:1394‑1398.

Wang, S., D. Cawthon, and W. G. Bottje. 1998. Age-related changes of plasma glutathione and cysteine in broilers: effect of dithiothreitol reduction in vitro on free and bound pools. Poult. Sci. 77:1234–1240.

Warnants, N., Millet, S., Van Oeckel, M. J., De Paepe, M., and D. L. L. De Brabander. 2008. Response of 40-70kg barrows and gilts to increasing ideal protein concentrations in the diet. Arch. Anim. Nutr. 62:127-140.

Watanabe, R, T. Iizuka, K. Kokawa, A. Yamamoto, and Ishibashi T. 1998. Quick responses of plasma amino acid concentration to changes in dietary amino acid levels in rats. Exp. Anim. 47:37-42.

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.

Wellock, I. J., P. D. Fortomaris, J. G. M. Houdijk, and I. Kyriazakis. 2006. The effect of dietary protein supply on the performance and risk of post-weaning enteric disorders in newly weaned pigs. J. Anim. Sci. 82:327-335.

Wellock, I. J., P. D. Fortomaris, J. G. M. Houdijk, and I. Kyriazakis. 2008. Effect of dietary protein supply, weaning age and experimental enterotoxigenic *Escherichia coli* infection on newly weaned pigs: performance. Animal 2:825-833.

Whitehead, T. R., and M. A. Cotta. 2001. Characterisation and comparison of microbial populations in swine faeces and manure storage pits by 16s rDNA gene sequence analyses. Anaerobe 7:181–187.

WHO/FAO/UNU. 2007. Expert Consultation. Protein and amino acid requirements in human nutrition. World Health Org. Tech. Rep. Ser Report No. 935.

Williams, N. H., T. S. Stahly, and D. R. Zimmerman. 1997. Effect of chronic immune system activation on body nitrogen retention, partial efficiency of lysine utilization, and lysine needs of pigs. J. Anim. Sci. 75:2472-2480.

Wiltafsky, M. K., M. W Pfaff, and F. X. Roth. 2010. The effects of branched-chain amino acid interactions on growth performance, blood metabolites, enzyme kinetics and transcriptomics in weaned pigs. Br. J. Nutr. 103:964-976.

Wu G. 2010. Functional amino acids in growth, reproduction, and health. Adv. Nutr. 1:31-37.

Wu, G., S. A. Meier, and D. A. Knabe. 1996. Dietary Glutamine supplementation prevents jejunal atrophy in weaned pigs. J. Nutr. 126:2578-2584.

Yi, G. F., A. M. Gaines, B. W. Ratliff, P. Srichana, G. L. Allee, K. R. Perryman, and C. D. Knight. 2006. Estimation of the true ileal digestible lysine and sulfur amino acid requirement and comparison of the bioefficacy of 2-hydroxy-4-(methylthio)butanoic acid and DL-methionine in eleven- to twenty-six-kilogram nursery pigs. J. Anim. Sci. 84:1709-1721.

Young, V. R., D. A. Wagner, R. Burrini, and K. J. Storch.1991. Methionine kinetics and balance at the 1985 FAO/WHO/UNU intake requirement in adult men studied with L-[2H3-methyl-113C] methionine as a tracer. Am. J. Clin. Nutr. 54: 377-385.

Zello, G. A., L. J. Wykes, R. O. Ball, and Paul B. Pencharz. 1995. Recent advances in methods of assessing dietary amino acid requirements for adult humans. Nutr. 125: 2907-2915.

Zhang, L. B., and Y. M. Guo. 2008. Effects of liquid dl-2-hydroxy-4-methylthio butanoic acid on growthperformance and immune responses in broiler chickens. Poult. Sci. 87:1370-1376.

Zhao, J., A. F. Harper, M. J. Estienne, K. E. Webb Jr., 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.

Zoetendal, E. G., C. T. Collier, S. Koike, R. I. Mackie, and H. R. Gaskins. 2004. Molecular Ecological Analysis of the Gastrointestinal Microbiota: A Review. J. Nutr. 134: 465–472.