EVALUATION OF STANDARDIZED ILEAL DIGESTIBLE THREONINE TO LYSINE RATIO AND TRYPTOPHAN TO LYSINE RATIO IN WEANED PIGS FED ANTIBIOTIC-FREE DIETS AND SUBJECTED TO IMMUNE CHALLENGE

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ABSTRACT

In animals, dietary amino acids (AA) are primarily important for maintenance and protein accretion. However, under immune challenge conditions, AA metabolism was altered, resulting in AA being redistributed away from growth and production functions to immune functions. Moreover, the AA requirements for weaned piglets might be higher with antibiotic growth promoter (AGP)-free starter diets than the NRC requirements for piglets, particularly if they are exposed to immune challenge conditions. Therefore, this project aimed at determining the optimal standardized ileal digestible threonine:lysine ratio (SID Thr:Lys) and tryptophan:lysine ratio (SID Trp:Lys) in weaned piglets fed AGP-free diets and subjected to immune challenge conditions (sanitation and enterotoxigenic *Escherichia coli* K88 (ETEC K88) models). The objective of study one was to determine the optimal SID Thr:Lys of weaned piglets fed AGP-free diets and reared under clean **(CL)** or unclean **(UCL)** conditions using growth as response criteria. An estimated SID Thr:Lys of 65% and 67% optimized feed efficiency for weaned pigs under CL and UCL conditions, respectively. The second study determined the optimal SID Trp:Lys in weaned piglets reared under CL or UCL conditions. Under CL conditions, the estimated optimal SID Trp:Lys for ADG was 19.7%, whereas under UCL conditions, these values were 20.5% and 19.0% for ADG and G:F, respectively. The third study was conducted to determine the optimal SID Trp:Lys and immune responses in weaned piglets fed AGP-free diets and challenged with ETEC K88. The optimal SID Trp:Lys determined using the linear-broken-line regression analysis for ADG and G:F was 21.7% and 20.1%, respectively. The objectives of the final experiment were to determine the optimal SID Thr:Lys and evaluate the effects of SID Thr:Lys on mucin gene expression and immune responses in weaned piglets challenged with ETEC K88. Based on ileal histomorphology as response criteria, the estimated optimal SID

Thr:Lys was 65.6% in weaned piglets challenged with ETEC K88. In conclusion, weaned piglets raised under sub-optimal health conditions (*E. coli* K88) and fed AGP-free diets need greater dietary Thr:Lys and Trp:Lys requirements than reported by NRC (2012). Ensuring enough AA in AGP-free starter diets would support in maintaining good performance under sub-optimal health conditions.

DEDICATION

Dedicated to my loving parents **(Jayaraman and Lalitha)**, adorable wife **(Uthra)** and my most loved sons **(Ashwin and Gautam)**

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FOREWORD

This thesis was prepared following a manuscript format. There are five manuscripts corresponding to five chapters. Manuscript I, II and IV are published in the Animal Nutrition Journal, Animal Science Journal, and Livestock Science Journal, respectively. Part of Manuscript V has been published in Advances in Animal BioSciences. A part of this thesis has been published as an extension article in Canadian Hog Journal. All manuscripts are formatted to meet the guidelines for the Journal of Animal Science.

The authors of the published manuscripts and extension article are as follows:

Manuscript I: Balachandar Jayaraman, John Htoo, and Martin Nyachoti.

Manuscript II: Balachandar Jayaraman, John Htoo, and Martin Nyachoti.

Manuscript IV: Balachandar Jayaraman, Alemu Regassa, John Htoo, and Martin Nyachoti.

Extension article: Balachandar Jayaraman, and Martin Nyachoti.

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LIST OF ABBREVIATIONS

AA	Amino acid
ADG	Average daily gain
ADFI	Average daily feed intake
BW	Body weight
CD	Crypt depth
CP	Crude protein
DM	Dry matter
DNA	Deoxyribonucleic acid
EAA	Essential amino acids
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
G:F	Gain-feed ratio
GIT	Gastrointestinal tract
Hp	Haptoglobin
IFN-γ	Interferon-gamma
IL-6	Interleukin-6
IL-10	Interleukin-10
LT	Heat labile enterotoxins
Lys	Lysine
ME	Metabolizable energy
mRNA	messenger ribonucleic acid

NE	Net energy
NEAA	Non-essential amino acids
PCR	Polymerase chain reaction
PUN	Plasma urea nitrogen
PW	Post weaning
PWD	Post-weaning diarrhea
PWC	Post-weaning coli-bacillosis
RNA	Ribonucleic acid
SBM	Soybean meal
SD	Standard deviation
SEM	Standard error of means
SI	Small intestine
Thr	Threonine
Trp	Tryptophan
TNF-α	Tumor necrosis factor-alpha
VH	Villus height

CHAPTER ONE

GENERAL INTRODUCTION

Post-weaning is one of the most stressful periods in pigs, which is generally associated with decreased feed intake, followed by diarrhea, and consequently resulting in reduced growth performance (Williams, 2003; Heo et al., 2013). Poor growth performance related with post-weaning is a result of multi-factorial stressors, including environmental, nutritional, and psychological (Lallès et al., 2004). These stress factors impede growth performance due to immature intestinal and immune system in weaned pigs (Lallès et al., 2007a). In addition, due to sudden withdrawal of sow's milk, piglets need to adapt to the plant-based ingredients, which are dry, less digestible and contains complex protein, and carbohydrate (Cranwell, 1995; Lallès et al., 2007a; Opapeju et al., 2009). In order to have efficient production, the piglets must rapidly adapt to all these stressors.

To counteract post-weaning diarrhea (PWD) in piglets and to optimize growth performance, antimicrobial growth promoters (AGP) have been included in starter diets (Verstegen and Williams, 2002). Due to the European Union's implementation of ban on AGP, there has been ongoing interest to reduce or completely exclude the addition of in-feed AGP in animal diets (Heo et al., 2013). In AGP-free starter diets, the requirements for specific nutrients demand (example. Amino acid (AA)) might be greater to meet the need of immune functions (Bikker et al., 2006).

In animals, AA are usually classified as either essential or non-essential. Nutritionally essential AA (EAA) are not synthesized by animals, and therefore, must be provided from the diet, whereas non-essential AA (NEAA) are synthesized *in vivo* in a species-dependant manner (Wu, 2010). Some NEAA have been categorized as conditionally essential because rates of use

are greater than rates of synthesis under certain conditions (e.g. early weaning, lactation, pregnancy, infection, stress conditions) (Wu, 2010). In animals, the primary functions of dietary AA are for maintenance and protein accretion (NRC, 2012). Currently, AA requirements for maintenance or production are expressed as ratio to lysine (Lys), which aim at an ideal balance of AA (van Milgen and Dourmad, 2015). Since the dietary proteins are not completely digested and AA absorbed by the pig's digestive tract, expressing dietary AA on standardized ileal digestible (SID) basis is regarded as the closest estimate of the AA requirement (Stein et al., 2007). In pigs, the SID is obtained from apparent ileal digestibility by correcting for basal endogenous losses (Stein et al., 2007).

A current interest in animal feeding program is to study the interaction of nutrition and immunity on animal health (Goodband et al., 2014). Stimulation of the inflammatory and immune systems is linked with interference in several metabolic pathways and alteration of nutrient utilization (Le Floc'h et al., 2004). Under disease challenge conditions, AA are shifted from growth and production towards tissues and cells involved in inflammatory and immune response (Le Floc'h et al., 2004). Modifications in AA metabolism due to immune response and inflammation could generate specific AA requirements. Therefore, AA requirements might be increased in piglets raised under sub-optimal health conditions (Le Floc'h et al., 2009; Kahindi, 2015).

Threonine (Thr) is considered as the second or third limiting AA in swine diets (Mao et al., 2014). In addition to protein synthesis, Thr is also highly essential in the maintenance of the integrity of the intestinal mucosa and barrier function (Wang et al., 2010; Mao et al., 2011). In particular, during intestinal infections, Thr requirement is increased due to augmented mucin protein synthesis in order to maintain gut mucosal integrity. Intestinal inflammation would

enhance synthesis of mucin to protect the gut which might necessitate a higher quantity of dietary Thr (Mao et al., 2011). Deficit of dietary Thr adversely affects integrity of the intestinal mucosa and barrier function (Wang et al., 2010; Mao et al., 2011). Thus, requirement of Thr is strongly associated with intestinal metabolism.

Tryptophan (Trp) is the fourth limiting AA in cereal-based swine diets (Le Floc'h and Sève, 2007), and an important nutrient for immune and nervous system (Yao et al., 2011). Previous studies demonstrated that Trp and its metabolic products (serotonin and melatonin) could be involved in appetite control, immune and neurological functions and anti-oxidative capacity (Le Floc'h and Sève, 2007; Li et al., 2007a; Capozzalo et al., 2015). Immune and inflammation stimulation lead to an increase in catabolism of Trp, consequently increases kynurenine plasma concentration (Takikawa et al., 1986). Melchior et al. (2004) demonstrated that pigs subjected to immune challenge had declining plasma Trp concentrations and increasing plasma kynurenine indicating that Trp requirement may be increased during disease challenge conditions in pigs.

In this thesis, it was hypothesized the requirements for dietary Thr:Lys and Trp:Lys are higher when piglets are subjected to an immunological challenge. Therefore, the main objective of this research was to determine the optimal SID Thr:Lys ratio and Trp:Lys ratio in weaned piglets fed antibiotic-free diets and subjected to immune challenge conditions.

CHAPTER TWO
LITERATURE REVIEW

2.1. WEANING AND ITS CONSEQUENCES

Weaning is the practise of removing young piglets from their mother and is an abrupt and stressful period in a pig's life (Pluske et al., 1997). Post-weaning growth-check is one of the most important constraints in swine production, which is characterized by reduced feed intake, followed by diarrhea, consequently leading to poor growth performance and death (Pluske et al., 1997; Heo et al., 2013). Diminished growth performance associated with weaning is due to multi-stress factors such as environmental, nutritional and psychological (**Fig 2.1**) (Pluske et al., 1997; Lallès et al., 2007a). At the time of weaning, piglets need to adapt to new environmental temperature, nutrition, housing, and separation from the sow and littermates (Lallès et al., 2007a). Nutritional changes include abrupt withdrawal of sow's milk and adaptation to physical and chemical composition of feed (Heo et al., 2013). Young pigs have a drastic drop in feed intake rapidly after weaning (Pluske et al., 1997; Jacobi and Odle, 2012), which would increase chances of susceptibility to pathogenic organisms. Further it might induce post-weaning diarrhea, finally resulting in poor growth performance.

2.1.1. Effects of weaning on gut health

Digestion, absorption and metabolism of dietary nutrients are the major functions of the gastrointestinal tract (GIT). In addition, the GIT serves as a barrier for allergens, bacteria and toxic products which might otherwise reach the systemic organs and tissues. Gut health has been defined as a generalized homeostasis condition of the gastrointestinal system of pig (Pluske et al., 2007). Key indices of gut health outcomes include gut structure, microbiota load, and incidences of diarrhea (Nyachoti and Jayaraman, 2016).

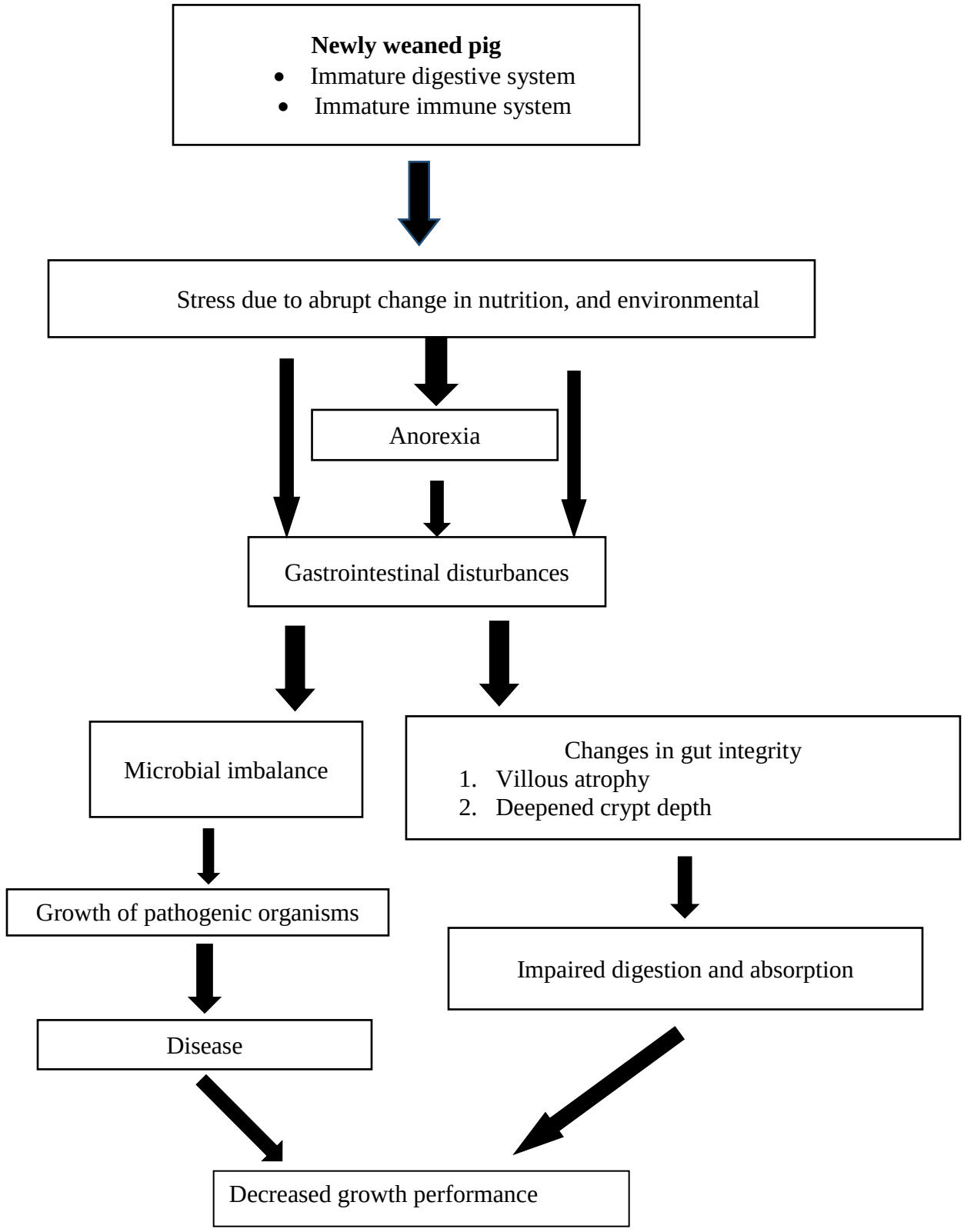


Figure 2.1. Weaning and its consequences in nursery pigs (Adapted from Kiarie, 2008)

2.1.1.1. Gut structure

Villi and crypts are the two major structures in the morphology of the small intestine. Villi are finger-like projections, which are responsible for digestion and absorption. Crypts are tubular glands and contain epithelial stem cells which are responsible for epithelial cell repopulation (Zhang and Xu, 2003). Longer villi and increased surface area are desirable as it facilitates digestive and absorptive capacity of the small intestine (Lieber-Tenorio et al., 1999). Weaning causes drastic changes in the small intestine of piglets (Pluske et al., 1997), which includes shortening of villi, crypt cell hyperplasia and epithelial cell mitosis (Nabuurs, 1998; Van Beers-Schreurs et al., 1998).

2.1.1.2. Gut barrier function

Intestinal barrier function maintenance is very important for proper absorption of nutrients and lessening contact with toxins or pathogens that may enter the gut lumen (Campbell et al., 2010). At the time of weaning, gut barrier function is generally affected by four different factors, which include weaning age, post-weaning stress, feed intake and diet composition (Wijtten et al., 2011). Intestinal barrier dysfunction and inflammation are accompanied with increased permeability due to toxins, allergens, viruses or bacteria (Verdonk et al., 2007). In a normal functional intestine, mucosal barrier restricts the paracellular and transcellular entry of antigens. Thus, increased permeability may lead to bacterial translocation and inflammatory bowel disease.

Weaning-related intestinal inflammation occurs in piglets. During inflammation, epithelial cells can signal the onset of the host intrinsic and adaptive immune response through the production of cytokines and chemokines (Stadnyk, 1994). Cytokines such as interleukin-1

(IL-1α), IL-6, and transforming growth factor (TGF-β) are collectively expressed by the gut epithelium and might favor the entry of immune cells across the basal membrane into the mucosa (Pie et al., 2004).

2.1.2. Weaning and immune system

During weaning, a major change in the degree and diversity of exposure to antigens derived from feed and pathogenic organisms occur (Lallès et al., 2007b). In addition, the immunoregulatory and immunoprotective portions of sow's milk are removed. At the time of weaning, the gut immune system stimulates immune responses, however, it is relatively immature (Stokes et al., 2004). Mucus secretion by intestinal goblet cells is considered as a primary host defense mechanism and secretion may be either base-line or accelerated. Baseline secretions maintain the mucus coat by replacing mucus lost by erosion, digestion and digesta flow, whereas accelerated mucosal secretions are increased in response to pathogenic, physical, and toxic load (Blok, 2002).

Host immune defense systems that are non-specific to pathogens are referred as innate immunity. When the innate immune system is activated by microbial or parasitic pathogens, it leads to inflammation which involves chemical signals and recruitment of phagocytic neutrophils, dendritic cells, and macrophages (Li et al., 2007b). In case of bacterial infections, neutrophils play a critical function and indicates inflammation (Li et al., 2007b).

Adaptive immune system is comprised of T lymphocytes, B lymphocytes and humoral factors which is referred to as an antigen specific response to infection. Antigens activate the immune system after initial stimulation and possess an immunological memory (Li et al., 2007a). When pathogens escape the humoral immunity, they are targeted by the cell-mediated immunity

which involves cytokine production (example. Interferon-γ (IFN-γ)) and other cytotoxic proteins by T lymphocytes. Both immune systems are highly dependent upon the adequate availability of AA for the synthesis of immunoglobulins and cytokines, and other molecules with great biological importance (Kim et al., 2007). These substances include nitric oxide, superoxide, hydrogen peroxide, histamine, glutathione and anthranilic acid. Collectively, the immune system in weaned piglets is juvenile with less capacity to differentiate harmful and innocuous antigens.

2.1.3. Weaning and expression of inflammatory cytokines in the gut

Cytokines are important mediators in the regulation of the immune and inflammatory responses (Pie et al., 2004). Besides lymphocytes and macrophages, cytokines are also produced by epithelial cells, endothelial cells, and fibroblasts, which are considered as effective sources and targets of cytokines (Pie et al., 2004). Cytokines such as TNF-α, IL-1β, and IL-8 are expressed by normal epithelial cells in the intestine but are significantly up regulated in response to pathogenic infection (Pie et al., 2004). Other cytokines, including transforming growth factor β (TGF-β), IL-1α, and IL-6 are constitutively expressed by the gut epithelium and may play a role in the basal influx of immune cells into the mucosa, in epithelial cell growth, and in homeostasis (Stadnyk, 1994). Previous studies demonstrated that widespread synthesis of pro-inflammatory cytokines could have a strong influence on gut integrity and epithelial functions, including permeability to macromolecules, and to the transport of nutrients and ions (Pie et al., 2004).

2.2. Post-weaning diarrhea in piglets

Post-weaning diarrhea (PWD) in piglets is often categorized by discharge of watery feces during the first 2 weeks and is considered as a major economic loss due to mortality and

morbidity (Pluske et al., 2002; Boudry et al., 2004; Fairbrother et al., 2005). Enterotoxigenic *E. coli* is the main cause of PWD and the combined infection with Rota virus also play an important role in PWD (Nabuurs, 1998). Other factors contributing to the occurrence of PWD include sudden removal of piglets from sow's milk, immature immune system, low levels of digestive enzymes, sudden diet change and poor hygiene (Pluske et al., 2002; Heo et al., 2013).

2.2.1. Pathogenesis of PWD caused by *E. coli*

Enterotoxigenic *E. coli* mostly carry the F4 (K88) or the F18 adhesin. The fimbriae of ETEC attach to the brush borders of the intestine and secrete heat labile toxins or heat stable toxins. Heat-labile toxins (LT) augment the secretion of sodium, chloride, hydrogen, and carbonate ions into the lumen, whereas heat-stable toxins (ST) decreases the absorption of liquid and salts (**Fig. 2.2**). Further, this would result in in hypersecretion of water and electrolytes into the intestinal lumen which in turn exceeds the absorptive capacity of the colon (Heo et al., 2013). These events collectively result in diarrhea, dehydration, decreased feed intake, lowered nutrient digestibility, reduced growth performance and mortality. Diarrhea results in injury to the intestinal epithelium which further weakens mucosal and cellular barrier functions (Pluske et al., 1997). Loss of gut integrity increase adhesion of pathogenic bacteria to the mucosal layer. A major goal of dietary interventions to minimise PWD is to reduce the total number of pathogenic *E. coli* or to prevent adhesion of the ETEC to enterocytes, or a combination of both.

In summary, weaning is one of the most critical phases in pig production which involves stress factors such as nutritional, social and environmental. Immune system stimulation during weaning phase could interfere with growth and protein accretion, which would disturb feed intake and modify nutrient metabolism (Le Floc'h et al., 2009). Among nutrients, AA are shifted

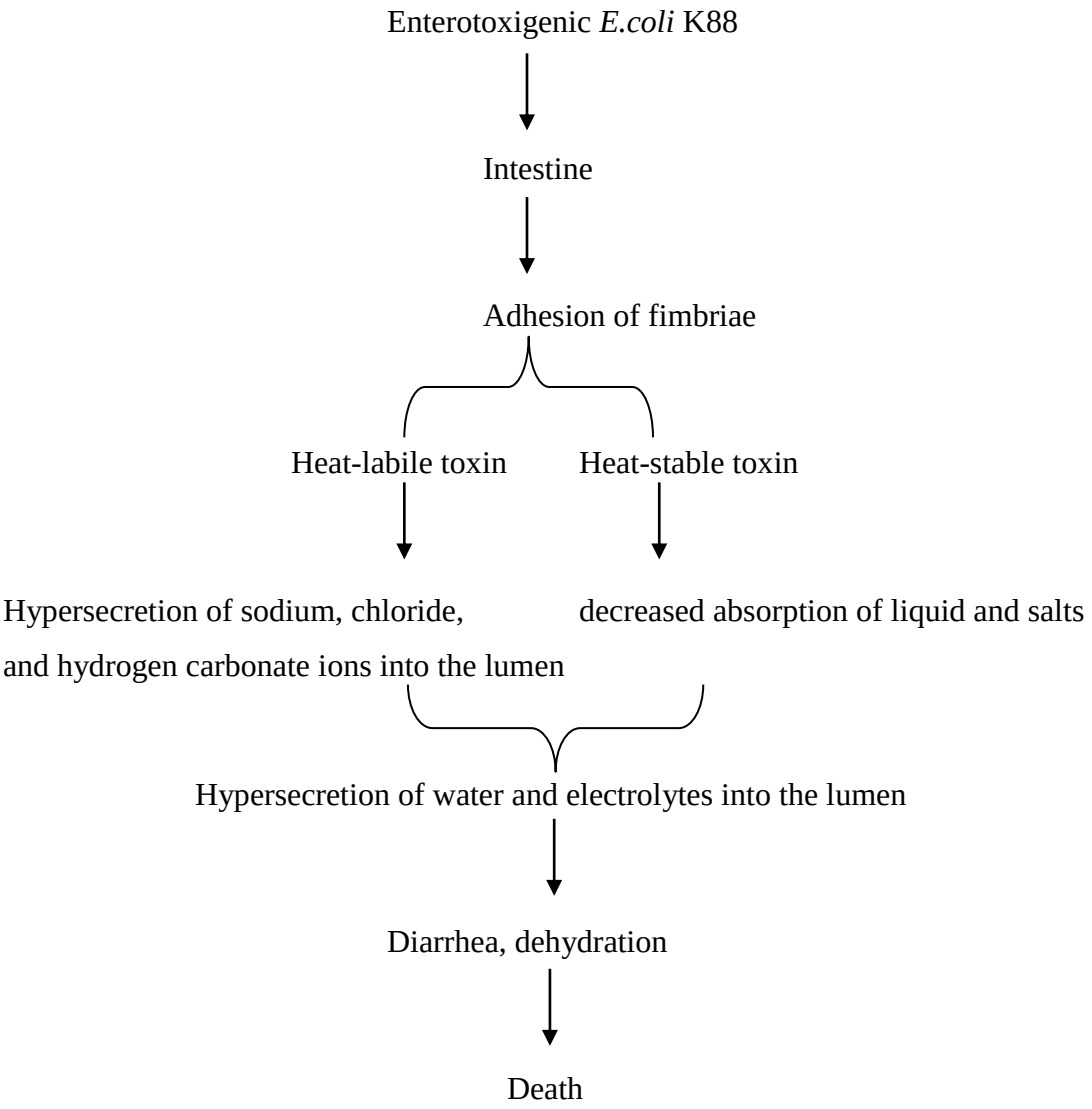


Figure 2.2. Outline of the steps in post-weaning diarrhoea caused by *Escherichia coli* K88 in piglets

(Adapted from Fairbrother et al., 2005; Heo et al., 2013).

from protein accretion to the cells involved in inflammatory and immune responses.

2.3. Introduction to amino acids

Amino acids comprise of both amino and acid groups. In nature, there are about 300 AA, among which only 20 AA are building blocks of proteins. Amino acids also function as substrates for synthesis of nucleic acids, neurotransmitters, hormones and glucose (Wu, 2010). In animals and humans, AA were conventionally classified as essential (indispensable) or non-essential (dispensable) (**Table 2.1**). Essential AA are AA that cannot be synthesized by the body relative to the needs and must be provided from the diet to meet optimal requirements. Non-essential AA can be synthesized *de novo* in adequate amounts by the body to meet optimal requirements. Conditionally EAA are those that normally can be synthesized in adequate amounts by animals, but it must be supplied from the diet under specific conditions where rates of utilization are greater than rates of synthesis. Glutamine, cysteine, proline, arginine and glycine are some of the examples of conditionally EAA (NRC, 2012). Since EAA and N act as a substrate for synthesis of non-essential AA and conditionally EAA, EAA requirements have been given major emphasis in swine nutrition (NRC, 2012). Determinants of EAA requirements include daily lean gain, sex, and environmental temperature (NRC, 2012). Health status impact AA requirements (Boisen et al., 2000), however, precise requirements have not been defined. In general, AA requirements are expressed in amounts or ratios applicable for maintenance, protein accretion or milk synthesis (NRC, 2012). The concept of expressing AA requirements on an ideal AA profile was later developed (NRC, 2012).

Table 2.1. Essentiality of amino acid in swine nutrition

Essential amino acid	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	
Leucine		
Valine		
Histidine		

NRC, 2012

2.3.1. Ideal protein ratio concept

The concept and applications of ideal protein in swine nutrition have recently been reviewed by Van Milgen and Dourmad (2015). Ideal protein has been described as a perfect balance of AA, both among the EAA and between EAA and NEAA. In an ideal protein ratio, each essential AA is equally limiting for maintenance and growth in the actual feeding situation and there is a minimal surplus of N (van Milgen and Dourmad, 2015). Currently, all EAA are expressed as a percentage of Lys, which is the reference AA because Lys is the first limiting amino acid in swine and Lys requirement is better defined compared to other AA (NRC, 2012). Using an established set of ideal ratios of other EAA to Lys (**Table 2.2**.), it is possible to formulate an ideal protein diet without having to independently establish requirements for each EAA.

2.3.2. Crystalline Amino Acids

Currently, crystalline AA are commonly used to balance AA in swine and poultry diets (Han and Lee, 2000). Crystalline AA are produced by chemical synthesis, chemical extraction, and fermentation processes. Chemical synthesis yields a racemic mixture (50% d- and 50% l-isomers), whereas chemical extraction and fermentative synthesis result in the l-isomer. The isomeric AA form must be converted to the l-isomeric form AA in the liver as only D-isomeric form can be used for protein synthesis (Han and Lee, 2000). D-amino oxidase and transaminase are the two enzymes which catalyze the conversion of d to l-isomeric form. D-amino acid oxidase catabolizes d-isomer AA to yield the keto analogue, and these keto acids can be transaminated by transaminase to yield the l-amino acids. The most commonly available CAA for commercial feed formulation are Lys, Met, Thr, Trp and Val. The major advantages of using

Table 2.2. Comparison of Ideal amino acid profile for piglets (10 – 25 kg)*

SID	BSAS (2003)[1]	NRC (2012)[2]	Gloaguen et al. (2013)
Lys	100	100	100
Met:Lys	30	29	30
(Met +Cys):Lys	59	55	60
Thr:Lys	65	59	65
Trp:Lys	19	16	22
Val:Lys	70	63	70
Ile:Lys	59	51	52
Leu:Lys	100	100	101
His:Lys	34	34	31
Phe:Lys	57	58	54
(Phe + Tyr):Lys	100	93	-

[1]BSAS-British Society of Animal Sciences, United Kingdom

[2]National Research Council (2012)

*All of the AA profile are factorially derived

these synthetic AA in swine diets include reduced production cost by lowering basic protein source (for e.g. soybean meal), increased digestibility, decreased N excretion, and increased formulation precision meeting AA requirements (Han and Lee, 2000).

2.4. Methods for determining amino acid requirements

The approaches for determining AA requirements are factorial and empirical. In the factorial approach, the daily requirements for an individual animal are obtained at a particular time point by adding the requirements for maintenance and production (Pomar et al., 2003). An important criterion for using this approach is that the selected animal must be the best representative of the population. Factorial approaches estimate the needs of an animal during a limited time period (Pomar et al., 2003).

In empirical approaches, AA requirement is defined as the minimal amount needed to maximize or minimize population responses for one or several response criteria during a given time period (Pomar et al., 2003). The basic fundamental for the empirical approach of AA requirement include appropriate animal models, appropriate environmental conditions, and suitable diets that allow significant extrapolation to commercial settings (NRC, 2012). The NRC (2012) outlines requisites for empirical determination of AA requirement: 1) a basal diet that is undersupplied in the test AA using feed ingredients deficient in the AA, 2) the basal diet has to contain sufficient levels of other nutrients except the test AA, 3) minimum of four graded levels of test AA (deficient to excess levels; two levels each above and below the estimated requirement), 4) sufficient time period, which depends on the response criteria, and 5) a proper statistical model.

A dose-response technique is used in the empirical approach. To achieve the optimal AA requirements, the response criteria are fitted to linear or curvilinear plateau models (Robbins et al., 2006). Response criteria include growth performance, plasma urea nitrogen (PUN), plasma AA concentrations, and N balance, and direct or indirect AA oxidation indicators. Growth performance and N retention are the most commonly used responses for growing pigs, and litter growth rate is by far the most common response criterion in lactating sows.

2.4.1. Growth performance

The response criteria for determining AA requirements based on growth performance include weight gain, feed intake and feed efficiency (NRC, 2012). The fundamental 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. Primarily, increasing levels of the limiting AA is provided to the animals, while CP and other nutrients are similar across the dietary treatments (Barea et al., 2009; Kahindi et al., 2014). It is anticipated that the effects of increasing dietary AA content should be reflected on the weight gain and feed efficiency. The optimum AA requirement determined may be determined using either non-linear regression or broken-line models (Robbins et al., 2006).

2.4.2. Plasma urea nitrogen

In animals, plasma urea nitrogen is inversely related to dietary level of first limiting AA. 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. A diet deficient in EAA leads to high N losses as compared to one with adequate EAA contents. 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 (Coma et al., 1995). This method has been effective in determining requirement for Lys (Coma et al., 1995), Thr (Pedersen et al., 2003), and Ile (Parr et al., 2003) in pigs. Similar to this method, AA requirement can be determined using plasma AA as a response criterion. In this method, the concentration of plasma AA with changing dietary level of the test AA is measured. The plasma AA concentration is constant in case of a limiting EAA but rise steadily once requirement is met. The major advantages of this method include repeatability of the sample collections, usage of few numbers of animals, and the requirement of several AA can be done within a short period. The limitation of this method is that the presence of related AA in the diet could influence the requirement of a test AA. Another major limitation includes, repeated blood sample collections which may cause stress in animals. Furthermore, plasma AA concentration differs between the fed and post-fed condition.

2.4.3. Isotope tracer technique

In a critical review, Zello et al. (1995) explained very well about isotope tracer techniques. In isotope tracer technique, radioactive or stable isotopes are used to label the substrates whose metabolism will be examined. An isotope is infused for a given duration until a steady state within the body is achieved. All isotope-dilution techniques measure the degree of isotopic enrichment of an AA after a challenge dose of labelled tracer. The method is established on the hypothesis that the partition of any essential AA between oxidation and protein synthesis is sensitive to the level of the most limiting AA in the diet (Zello et al., 1995). When an EAA is limiting for protein synthesis, then all other AA which are in surplus should be oxidized. This indicates that increasing the dietary level of limiting AA in graded amounts will increase the uptake of all dietary AA for protein synthesis, which in turn reduces their oxidation, until the

requirement point is attained, where thereafter oxidation is constant. The types of isotope tracer method are direct AA oxidation and balance, and indirect AA oxidation and balance (Zello et al., 1995). In swine, indicator AA oxidation has been used in neonatal pigs (House et al., 1997; Shoveller et al., 2003) and growing pigs (Bertolo et al., 1998; Moehn et al., 2008).

In summary, AA requirements could be affected by different factors such as animal (genetic, age, sex, and health status), feed (allowance, nutrient composition, and digestibility) and environment (housing conditions, temperature, and space allowance) (Brossard et al., 2017). Different methods of determination of AA requirement have their own advantages and disadvantages. The following review focus on AA and immune functions in animals.

2.5. Amino acids and immune functions

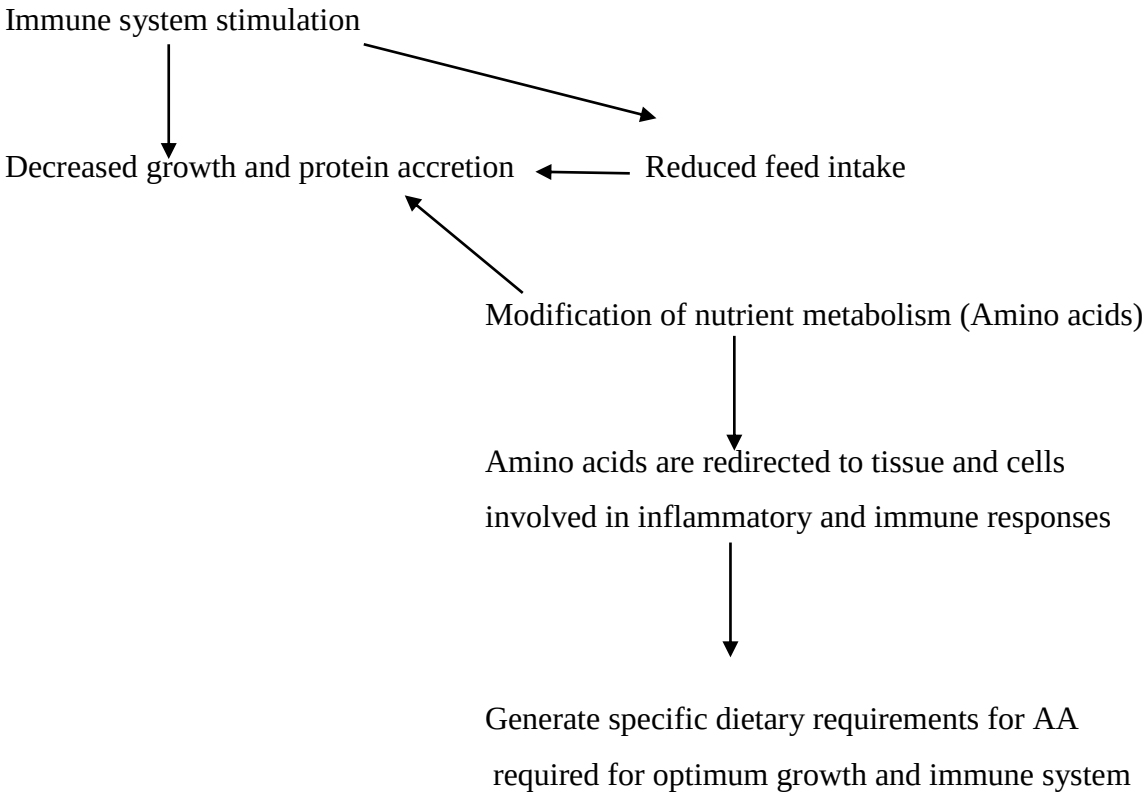
The functions of AA in animals have been extensively reviewed by Wu (2010) and Li et al. (2007b). In animals and humans, protein deficiency due to malnutrition and diseases are predominant barriers to health, growth and reproduction (Li et al., 2007b). The role of AA on immune functions are outlined in **Table 2.3**. The fundamental mechanisms may involve mammalian target of rapamycin (mTOR) activation, nitric oxide (NO) and glutathione synthesis, hydrogen sulphide (H_2S) signaling and cellular redox state. Over the years, studies in pigs and poultry have demonstrated that metabolism and requirement for EAA (Thr, Met, Trp) and conditional EAA (Glu, Pro, Arg) change during a disease challenge (Le Floc'h et al., 2009; Capozzalo et al., 2015). It is clear that immune challenge conditions alter AA metabolism which could divert AA away from growth and production functions to immune functions (**Fig 2.3**). Hence, the dietary AA recommendations provided for healthy animals may not be enough during immune challenge conditions. There is a need to determine AA recommendations for animals maintained under immune-challenged or sub-optimal health conditions.

Table 2.3. Amino acids and immune functions

Amino acid	Immune functions
Sulphur AA (Methionine and Cysteine)	• Precursor for the synthesis of homocysteine. Homocysteine is an oxidant and inhibitor of nitric oxide (NO) synthesis. • Precursor for the synthesis of betaine.
Threonine	• Synthesis of mucin proteins which are needed for the maintenance of gut immune functions.
Tryptophan	• Precursor for serotonin, a neurotransmitter • Inhibits production of inflammatory cytokines and superoxide
Phenylalanaine	• Regulation of tetrahydrobiopeterin (a cofactor of NO synthesis) synthesis • Tyrosine, the product of Phe participates in the synthesis of neurotransmitters that regulate neuronal function and cell metabolism
Arginine	• Direct precursor for NO synthesis • Regulation of cytokine production and mediator of autoimmune diseases
Tyrosine	• Precursor of dopamine - Neurotransmission - Inhibition of production of inflammatory cytokines and superoxide
Glutamine	• Neurotransmitters • Inhibition of T-cell response and inflammation
Serine	• Stimulation of lymphocyte proliferation

Adapted from Li et al. (2007b)

Figure 2.3. Illustration on impact of immune system stimulation on amino acid requirements in animals



(Adapted from Le Floc'h and Sève, 2007)

2.6. Disease challenge models in swine

Disease challenge models have been used in swine nutrition studies to evaluate the impact of disease on nutritional needs of growing pigs (**Table 2.4**; Adewole et al., 2016). The criteria for selecting a disease challenge model include relative easy of use, predictability and simulation of chronic disease (Rakhshandeh and de Lange, 2012). Disease challenge models used in young piglets include oral-challenge with ETEC (Owusu-Asiedu et al., 2002; Opapeju et al., 2009; Nyachoti et al., 2012; Khafipour et al., 2014), intra-peritoneal injection of LPS (Wang et al., 2011), oral challenge with *Salmonella enteric* serotype *typhimurium* (Balaji et al., 2000; Turner et al., 2002), sanitation challenge (Lee et al., 2005; Le Floc'h et al., 2006; Le Floc'h et al., 2009; Kahindi et al., 2014; van der Meer et al., 2016), turpentine injections (Rakhshandeh and de Lange, 2012), and feeding mycotoxins (Rakhshandeh and de Lange, 2012).

In the ETEC challenge model, the most commonly used response criteria include growth performance, fecal consistency score, PUN, fecal bacteria count, intestinal morphology, serum cytokines concentration, rectal temperature, count of intestinal adherent bacteria and mortality (Adewole et al., 2016). A number of factors that influence the success of the ETEC challenge model, including breed, age, variation in the volume and dosage of inoculants used, buffer type and method of delivery of the inoculants into the oral cavity, and health status of pigs prior to the ETEC challenge (Adewole et al., 2016).

In a recent meta-analysis covering 121 different studies, Pastorelli et al. (2012) evaluated the impact of an immune system challenge on growth response criteria. The immune system challenges models include digestive bacterial infections, sanitation, LPS challenge, mycotoxicoses, parasitic infections, and respiratory disease. The greatest negative impact on

Table 2.4. Impact of different disease challenge models on response criteria in weaned piglets

Disease challenge model	Key findings	References
ETEC K88	• Tendency of reduction in overall ADG and ADFI.	Owusu-Asiedu et al. (2002)
	• Reduced growth performance after challenge.	Trevisi et al. (2009); Lee et al. (2012)
	• No change in growth.	Bhandari et al. (2008)
	• Severe to moderate diarrhea within 10 h after oral challenge.	Marquardt et al. (1999)
E. coli K88 lipopolysaccharide challenge (LPS)	• Reduced feed intake.	Wang et al. (2011)
	• Reduced ADG and ADFI.	Liu et al. (2003); Waititu et al. (2016)
	• Increased crypt depth of the duodenum and decreased. villus height:crypt depth of ileum.	Jiang et al. (2009)
	• Reduction in ADG and tendency of reduction in ADFI.	
Oral challenge with *Salmonella typhimurium* enteric serotype	• Reduced feed intake and ADG.	Balaji et al. (2000)
	• Reduced growth performance.	Turner et al. (2002)
	• Elevated rectal temperature in pigs after challenge.	Gebru et al. (2010)
Sanitation challenge	• Reduced G:F in pigs reared under poor sanitary conditions.	Montagne et al. (2012)
	• Reduced growth performance in pigs reared under poor sanitary conditions.	Le Floc'h et al. (2006); Le Floc'h et al. (2009); Kahindi et al. (2014); van der Meer et al. (2016)

Modified from Adewole et al. (2016).

growth responses were observed in pigs challenged with bacterial infections, followed by unsanitary conditions and LPS. Under these conditions, there may be temporary changes in AA requirement for maintaining the immune system (Goodband et al., 2014).

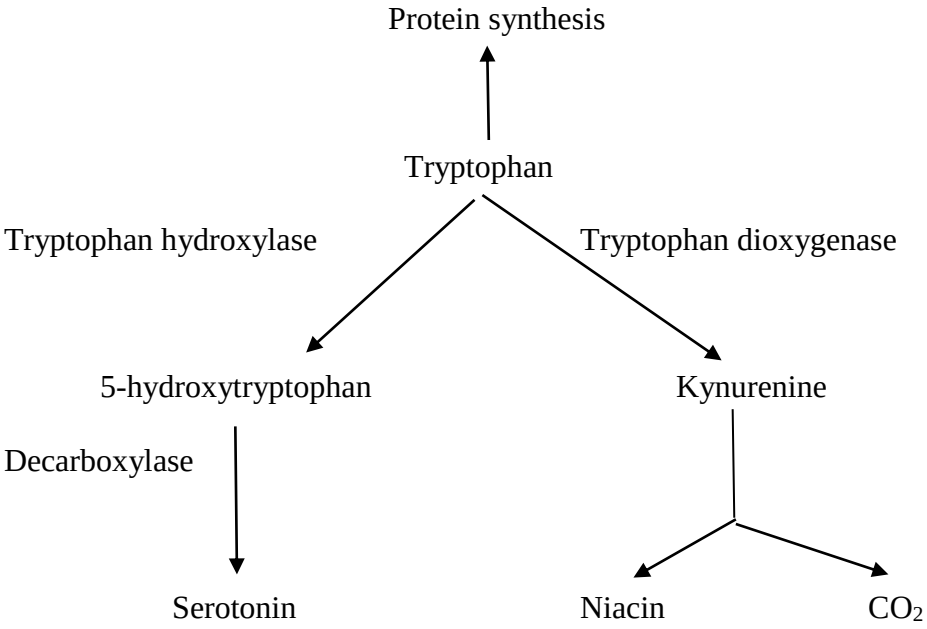
2.7. Tryptophan

In swine, Trp is the second or third limiting AA in cereal-based diets (Guzik et al., 2002). Tryptophan is a substrate for synthesis of serotonin, a neuro-mediator synthesized mainly in the gut and also in platelets and brain (Le Floc'h and Sève, 2007) and also the substrate for synthesis of nicotinamide adenine dinucleotide (Moffett and Namboodiri, 2003; Le Floc'h et al., 2008). In animals, the metabolic fates of Trp occur via two major pathways, kynurenine and serotonin pathway (**Fig 2.4**) (Le Floc'h and Sève, 2007). In the kynurenine pathway, Trp is degraded either through the activation of indoleamine 2, 3-dioxygenase (IDO) by all tissues or 2, 3 tryptophan dioxygenase in the liver (Le Floc'h and Sève, 2007). During inflammation and immune system activation, inflammatory cytokine interferon-γ (IFN-γ) induces IDO thereby leading to an increase in kynurenine plasma concentration and kynurenine excretion in urine as xanthurenic acid (Le Floc'h and Sève, 2007). Thus, Trp requirements for swine could be modified under immune challenge conditions.

2.7.1. Effect of tryptophan on appetite and feed intake in pigs

In pigs, the effect of Trp on appetite control has been well documented (Le Floc'h and Sève, 2007). Numerous studies demonstrated that Trp-deficient swine diets resulted in reduced growth performance (Henry et al., 1992; Eder et al., 2001). The reduced growth performance due to Trp deficiency is in part related to reduced appetite and feed intake (Henry et al., 1992; Zhang et al., 2007; Le Floc'h et al., 2009). Previous studies demonstrated that Trp-deficient diets

Figure 2.4. Metabolism of tryptophan in animals.



Adapted from Le Floc'h and Sève, 2007.

resulted in reduced feed intake in weaned piglets (Ettle and Roth, 2004), growing pigs (Henry et al., 1996) and finishing pigs (Henry et al., 1992). The underlying physiological mechanism through which Trp affects appetite are not yet fully demonstrated. However, several hypotheses were presented in the review by Le Floc'h and Sève (2007). One hypothesis is that, Trp is the precursor of serotonin, a nerumediator involved in appetite control in animals. Another hypothesis is that role of Trp in the peripheral control of appetite via modulation of rate of gastric emptying, and secretion of insulin. Effect of Trp on appetite control could be facilitated by ghrelin, a gut hormone involved in feed intake. Collectively, Trp-deficient diets could result in reduced feed intake in pigs.

2.7.2. Tryptophan and inflammatory response

In animals, Trp concentration in plasma and body tissues is low (Le Floc'h et al., 2004). During inflammation (natural or induced) and infections, depletion of plasma Trp occurs in pigs (Melchior et al., 2004; Le Floc'h et al., 2008) and mice, indicating increased utilization of Trp. The decrease in plasma Trp could be attributed to the synthesis of acute phase proteins, which are considered to have high Trp content, and /or to an increase in catabolism of Trp (Le Floc'h et al., 2006). Indoleamine dioxygenase is a rare-limiting enzyme which is activated by IFN-γ. During inflammation and immune system activation, kynurenine plasma concentration and its excretion as xanthurenic acid (Takikawa et al., 1986).

Degradation of Trp to Kyn has three major functions. The first one is that IDO was reported to have antimicrobial properties which would be the principle behind restricting proliferation of bacteria, virus and parasite. Secondly, cells that express IDO (i.e. dendritic cells and macrophages) can inhibit T cell proliferation and thus control lymphocyte production (Le Floc'h et al. 2004). Finally, IDO induction during immune activation may protect cells from

oxidative damage. Indeed, 3-hydroxyanthranilic acid and 3-hydroxykynurenine, produced from Trp through the IDO–kynurenine pathway, have antioxidant properties (Le Floc'h et al., 2004). Collectively, it implies that dietary Trp could influence the inflammatory response and the animal health.

Previous studies demonstrated that Trp requirement increased in piglets reared under poor sanitation, which triggered a low-grade inflammatory response (Le Floc'h et al., 2004; Le Floc'h and Sève, 2007). Increased catabolism of Trp occurred during lung infection in piglets (Melchior et al. 2004), under conditions of inflammation (Le Floc'h et al., 2004), sub-optimal health (Melchior et al. 2005) and oxidative stress induced by diquat injection. Mortality rate of pigs orally infected with ETEC was lower in pigs supplemented with Trp (Trevisi et al., 2009). In pigs susceptible to ETEC, genes related to the innate immune system and bacterial response (such as LPS-binding protein, TLR4 and IL-8) were up-regulated, but when supplemented with Trp, this effect was reversed (Trevisi et al., 2010). De Ridder et al. (2012) suggested an increase of 7% dietary Trp would maintain growth in pigs suffering from inflammation and immune stimulation.

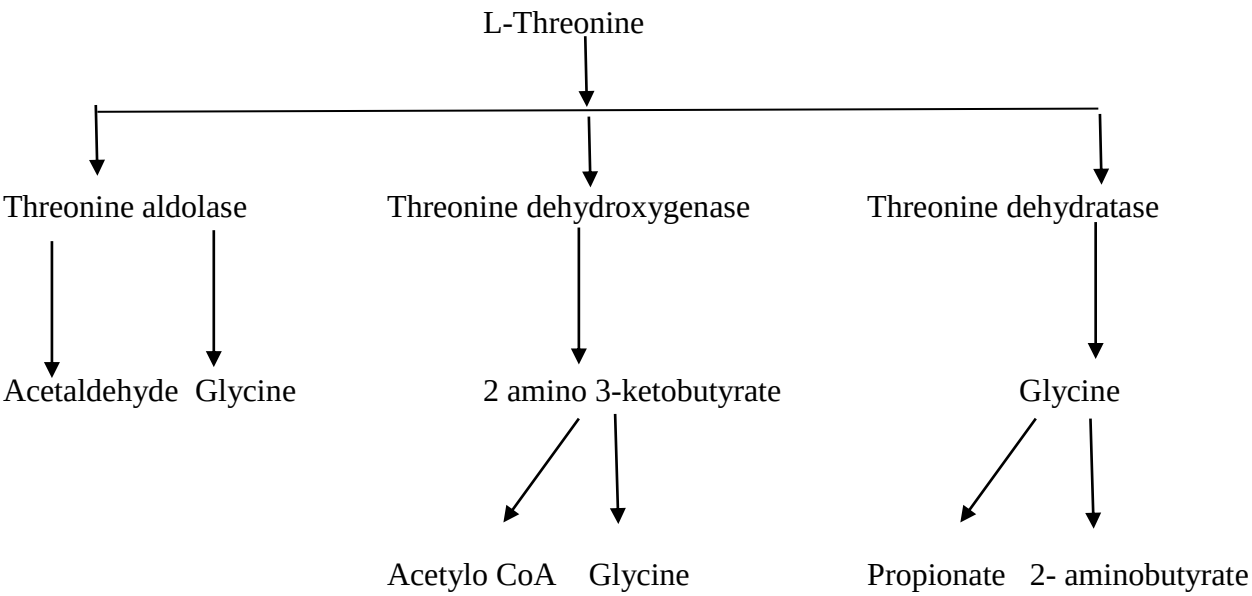
2.8. Threonine

Threonine is a polar, short-chain AA with a hydroxyl group attached to the single carbon of the R-group and based on its structure, it is also known as α-amino β-hydroxybutyric acid. In swine diets, Thr may be the second or third limiting (Mao et al., 2014)

2.8.1. Threonine metabolism

Threonine is both gluconeogenic and ketogenic (Wu, 2013). In mammals, Thr degradation occurs in the liver via three pathways 1) threonine dehydratase, 2) threonine dehydrogenase (a mitochondrial enzyme) and 3) threonine aldolase (**Fig 2.5**). The products of

Figure 2.5. Metabolism of threonine in monogastric animals



Adapted from Mathai et al. (2016)

Thr catabolism include glycine, pyruvate, and propionyl-Co A (Davis and Austic, 1997). In growing pigs and rats, the threonine dehydrogenase pathway accounts for approximately 80% of threonine catabolism (Wu, 2013).

2.8.2. Roles of Thr in maintaining intestinal mucosal integrity and barrier function

Threonine plays a critical role in maintaining intestinal mucosal integrity (Schaart et al., 2005; Wang et al., 2010; Wu, 2013). A large proportion of dietary Thr is retained by the intestine of healthy pigs and humans during first-pass metabolism (Wang et al., 2010) and utilized for maintenance of gut integrity and function (Mao et al., 2011). Previous studies support the concept that availability of dietary Thr could affect intestinal morphology. Hamard et al. (2009) found that weaned pigs fed Thr deficient diet had reduced villous height. Another study indicated that a Thr-deficient diet (6.5 g Thr/kg diet) fed to early-weaned pigs induced villous atrophy, reduced VH, CD and increased VH:CD (Hamard et al., 2010). In addition, Wang et al. (2010) suggested that a deficiency of dietary Thr could drastically reduce villous area and CD, and induced villous atrophy.

2.8.3. Role of Threonine in Mucin synthesis

Mucins are highly glycosylated large glycoproteins with protein backbone structures rich in Thr and serine (**Table 2.5**). These AA are linked to a wide variety of O-linked oligosaccharide chains that make up more than 70% of the weight of the molecule (Kim and Ho, 2010). The *de novo* synthesis of intestinal mucins requires Thr in the intestinal lumen (Mao et al., 2011). The role of mucins in gut physiology and health is presented in **Table 2.6**.

Table 2.5. Amino acid composition of intestinal mucins of pig

Amino acid	nmol/g dry weight of glycoprotein	Mol/100 mol protein
Lys	39	2.22
His	24	1.37
Arg	39	2.22
Asp	81	4.63
Thr	**464**	**26.53**
Ser	182	10.4
Glu	74	4.2
Pro	270	15.5
Gly	98	5.6
Ala	66	3.77
Cys	75	4.28
Val	125	7.15
Met	22	1.25
Ile	55	3.14
Leu	55	4.28
Tyr	26	1.46
Phe	35	2.00

(Adapted from Mantle and Allen, 1981)

Table 2.6. Functional role of mucins in gut physiology and health

Gut physiology	Gut health
1. Lubrication of gut epithelium	1. Fixation of commensal bacteria permitting colonization resistance
2. Protection of gut epithelium	2. Fixation of pathogens, bacteria, viruses and parasites
3. Protection against endogenous and bacterial proteases	3. Epithelial reparation
4. Selective diffusive barrier permeable to nutrients but not to macromolecules	4. Component of gut-associated lymphoid tissue

Adapted from Montagne et al. (2004)

To date, 20 different genes encoding mucin proteins have been identified, MUC1 to MUC20 according to their order of discovery. Mucin genes are broadly classified into two types, secretory and membrane-associated. Mucin 2 gene encodes for the major secretory mucin protein synthesized and secreted by goblet cells, whereas goblet and absorptive cells express apical membrane-bound mucins, i.e., MUC1, MUC3, MUC4, MUC13, and/or MUC17 (Montagne et al., 2004). Intestinal mucosal absorptive cells also express epithelial membrane-bound mucins, MUC1, MUC3, MUC4, MUC12, MUC13 and MUC17, which have structural similarity.

2.9. SUMMARY AND PERSPECTIVES

One of the major challenges in the nutritional management of piglets occur in the immediate post-weaning period, which has a direct impact on the growth performance in the nursery through market weight. This period is characterized by poor feed intake, with an immature digestive and immune systems which predispose the piglet to intestinal disturbances, especially proliferation of enteric pathogens such as *E. coli* K88. Antibiotic growth promoters (AGP) have been used in the swine industry to minimize predisposition following to weaning. However, European Union banned AGP in 2006 due to the threat of antimicrobial resistance in animals and humans. Ban on AGP also had significant impact in North America where consumer pressure demanding swine production without AGPs (Huyghebaert et al., 2011). Therefore, a major challenge for the swine industry is to formulate starter diets that primarily suit the digestive capacity, maintain gut health and promote growth. Piglets raised on diets without AGP experience higher incidences of intestinal health problems and prolonged period of immunological challenge, which consequently could have an impact on the amino acid requirements for pigs. Therefore, new AA requirements must be generated in non-AGP fed pigs.

A potentially essential means to minimize the negative impact of immune system stimulation, due to disease, is the fortification of swine diets with nutrients at levels exceeding recommendations that have been established for pigs fed AGP containing diets.

In pigs, Trp is involved in appetite control, inflammatory and immune functions. Threonine aids in the synthesis of mucin and maintenance of intestinal health. During an immune challenge, the requirements of AA (Trp and Thr) might be increased in weaned piglets. Thus, there is a need to establish optimal dietary requirements of the essential AA such as, Thr and Trp in piglets under general immune challenge.

CHAPTER THREE
HYPOTHESIS AND OBJECTIVES

Overall Hypothesis

Weaned piglets subjected to immune challenge conditions have higher dietary Thr:Lys and Trp:Lys requirements compared to those raised under optimal health conditions.

Overall Objective

To estimate the optimal Thr:Lys and Trp:Lys requirements for piglets raised under optimal health conditions (clean conditions) and subjected to sub-optimal health conditions (unclean conditions or *E. coli* K88 challenge)**.**

Specific objectives

1. To determine the optimal dietary Thr:Lys requirement of weaned pigs fed AGP- free diets and subjected to clean or unclean sanitary conditions.
2. To determine the optimal dietary Trp:Lys requirement of weaned pigs fed AGP-free diets and subjected to clean or unclean sanitary conditions.
3. To determine the optimal dietary Trp:Lys requirement of weaned pigs fed AGP-free diets and subjected to enterotoxigenic *E. coli* K88 challenge.
4. To determine the optimal dietary Thr:Lys requirement of weaned pigs fed AGP-free diets and subjected to enterotoxigenic *E. coli* K88 challenge.

CHAPTER FOUR

MANUSCRIPT 1

Effects of dietary threonine to lysine ratio and sanitary conditions on performance, plasma urea nitrogen, plasma-free threonine and lysine of weaned pigs

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4.1. ABSTRACT

This experiment tested the hypothesis that weaned piglets raised under poor sanitation conditions have increased dietary threonine to lysine (Thr:Lys) requirements compared to clean sanitation conditions and the objectives were to determine the optimal SID Thr:Lys in weaned piglets raised under clean (CL) and unclean (UCL) sanitation conditions. A total of 180 mixed-sex pigs (Duroc × [Yorkshire×Landrace]; average initial BW 7.2 ± 0.3 kg) weaned at 21 ± 1 days were randomly assigned to 2 × 5 factorial settings (two sanitation conditions; CL and UCL, and 5 dietary treatments (SID Thr to Lys ratios; 55, 59, 63, 67 and 71%) each with 6 replicates (3 pigs per pen). Diets were corn-wheat-soybean meal-based with a constant SID Lys of 1.18% that was set to be the second limiting amino acid. In each sanitation condition, blood was collected on d 0 and 14 to determine plasma urea nitrogen (PUN) and plasma-free Thr and Lys. Pigs raised under UCL conditions had lower growth rate and reduced feed intake ($P < 0.05$) compared to pigs raised under CL conditions. Increasing dietary SID Thr:Lys improved ($P < 0.05$) G:F in piglets raised under UCL conditions. Under CL and UCL conditions, the estimated optimal SID Thr:Lys for G:F was 65% and 67%, respectively. Pigs raised under UCL had higher plasma-free Lys than pigs under CL conditions. Plasma-free Thr increased ($P = 0.05$) with increasing dietary SID Thr:Lys. In conclusion, an average estimated SID Thr:Lys of 65% and 67% optimized feed efficiency for weaned pigs under clean and poor sanitation conditions, respectively.

Keywords: Thr to Lys ratio, sanitation, weaned pigs, growth performance

4.2. INTRODUCTION

Threonine (Thr) is the second-limiting amino acid (AA) after Lys in pigs when fed wheat and barley-based diets and the third-limiting AA in corn-based diets (Adeola et al., 1994; Saldana et al., 1994). Besides protein synthesis, the major functions of Thr include maintenance of gut integrity and immunity (Ruth and Field, 2013). Hence, the requirement for Thr is likely to vary according to the importance of each of its functions. The BSAS (2003) and NRC (2012) requirements for standardized ileal digestible (SID) Thr:Lys in young pigs (7 and 25 kg BW) are 65% and 59%, respectively. These levels might not be adequate for pigs reared in commercial production conditions, where the occurrence of clinical and sub-clinical infections are high.

Weaned pigs reared under poor sanitary conditions are used as a model to provoke a low-grade inflammatory response (Le Floc'h et al., 2009; Kahindi et al., 2014; van der Meer et al., 2016) and those pigs had reduced growth performance (Kahindi et al., 2014; van der Meer et al., 2016) and activated immune system (Williams et al., 1997) that would interfere with growth because of competition between protein deposition in structural tissues and immune function (Le Floc'h et al., 2009).

A current interest in the nutritional management of nursery pigs is to utilize nutritional programs without in-feed antibiotic growth promoter (AGP, Nyachoti et al., 2006; de Lange et al., 2010), which may alter the Thr:Lys requirement for optimal performance, especially when piglets are exposed to an immunological challenge as is often the case under unsanitary conditions. For example, it has been shown that metabolism and requirement for Thr changes in pigs during an immune challenge (Le Floc'h et al., 2004; Ren et al., 2014). Furthermore, exposing piglets to unclean conditions is considered as a predisposing factor for weaning-related disorders (Williams et al., 1997).

Information about Thr:Lys for piglets fed AGP-free diets and subjected to an immunological challenge is limited. Thus, it was hypothesized that the optimal SID Thr:Lys ratio is higher for piglets subjected to unclean sanitation conditions compared to that of pigs raised under a clean environment. The objective of this study was to determine the optimum standardized ileal digestible (SID) Thr to Lys ratio for weaned piglets reared under clean or unclean sanitary conditions.

4.3. MATERIALS AND METHODS

4.3.1 Animal care

The experimental protocol (F10-041/1/2) was reviewed and approved by the Animal Care Committee of University of Manitoba and pigs were cared for in accordance with the guidelines of the Canadian Council on Animal Care (2009).

4.3.2. Experimental diets

Diets were corn (*Zea mays*), wheat (*Triticum aestivum*) and soybean (*Glycine max*) meal-based with a constant SID Lys of 1.18% that was set to be the second limiting AA (**Table 4.1**). Ingredients contributing AA (Corn, Wheat and Soybean meal) were analyzed for AA composition and the values were used in diet formulation. The diets contained graded levels of SID Thr:Lys (55, 59, 63, 67 and 71%). All other nutrients were provided in quantities meeting or exceeding NRC (2012) requirements for a 6 to 10 kg pig. All diets were fed in mash form and did not contain any AGP.

4.3.3. Animals and experimental design

A total of 180 piglets (Duroc × [Yorkshire×Landrace]; mixed sex) weaned at 21±1 d of age and housed three per pen were fed a corn-soybean meal-based starter diet (21% CP;

Table 4.1. Ingredient and nutrient composition of experimental diets (as-is-basis)

	Dietary SID Thr:Lys, %				
Item	55	59	63	67	71
Ingredients, %					
Corn	46.20	46.20	46.20	46.20	46.20
Wheat	15.00	15.00	15.00	15.00	15.00
Soybean meal	30.00	30.00	30.00	30.00	30.00
Vegetable oil	3.85	3.85	3.85	3.85	3.85
Corn starch	0.50	0.45	0.40	0.36	0.31
Limestone	1.08	1.08	1.08	1.08	1.08
Salt	0.30	0.30	0.30	0.30	0.30
Min-Vit premix[1]	1.00	1.00	1.00	1.00	1.00
L-Lys HCl	0.35	0.35	0.35	0.35	0.35
L-Trp	0.05	0.05	0.05	0.05	0.05
DL-Met	0.14	0.14	0.14	0.14	0.14
L-Thr	-	0.05	0.09	0.14	0.19
Calculated nutrient composition[2]					
NE, Kcal/kg	2,484	2,484	2,484	2,484	2,484
Crude protein, %	22	22	22	22	22
Crude fat, %	3.10	3.10	3.10	3.10	3.10
NDF, %	9.30	9.30	9.30	9.30	9.30
ADF, %	3.51	3.51	3.51	3.51	3.51
Crude fiber, %	3.70	3.70	3.70	3.70	3.70
Calcium, %	0.80	0.80	0.80	0.80	0.80
Total phosphorus, %	0.61	0.61	0.61	0.61	0.61
Available phosphorus, %	0.32	0.32	0.32	0.32	0.32

SID Lys, %	1.18	1.18	1.18	1.18	1.18
SID Met, %	0.49	0.49	0.49	0.49	0.49
SID Cys, %	0.23	0.23	0.23	0.23	0.23
SID Met + Cys, %	0.72	0.72	0.72	0.72	0.72
SID Trp, %	0.26	0.26	0.26	0.26	0.26
SID Thr, %	0.65	0.70	0.75	0.79	0.84
SID Ile, %	0.76	0.76	0.76	0.76	0.76
Analyzed CP and AA, %					
Crude protein	21.29	22.19	21.68	22.01	22.26
Lys	1.26	1.29	1.30	1.29	1.31
Thr	0.77	0.82	0.87	0.89	0.94
Met + Cys	0.77	0.79	0.80	0.80	0.81
Ile	0.89	0.91	0.91	0.90	0.92
Val	0.99	1.01	1.01	0.99	1.01
Trp	0.28	0.29	0.29	0.29	0.29
SID Thr:Lys[3]	61.00	64.00	67.00	69.00	72.00

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

[2]The calculated total contents of Lys, Thr, Met + Cys and Trp in the basal diet were 1.32, 0.77, 0.82, and 0.30, respectively.

[3]Corrected dietary SID Threonine: Lysine = (calculated SID Thr:Lys × analyzed total Thr:Lys)/calculated total Thr:Lys.

59% SID Thr:Lys) for 6 d adaptation period. On d 7, piglets (initial BW 7.36 ± 0.2 kg) were randomly assigned to 1 of 5 dietary treatments with SID Thr to Lys ratio (55, 59, 63, 67 and 71%) each with 6 replicates (3 pigs per pen). This experimental design was considered as factorial arrangement in a completely randomized design, since all the external factors such as housing management (lighting, humidity, temperature) for clean and unclean conditions were similar. Under CL conditions, 90 piglets were housed in a clean room that had been cleaned and disinfected before the arrival of the piglets and the room was cleaned once weekly. Under UCL conditions, 90 piglets were housed in the same room which was not cleaned and disinfected at the end of week 3 from the first batch to allow the build-up of manure. Moreover, manure from the swine herd was added (5 kg per pen) to the pens on d 0 and d 7 of the UCL conditions. Pigs were provided *ad libitum* access to feed and water. Body weight and pen feed disappearance were recorded weekly to determine ADG, ADFI and G:F calculated on per pen basis. Blood was collected on d 0 and d 14 and analyzed for plasma urea nitrogen (PUN) and plasma-free Thr and Lys. Faecal consistency scoring (0=normal, 1=soft faeces, 2=mild diarrhoea, and 3=severe diarrhoea) was done as described by Marquardt et al. (1999) by 2 independent trained individuals with no prior knowledge of the treatment allocation.

4.3.4 Sample preparation and laboratory analyses

Diet samples were ground through a 1-mm mesh screen. The DM content was determined according to AOAC (2000). The N content of the diets was determined with a gas combustion method using a Leco FP-2000 Nitrogen Analyzer (Leco Corp., St. Joseph, MI). Amino acid analyses were carried out at the lab of Evonik Industries AG, Hanau-Wolfgang, Germany. Dietary concentrations of all essential and non-essential AA, except for tryptophan and tyrosine, were determined by ion-exchange chromatography with post-column derivatization with ninhydrin. Amino acids were oxidized with performic acid, which was neutralized with Na metabisulfite (Llames and Fontaine, 1994; Commission Directive, 1998). Amino acids were liberated from the protein by hydrolysis with 6 mol/L 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).

4.3.5 Plasma urea nitrogen

Blood sampling and PUN analysis were performed according to Nyachoti et al. (2006). Briefly, on d 0 and 14, a 10-mL blood sample was collected from one pig per pen via jugular venipuncture into heparinized Vacutainer tube (Becton Dickinson, Rutherford, US) and stored on ice for 20 min before being centrifuged at 2,000 × *g* for 10 min at 4˚C to recover plasma. Plasma samples were stored at -20˚C until used for further analysis. Plasma samples were thawed and then analyzed for PUN using a Nova Stat Profile M blood gas and electrolyte analyzer (Nova Biomedical Corporation, Waltham, MA).

4.3.6 Plasma-free threonine and lysine

Plasma-free Thr and Lys concentrations were determined using amino acid analyzer (Skykam Amino Acid Analyzer, Germany) after being deproteinized with 4% sulfosalicylic acid.

4.3.7 Statistical Analysis

Data were subjected to ANOVA using the Proc mixed procedure of SAS 9.2 (SAS Inst. Cary. NC). The data were analyzed as 2 × 5 factorial arrangement with the factors being sanitation conditions (CL and UCL) and dietary SID Thr:Lys levels (61, 64, 67, 69 and 72%). Since, the housing management (lighting, humidity, temperature) for clean and unclean conditions were similar, this was considered as factorial arrangement in a completely randomized design. Each experimental unit was a pen of 3 piglets and there were 6 experimental units per treatment and thus 60 experimental units in total. Orthogonal polynomial contrasts were used to determine the linear and quadratic effects of increasing levels of SID

Thr to Lys ratio. Statistical significance was accepted at $P < 0.05$ and $0.05 < P < 0.10$ was considered a trend. The statistical model was $Y_{ij} = \mu + Sani_i + Thr_j + (Sani \times Thr)_{ij} + €_{ijk}$,

Where,

Y_{ijk} is an observation on the variable of interest on the k^{th} experimental unit receiving the j^{th} level of factor Thr and i^{th} level of factor Sani,

μ - overall mean,

$_i$ = CL and UCL,

$_j$ = 61, 64, 67, 69, and 72%,

$Sani_i$ – effect of level $_i$ of factor sanitation,

Thr_j – effect of level $_j$ of factor dietary SID Thr:Lys

$(Sani \times Thr)_{ij}$ – effect of the interaction of level i of factor Sani with j level of factor Thr,

$€_{ijk}$ – random error due to $_k$th experimental unit in the $_j$th level of Thr and $_i$th level of sanitation.

To determine the optimal SID Thr:Lys level, data were subjected to broken-line analysis (Robbins et al., 2006) using the Proc NLIN of SAS (SAS Inst. Inc. Cary, NC). Estimates of R (requirement) for CL and UCL environments were derived in separate analysis. The R-values for 2 sanitation environments were compared using a t-test. The estimated SID Thr:Lys requirements was compared with the current recommendations (NRC, 2012) using pooled t-test.

4.4. RESULTS

The analyzed AA and crude protein contents of the experimental diets were presented in **Table 4.1**. The SID Thr to Lys ratios were then corrected based on the analyzed contents using the following formula: corrected SID Thr: Lys = ((calculated SID Thr:Lys × analyzed total Thr:Lys) / calculated total Thr:Lys). The corrected SID Thr:Lys in the diets were 61, 64, 67, 69 and 72% which were used for the regression analysis.

4.4.1. Growth performance

Growth performance values are shown in **Table 4.2**. There were no interactions between sanitary conditions and dietary SID Thr:Lys on any of the response criteria. Piglets raised under CL conditions had higher ($P < 0.05$) ADG and lower ADFI compared to those pigs raised under UCL conditions. During week 1, 2 and overall experimental period, piglets raised under UCL conditions had improved G:F than

Table 4.2. Effects of dietary threonine to lysine ratio and sanitary conditions on performance of weaned piglets[1]

Item	Clean sanitary condition[2]					Unclean sanitary condition[3]					SEM[5]	*P* value[6]						
	Corrected SID Thr:Lys, %[4]					Corrected SID Thr:Lys, %						S	Thr	S × Thr	Clean		Unclean	
	61	64	67	69	72	61	64	67	69	72					L	Q	L	Q
Initial BW, kg	7.37	7.16	7.33	7.40	7.30	7.18	7.03	7.05	7.11	7.12	0.34	-	-	-	-	-	-	-
Final BW, kg	17.5	17.3	17.7	17.6	18.0	15.7	16.0	15.1	15.7	15.8	0.81	**<0.001**	0.966	0.901	0.166	0.509	0.940	0.637
Week 1 (d 0 to 7)																		
ADG, g	301^{abcd}	315^{abc}	314^{abc}	320^{ab}	353^{a}	209^{cd}	214^{cd}	221^{bcd}	234^{bcd}	197^{d}	25	**<0.001**	0.868	0.524	0.171	0.638	0.947	0.283
ADFI, g	452	438	431	450	431	322	348	303	345	299	36	**<0.001**	0.805	0.960	0.712	0.922	0.533	0.576
G:F	0.66	0.74	0.73	0.73	0.82	0.66	0.65	0.73	0.69	0.65	0.05	**0.043**	0.469	0.349	**0.033**	0.835	0.879	0.343
Week 2 (d 8 to 14)																		
ADG, g	500^{a}	440^{ab}	458^{ab}	512^{a}	510^{a}	409^{ab}	411^{ab}	405^{ab}	450^{ab}	373^{b}	29	**<0.001**	0.254	0.321	0.352	0.166	0.555	0.313
ADFI, g	827^{a}	746^{ab}	797^{a}	836^{a}	812a	577^{bc}	534c	538c	613bc	558^{bc}	43	**<0.001**	0.275	0.969	0.750	0.575	0.655	0.358
G:F	0.60^{cd}	0.59cd	0.59d	0.62^{bcd}	0.62^{bcd}	0.71^{abcd}	0.78^{a}	0.75^{ab}	0.74^{abc}	0.67^{abcd}	0.03	**<0.001**	0.786	0.223	0.464	0.487	0.247	0.066
Week 3 (d 15 to 21)																		
ADG, g	665^{abc}	691^{abc}	729^{a}	673^{abc}	698^{ab}	544^{c}	571^{bc}	574^{bc}	589^{abc}	590^{abc}	35	**<0.001**	0.643	0.864	0.997	0.265	0.184	0.842
ADFI, g	1002^{a}	952^{ab}	998^{a}	997^{a}	985^{ab}	875^{ab}	805^{ab}	776^{b}	829^{ab}	855^{ab}	50	**<0.001**	0.687	0.828	0.556	0.732	0.929	**0.007**
G:F	0.67	0.73	0.74	0.68	0.71	0.63	0.71	0.75	0.71	0.69	0.03	0.868	0.077	0.841	0.581	0.283	0.322	**0.024**
Overall (d 0 to 21)																		
ADG, g	489^{ab}	482^{abc}	500^{abc}	502^{ab}	520^{a}	387^{d}	398^{cd}	400^{cd}	424^{bcd}	387^{d}	21	**<0.001**	0.679	0.632	0.154	0.651	0.651	0.233
ADFI, g	760^{a}	712^{ab}	742^{a}	761^{a}	743^{a}	591^{bc}	562^{c}	539^{c}	596^{bc}	571^{bc}	33	**<0.001**	0.537	0.939	0.928	0.684	0.870	0.245
G:F	0.64^{b}	0.68^{ab}	0.68^{ab}	0.67^{ab}	0.70^{ab}	0.66^{ab}	0.71^{ab}	0.75^{a}	0.72^{ab}	0.68^{ab}	0.02	**0.041**	0.078	0.312	0.138	0.822	0.440	**0.006**

[1]Values are least square means; *n* = 6 per treatment. Bold *P* values are significant and underlined *P* values are considered as tendency. Means with a different superscript letters indicate a significant difference among treatments.

[2]Clean sanitary condition: piglets subjected to clean sanitary conditions were housed in cleaned and disinfected rooms and fed antibiotic-free diets

[3]Unclean sanitary conditions: piglets subjected to unclean sanitary conditions were housed in a room where cleaning and disinfection was not done, moreover, manure from swine herd was added (5 kg per pen) to the pens on d 0 and d 7 of the experiment.

[4]SID Thr:Lys, %: corrected standardized ileal digestible threonine:lysine, %

[5]SEM: standard error of mean that applies to the statistical model

[6]Probability values of fixed effects (S-sanitation; Thr- dietary SID Thr:Lys; S × Thr –interaction effect between S and Thr; and linear (L) and quadratic (Q) effects for dietary SID Thr:Lys, %

those raised under CL conditions. Increasing dietary SID Thr:Lys tended to improve (P = 0.078) the overall G:F of pigs. During week 1, piglets raised under CL conditions had improved (linear; P = 0.033) G:F by increasing Thr:Lys. However, overall growth performance was not affected by increasing Thr:Lys in piglets raised under CL conditions. Under UCL condition, increasing dietary Thr:Lys improved (quadratic; $P < 0.05$) feed efficiency during overall and week 3 experimental periods. Under CL and UCL conditions, the estimated optimal SID Thr:Lys using the curvilinear regression and G:F as response criteria was 65% (**Fig 4.1**) and 67% (**Fig. 4.2**), respectively. The estimated optimal SID Thr:Lys was not different ($P > 0.05$) between CL and UCL conditions.

4.4.2. Plasma-free Thr and Lys and plasma urea nitrogen

There were no interactions ($P > 0.05$) between sanitary conditions and dietary SID Thr:Lys on PUN and plasma-free Thr and Lys (**Table 4.3**). Piglets raised under UCL conditions had higher ($P < 0.05$) plasma-free Lys compared to pigs raised under CL conditions, however, plasma-free Thr was not affected ($P > 0.05$) by sanitation conditions. Plasma-free Thr increased (linear; $P \leq 0.05$) in piglets raised under both CL and UCL conditions, whereas, plasma-free Lys did not differ ($P > 0.05$) due to dietary SID Thr:Lys.

4.5. DISCUSSION

The goal of this study was to determine the optimal standardized ileal digestible Thr to Lys ratio for weaned pigs reared under clean (CL) or unclean (UCL) sanitary conditions using growth performance as response criteria. In this study, diets were corn-wheat-soybean meal-based with a constant SID Lys content of 1.18% was set to be 10% lower than the requirement level that was established in our lab (Kahindi et al., 2014).

Under CL conditions, increasing the SID Thr:Lys improved the overall G:F but had no effect on ADG during week 1, which is in agreement with Fernandez and Strathe (2009). Based on overall feed

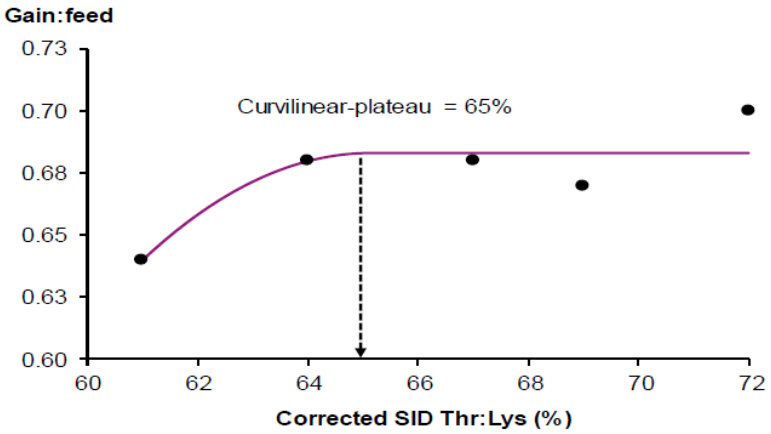

Fig 4.1. The optimal dietary standardized ileal digestible (SID) threonine (Thr): lysine (Lys) for gain to feed ratio (G:F) in weaned pigs reared under clean sanitary conditions determined using regression analysis was 65% ± 4.14 [Y= 0.68 – 0.0025 (65- x)^2] (SE = 4.14; R^2 = 0.76). Data points ( ) represent least square means of dietary treatments (n = 6).

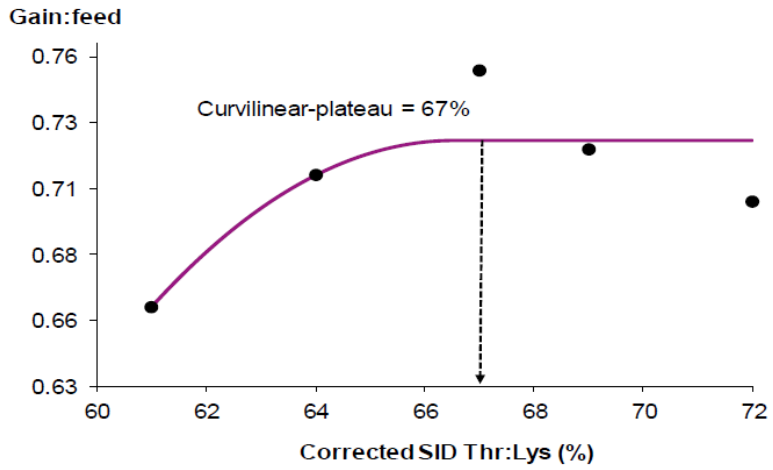

Fig 4.2 The optimal dietary standardized ileal digestible (SID) threonine (Thr): lysine (Lys) for gain to feed ratio (G:F) in weaned pigs reared under unclean sanitary conditions determined using regression analysis was 67% [Y= 0.72 – 0.0021 (67- x)^2] (SE = 4.96; R^2 = 0.70). Data points ( ) represent least square means of dietary treatments (n = 6).

Table 4.3. Effects of dietary threonine:lysine ratio (Thr:Lys) and sanitation on plasma-free threonine, lysine and plasma urea nitrogen of weaned pigs[1]

Item	Clean sanitary condition[2]					Unclean sanitary condition[3]					SEM[5]	*P* value[6]						
	Corrected SID Thr:Lys, %[4]					Corrected SID Thr:Lys, %						S	Thr	S × Thr	Clean		Unclean	
	61	64	67	69	72	61	64	67	69	72					L	Q	L	Q
Thr, mmol/L	535	627	612	648	627	422	509	632	587	640	53	0.101	0.020	0.464	**0.050**	0.185	**0.010**	0.241
Lys, mmol/L	139	119	129	121	125	197	197	166	169	207	29	**<0.001**	0.840	0.841	0.570	0.581	0.938	0.399
PUN, mmol/L	3.57	3.72	2.94	2.64	3.37	3.74	4.12	3.43	3.99	3.84	0.33	0.271	0.378	0.476	0.226	0.207	0.980	0.871

[1]Values are least square means; $n = 6$ per treatment. Bold *P* values are significant.

[2]Clean sanitary condition: piglets subjected to clean sanitary conditions were housed in cleaned and disinfected rooms and fed antibiotic-free diets

[3]Unclean sanitary conditions: piglets subjected to unclean sanitary conditions were housed in a room where cleaning and disinfection was not done, moreover, manure from swine herd was added (5 kg per pen) to the pens ond 0 and d 7of the experiment.

[4] SID Thr:Lys, %: corrected standardized ileal digestible threonine:lysine, %

[5]SEM: standard error of mean that applies to the statistical model

[6]Probability values of fixed effects (S-sanitation; Thr- dietary SID Thr:Lys; S × Thr –interaction effect between S and Thr; and linear (L) and quadratic (Q) effects for dietary SID Thr:Lys, %

efficiency, SID Thr:Lys of 65% was optimized using curvilinear regression model, which is consistent with previous studies (James et al., 2003; Lenehan et al., 2004). The estimated SID Thr:Lys requirement for weaned pigs was higher than current recommendations (NRC, 2012).

Weaned pigs subjected to unclean sanitary condition was used as a model of moderate immune system stimulation (Le Floc'h et al., 2006), which was in turn anticipated to have effect on partitioning of AA between lean tissue deposition and supporting the immune system (Williams et al., 1997). Piglets raised under UCL conditions had reduced growth performance compared with those raised under CL conditions. This suggests that the sanitation model of immune challenge was effective, which is consistent with the findings of others (Lee et al., 2005; Le Floc'h et al., 2006; Kahindi et al., 2014; van der Meer et al., 2016) that showed decreased growth performance when piglets were subjected to poor sanitary conditions.

Under UCL conditions, the estimated SID Thr:Lys using regression was 67% for feed efficiency. Immune system stimulation redirects AA for immune response instead of protein deposition which could result in reduced growth performance (Le Floc'h et al., 2004; Pluske et al., 2018). Trevisi et al. (2015) demonstrated increasing dietary Thr (9 vs. 8.5 g/kg diet) improved growth performance in weaned piglets challenged with ETEC K88 challenge. In a recent study, van der Meer et al. (2016) indicated that dietary AA supplementation above the basal requirement would be advantageous for growth performance of pigs, particularly when raised under poor sanitary conditions.

Plasma urea nitrogen has often been used as a response criterion for determining AA requirements since PUN can be used as an indicator of protein utilization efficiency (Coma et al., 1995). When there is an excess AA, PUN is known to increase because excess AA cannot be stored and therefore they are degraded with the production of urea (Heo et al., 2009; Waguespack et al., 2011). In this study, both under CL and UCL conditions, dietary Thr:Lys did not affect ($P > 0.10$) PUN concentration and therefore, the optimal SID Thr:Lys could not be determined using PUN as response criteria.

Under both CL and UCL, increasing levels of dietary SID Thr:Lys increased (linear, $P < 0.05$) concentration of plasma-free Thr. This result is consistent with the previous studies (Li et al., 1999; Wang et al., 2006) who also reported that increasing levels of dietary Thr increased serum free Thr. The increase in plasma-free Thr could be due to increasing levels of dietary SID Thr:Lys which is in agreement with Zhang et al. (2013). Piglets raised under poor sanitation conditions had higher plasma-free Lys concentration on d 14 compared to pigs raised CL conditions which is in line with Le Floc'h et al. (2007). Previous studies demonstrated that pigs maintained under poor sanitation conditions needed less Lys for protein deposition because of their reduced growth performance (Le Floc'h et al., 2007; William et al., 1997).

4.6. CONCLUSIONS

Weaned pigs raised under poor sanitation conditions had reduced growth performance compared to pigs raised under clean conditions**.** Based on feed efficiency, the estimated SID Thr:Lys requirement for weaned pigs raised under clean and poor sanitation conditions was 65% and 67%, respectively. The estimated SID Thr:Lys requirements for weaned piglets was not different between clean and poor sanitation conditions. The estimated SID Thr:Lys requirement for weaned pigs was higher than current recommendations (NRC, 2012).

CHAPTER FIVE

MANUSCRIPT 2

Effects of different dietary tryptophan:lysine ratio and sanitary conditions on growth performance, plasma urea nitrogen, serum haptoglobin and ileal histomorphology of weaned pigs

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5.1. ABSTRACT

A total of 180 mixed-sex pigs (Duroc × [Yorkshire × Landrace]; average initial BW of 7.36 ± 0.2 kg) weaned at 21 ± 1 d were fed corn-soybean meal-wheat based-diets to determine the optimal standardized ileal digestible (SID) tryptophan to lysine ratio (Trp:Lys) in a 2 × 5 factorial arrangement [2 sanitary conditions: clean (CL) and unclean (UCL), and 5 dietary treatments [SID Trp:Lys (16, 18, 20, 22 and 24%)]. In each sanitary condition, blood was collected on d 0 and d 14 to determine plasma urea nitrogen and on d 14, ileal tissue (one pig per pen) was collected for the measurement of gut morphology. Pigs kept under UCL conditions had lower growth rate ($P < 0.05$) than under CL conditions. Under CL conditions, the estimated optimal SID Trp:Lys for ADG was 19.7% whereas under UCL conditions these values were 20.5% and 19.0% for ADG and G:F, respectively. Under CL and UCL conditions, increasing SID Trp:Lys reduced (linear, $P = 0.05$) PUN concentration but had no effect ($P > 0.10$) on villous height (VH), crypt depth (CD) and VH:CD. In conclusion, estimated optimal Trp:Lys requirements based on ADG for clean and unclean sanitation conditions were 19.7% and 20.5%, respectively.

Keywords: Piglets, sanitation, standardized ileal digestible, tryptophan.

5.2. INTRODUCTION

Tryptophan (Trp) is the second or third dietary limiting amino acid (AA) in pig diets (Guzik et al., 2005). Besides protein synthesis, Trp is involved in many important biological functions. Among those functions, Trp is the precursor for serotonin, a neurotransmitter which is involved in feed intake (Henry et al., 1992) and stress response (Sève et al., 1991). Tryptophan is also important for immunity because the conversion of Trp into kynurenine is increased under inflammation or immune challenge (Le Floc'h et al., 2008). Thus, providing sufficient Trp in the diet is important to achieve optimal growth performance in pigs (Guzik et al., 2002).

Dietary requirement of SID Trp:Lys for pigs between 7 and 25 kg is 16% (NRC, 2012). However, because of its involvement in the immune response this level of Trp might not be adequate for pigs raised in commercial production conditions, whereby incidences of clinical and subclinical infections are high, especially during the immediate post-weaning period. Indeed, piglets raised under unclean (UCL) sanitary conditions had reduced growth performance (Kahindi et al., 2014) and an activated immune system (Williams et al., 1997), which also induced an inflammatory response (Le Floc'h et al., 2006). Anti-inflammatory response interferes with growth in part because of the competition for AA between protein accretion and the immune system (Le Floc'h et al., 2009).

A growing interest in the nutritional management of nursery pigs is to utilize nutritional programs without in-feed antibiotic growth promoters (AGP) (de Lange et al., 2010; Heo et al., 2013). It is prudent to expect that this development may alter the Trp:Lys requirement for optimal performance, especially when piglets are exposed to an immunological challenge as is often the case under UCL conditions. For example, it has been shown that the metabolism and requirement for Trp are modified in pigs during an immune challenge (Le Floc'h and Sève,

2007; Ren et al., 2013). Furthermore, exposing piglets to unclean conditions is considered as a predisposing factor for weaning disorders (Le Floc'h and Sève, 2007; Williams et al., 1997).

Information about the Trp:Lys ratio for piglets fed AGP-free diets and subjected to an immunological challenge is limited. It was hypothesized that weaned piglets subjected to UCL conditions have increased Trp:Lys requirement compared to CL conditions. Thus, the objective of this study was to determine the standardized ileal digestible (SID) Trp:Lys requirement for weaned piglets reared under clean (CL) or UCL sanitary conditions using growth as response criteria.

5.3. MATERIALS AND METHODS

The experimental protocol was reviewed and approved by the University of Manitoba Animal Care Committee and pigs were cared for in accordance with the guidelines of the Canadian Council on Animal Care (2009).

5.3.1 Experimental diets

Diets were corn, wheat and soybean meal-based with a constant SID Lys of 1.18% that was set to be the second limiting AA and formulated to contain graded levels of SID Trp:Lys (16, 18, 20, 22 and 24%; **Table 5.1**). Analyzed AA composition of protein-containing ingredients (i.e. corn, wheat and soybean meal) were used in diet formulation. All other nutrients were provided in quantities to meet or exceed NRC (2012) requirements for a 6 to 10 kg pig. All diets were in mash form and did not contain any AGP.

Table 5.1. Ingredient composition and nutrient contents of experimental diets (g/kg, as-is-basis)

	Dietary SID Trp:Lys, %				
Items	16	18	20	22	24
Ingredients					
Corn	473.5	473.5	473.5	473.5	473.5
Wheat	210.0	210.0	210.0	210.0	210.0
Soybean meal	233.1	233.1	233.1	233.1	233.1
Vegetable oil	29.2	29.2	29.2	29.2	29.2
Corn starch	5.0	4.8	4.6	4.3	4.1
Monocalcium phosphate	15.6	15.6	15.6	15.6	15.6
Limestone	11.0	11.0	11.0	11.0	11.0
Salt	3.0	3.0	3.0	3.0	3.0
Vitamin-mineral premix*	10.0	10.0	10.0	10.0	10.0
L-Lys HCl	5.4	5.4	5.4	5.4	5.4
L-Thr	1.9	1.9	1.9	1.9	1.9
DL-Met	1.7	1.7	1.7	1.7	1.7
L-Val	0.6	0.6	0.6	0.6	0.6
L-Trp	0.0	0.2	0.5	0.7	0.9
Total	100.0	100.0	100.0	100.0	100.0
Calculated nutrient content in the diet					
NE (MJ/kg)	10.4	10.4	10.4	10.4	10.4
Crude protein, g/kg	197.6	197.6	197.6	197.6	197.6
Ether extract, g/kg	29.7	29.7	29.7	29.7	29.7
Crude fiber, g/kg	40.4	40.4	40.4	40.4	40.4
Total Lys, g/kg	13.0	13.0	13.0	13.0	13.0
Total Trp, g/kg	2.2	2.5	2.7	2.9	3.1

Total Ca, g/kg	8.0	8.0	8.0	8.0	8.0
Available P, g/kg	4.5	4.5	4.5	4.5	4.5
SID Lys, g/kg	11.8	11.8	11.8	11.8	11.8
SID Met, g/kg	4.5	4.5	4.5	4.5	4.5
SID Cys, g/kg	2.7	2.7	2.7	2.7	2.7
SID Met+Cys, g/kg	7.2	7.2	7.2	7.2	7.2
SID Thr, g/kg	7.6	7.6	7.6	7.6	7.6
SID Trp, g/kg	1.9	2.1	2.4	2.6	2.8
SID Val, g/kg	8.2	8.2	8.2	8.2	8.2
SID Arg, g/kg	10.6	10.6	10.6	10.6	10.6
SID SAA:Lys, g/kg	6.1	6.1	6.1	6.1	6.1
SID Trp:Lys	0.16	0.18	0.20	0.22	0.24
Analyzed CP and amino acid composition (g/kg)					
Crude protein	201.0	202.0	200.0	199.0	204.0
Lys	12.8	13.3	12.9	12.8	12.3
Trp	2.3	2.5	2.6	2.9	3.0
Met	4.5	4.7	4.6	4.5	4.4
Cys	3.4	3.4	3.4	3.4	3.4
Met+ Cys	7.9	8.1	8.0	7.9	7.9
Ile	7.8	7.9	7.8	7.7	7.8
Val	9.4	9.6	9.5	9.4	9.5
Thr	8.4	8.7	8.5	8.5	8.3
Arg	11.7	12.0	11.7	11.7	11.8

* Supplied the following per kg of diet: 8250 IU of vitamin A, 835 IU of vitamin D3, 40 IU of vitamin E, 25µg 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.

5.3.2 Animals and experimental design

A total of 180 piglets (Duroc × [Yorkshire × Landrace], mixed sex) weaned at 21 ± 1 d of age and housed 3 per pen were fed a corn-soybean meal-based starter diet (20% CP; 16% SID Trp:Lys) for a 6-d adaptation period. On d 7, piglets (average initial BW of 7.36 ± 0.2 kg) were randomly assigned to 1 of 5 dietary SID Trp:Lys (16, 18, 20, 22 and 24%) to give 6 replicates per treatment. Under CL conditions, 90 piglets were housed in a room that had been cleaned and isinfected before the arrival of the piglets and that was cleaned once weekly thereafter. Under UCL conditions, 90 piglets were housed in the same room occupied by the CL group without cleaning and disinfection. Moreover, manure from the swine herd was added (5 kg per pen) to the pens on d 0 and d 7 to further enhance the unsanitary conditions for the UCL group. Body weight and pen feed disappearance were recorded weekly to determine ADG, ADFI and G:F.

5.3.3. Sample preparation and analysis

Prior to analysis, ingredient and diet samples were ground through a 1-mm mesh screen. Dry matter content was determined according to AOAC (2000) whereas dietary N content was determined with a gas combustion method using a Leco FP-2000 Nitrogen Analyzer (Leco Corp., St. Joseph, MI). Amino acid analysis for the feed ingredients and diets were carried out at the lab of Evonik Industries AG, Hanau-Wolfgang, Germany, using the method described by Llames and Fontaine (1994).

5.3.4. Measurement of hydrogen sulphide and ammonia in the air

Under CL and UCL conditions, air quality status in the rooms was analyzed thrice per week. Hydrogen sulphide (H_2S) concentration in the air was measured using a JEROME-631-X

instrument (Arizona Instrument Corporation, Phoenix, AZ, USA), whereas ammonia (NH_3) concentration was measured using detector tubes (RAE systems, San Jose, CA, USA). Ammonia detector tubes (5 tubes for each measurement day) were placed on animal level in the pens and measurements taken.

5.3.5. Plasma urea nitrogen

On d 0 and 14, a 10-mL blood sample was collected from one pig per pen via jugular venipuncture into heparinized vacutainer tubes (Becton Dickinson, Rutherford, US) and stored on ice for 20 min before being centrifuged at 2000 × g for 10 min at 4˚C to recover plasma. Plasma samples were stored at -20˚C until used for further analysis. Plasma urea nitrogen (PUN) was determined using Nova Stat Profile M blood gas and electrolyte analyzer (Nova Biomedical Corporation, Waltham, MA, USA).

5.3.6. Serum haptoglobin

On d 0 and d 14, a 10 mL blood samples were collected via jugular venipuncture into serum tubes (Becton Dickinson and company, Rutherford, NJ). 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 C and serum stored at -80 C until analyses. For the determination of serum haptoglobin, a commercial ELISA kit was used according to the Manufacturer's recommendations (Life diagnostics, Inc, PA, USA). Serum samples were tested in duplicate and samples were diluted 1:20,000 prior to analysis.

5.3.7. Ileal tissue collection and histomorphology measurement

Under both the CL and UCL conditions, one pig per pen was killed on d 14 as described by Kiarie et al. (2007) and the abdominal cavity was opened from the sternum to pubis to expose

the whole gastrointestinal tract. Approximately 5 cm of ileal tissue was removed and fixed in a 10% formalin solution for morphological analysis. Villous height (VH), crypt depth (CD), and VH:CD ratio were determined as described by Nyachoti et al. (2012).

5.3.8. Statistical analysis

Data were subjected to ANOVA using the Proc mixed procedure of SAS 9.2 (SAS Inst. Cary. NC). The data were analyzed as 2 × 5 factorial arrangement with the factors being sanitation conditions (CL and UCL) and dietary SID Trp:Lys levels (18, 19, 20, 22, and 24%). Since, the housing management (lighting, humidity, temperature) for clean and unclean conditions were similar, this was considered as factorial arrangement in a completely randomized design. Each experimental unit was a pen of 3 piglets and there were 6 experimental units per treatment and thus 60 experimental units. Orthogonal polynomial contrasts were used to determine the linear and quadratic effects of increasing levels of SID Trp to Lys ratio. Statistical significance was accepted at $P < 0.05$ and $0.05 < P < 0.10$ was considered a trend.

The statistical model is $Y_{ij} = \mu + Sani_i + Trp_j + (Sani \times Trp)_{ij} + €_{ijk}$, where,

Y_{ijk} is an observation on the variable of interest on the kth experimental unit receiving the jth level of factor Trp and ith level of factor Sani,

μ - overall mean,

$_i$ = CL and UCL

$_j$ = 18, 19, 20, 22, and 24%;

$Sani_i$ – effect of level $_i$ of factor Sanitation,

Trp_j – effect of level $_j$ of dietary SID Trp:Lys

$(Sani \times Trp)_{ij}$ – effect of the interaction of level $_i$ of factor Sani and with level $_j$ of factor Trp,

$€_{ijk}$ – random error due to $_k$th experimental unit in the jth level of Trp and $_i$th level of

sanitation.

To determine the optimal SID Thr:Lys level, data were subjected to broken-line analysis (Robbins et al., 2006) using the Proc NLIN of SAS (SAS Inst. Inc. Cary, NC). Estimates of R (requirement) for CL and UCL environments were derived in separate analysis. The R-values for 2 sanitation environments were compared using a t-test. The estimated SID Trp:Lys requirements was compared with the current recommendations (NRC, 2012) using pooled t-test.

5.4. RESULTS

The analyzed AA and crude protein contents of the experimental diets are presented in **Table 5.1**. The SID Trp:Lys ratios were then corrected based on the analyzed contents using the following formula; Corrected SID Trp:Lys = (calculated SID Trp:Lys × analyzed total Trp:Lys) / calculated total Trp:Lys. The corrected SID Trp:Lys in the diets were 18, 19, 20, 22 and 24% which were used for the regression analysis. All animals remained healthy throughout the experimental period.

5.4.1. Concentrations of ammonia and hydrogen sulphide

Under UCL condition**,** NH_3 and H_2S concentrations in the air were higher ($P < 0.05$) than under CL condition (**Table 5.2**). Under UCL, the concentration of H_2S during week 2 was higher ($P < 0.01$) than in week 1.

5.4.2. Growth performance

Growth performance values are shown in **Table 5.3**. There were no interactions ($P > 0.10$) between sanitary conditions and dietary SID Trp:Lys on any of the response criteria. The initial average BW was similar for pigs reared under CL and UCL conditions. Pigs raised under

CL conditions had a higher ($P < 0.05$) growth rate compared to those pigs raised under UCL conditions. Regardless of sanitary conditions, during week 1 and the overall experimental period, increasing SID Trp:Lys improved (linear; $P < 0.05$) ADG and feed efficiency. During the overall experimental period, feed intake tended to increase ($P = 0.06$) with increasing dietary SID Trp:Lys ratio. The final BW of pigs under CL was higher ($P < 0.05$) than that of pigs raised under UCL condition.

Under CL conditions, the optimal SID Trp:Lys was estimated to be 19.7% using the broken-line quadratic (BLQ) model and ADG as response criteria (**Fig. 5.1**). Under UCL conditions, the estimated optimal SID Trp:Lys for ADG and G:F using the BLQ model were 20.5% (**Fig. 5.2**) and 19.0% (**Fig. 5.3**), respectively. The estimated SID Trp:Lys requirement was not different ($P > 0.05$) between CL and UCL conditions.

5.4.3. Plasma urea nitrogen and serum haptoglobin

The PUN concentration was not affected by sanitary conditions (**Table 5.4**). However, increasing dietary SID Trp:Lys linearly reduced ($P = 0.05$) PUN on d 14. Based on PUN as a response criterion, the optimal SID Trp:Lys could not be determined using regression analysis.

On d 14, serum haptoglobin concentration tended to increase ($P = 0.087$) in pigs under UCL conditions (**Table 5.4**), whereas dietary SID Trp:Lys had no effect on serum haptoglobin.

5.4.4. Ileal histomorphology

There were no interactions ($P > 0.10$) between sanitary conditions and dietary SID Trp:Lys (**Table 5.5**) on ileal histomorphology (**Fig. 5.4**). Pigs raised under CL conditions had

Table 5.2. Effect of sanitary conditions on concentrations of ammonia and hydrogen sulphide in room air[1]

Item	Clean conditions		Unclean conditions		*P* value		
	Week 1	Week 2	Week 1	Week 2	S[3]	Time	S × Time
Ammonia, ppm[2]	18.67 ± 1.08[4]	17.67 ± 1.08	26.67 ± 1.08	26.63 ± 0.94	**<0.001**	0.631	0.659
Hydrogen sulphide, ppm	0.0005 ± 0.03	0.02 ± 0.03	0.035 ± 0.03	0.163 ± 0.02	**< 0.050**	**0.009**	0.070

[1] Under CL and UCL conditions, air quality status in the rooms was analyzed thrice per week. Hydrogen sulphide concentration in the air was measured using a JEROME-631-X instrument (Arizona Instrument Corporation, Phoenix, AZ) whereas ammonia was measured using detector tubes (RAE systems, San Jose, CA).

[2]parts per million

[3] sanitation

[4] values are least square means ± standard error of mean. Bold *P* values are significant.

Table 5.3. Effects of different standardized ileal digestible tryptophan:lysine ratio and sanitation on growth performance in weaned pigs[1]

Dietary	Clean sanitary conditions					Unclean sanitary conditions						*P*-value[5]						
SID	18	19	20	22	24	18	19	20	22	24	SEM[3]	S	Trp	S × T	CL		UCL	
Trp:Lys[2]															L	Q	L	Q
Week 1																		
ADG, g	197^{ab}	213a	204^{ab}	197^{ab}	215^{a}	111^{b}	149^{ab}	198^{ab}	186^{ab}	174^{ab}	18.9	**0.001**	0.204	0.213	0.681	0.900	**0.041**	0.080
ADFI, g	313	269	283	333	262	318	298	300	311	306	27.9	0.413	0.506	0.799	0.405	0.715	0.932	0.792
G:F	0.63^{abc}	0.79^{a}	0.72^{ab}	0.60^{abc}	0.83^{a}	0.36^{c}	0.52^{bc}	0.66^{abc}	0.59^{abc}	0.60^{abc}	0.06	<**0.001**	**0.015**	0.100	0.228	0.696	**0.030**	0.094
Week 2																		
ADG, g	423	427	437	449	454	299	365	359	371	391	37.7	**0.002**	0.669	0.950	0.549	0.975	0.125	0.602
ADFI, g	577	547	564	622	562	510	496	658	598	613	42.3	0.972	0.116	0.270	0.709	0.887	0.074	0.402
G:F	0.75^{ab}	0.78^{a}	0.77^{ab}	0.72^{ab}	0.81^{a}	0.60^{ab}	0.73^{ab}	0.56^{b}	0.62^{ab}	0.64^{ab}	0.05	<**0.001**	0.217	0.415	0.703	0.695	0.874	0.993
Overall																		
ADG, g	310^{ab}	320^{ab}	320^{ab}	323^{ab}	335^{a}	205^{b}	257^{ab}	279^{ab}	278^{ab}	283^{ab}	22.2	<**0.001**	0.292	0.713	0.455	0.936	**0.041**	0.227
ADFI, g	445	408	424	477	412	414	397	479	454	459	25.7	0.605	0.060	0.217	0.961	0.791	0.123	0.567
G:F	0.68^{abcd}	0.79^{ab}	0.75^{abc}	0.66^{abcd}	0.82^{a}	0.48^{d}	0.62^{bcd}	0.61^{bcd}	0.60^{cd}	0.62^{bcd}	0.04	<**0.001**	**0.012**	0.333	0.194	0.560	0.098	0.181
Initial BW, kg	7.67	7.34	7.30	7.61	7.45	7.55	7.42	7.24	7.26	7.26	0.19	0.319	0.545	0.845	-	-	-	-
Final BW, Kg	11.76	11.38	11.35	11.68	11.38	10.78	11.01	11.14	11.15	11.21	0.37	**0.041**	0.953	0.894	0.721	0.704	0.915	0.646

[1]values are least square means, n=6 per treatment. Bold *P* values are significant and underlined *P* values are considered as tendency.

Means with a different superscript letters indicate a significant difference among treatments.

[2] corrected standardized ileal digestible tryptophan:lysine%

[3] standard error of mean that applies to the statistical model

[4]Probability values of linear effects for dietary SID Trp:Lys%

[5]Probability values of quadratic effects for dietary SID Trp:Lys%

Table 5.4. Effects of different standardized ileal digestible tryptophan:lysine ratio and sanitation on plasma urea nitrogen (PUN), serum haptoglobin and ileal histomorphology in weaned pigs[1]

Dietary SID	Clean sanitary conditions					Unclean sanitary conditions						*P*-value[4]						
Trp:Lys[2]	18	19	20	22	24	18	19	20	22	24	SEM[3]	S	Trp	S × T	CL		UCL	
															L	Q	L	Q
PUN, mmol/L																		
d 0	3.92	3.44	3.92	4.00	4.18	3.76	3.84	3.36	3.82	3.95	0.32	0.471	0.602	0.665	-	-	-	-
d 14	3.64	3.44	3.20	3.13	3.10	3.64	3.62	3.56	3.52	2.80	0.36	0.576	0.343	0.855	0.110	0.606	0.183	0.382
Serum haptoglobin, ng/mL																		
d 0	86.1	97.7	102.0	82.9	93.8	108.0	106.3	94.6	90.9	111.0	12.8	0.251	0.655	0.862	-	-	-	-
d 14	94.2	112.4	138.3	105.0	101.7	147.2	138.9	112.3	137.0	184.1	29.4	0.087	0.915	0.481	0.820	0.227	0.530	0.232
Ileum histomorphology																		
VH, µm	600^{ab}	616^{a}	623^{a}	594^{ab}	618^{a}	457^{ab}	427^{b}	508^{ab}	481^{ab}	481^{ab}	37.08	**<0.001**	0.768	0.837	0.923	0.919	0.222	0.621
CD, µm	404^{abc}	437^{abc}	473^{a}	393^{abc}	459^{ab}	299^{c}	301^{c}	337^{abc}	323^{abc}	356^{abc}	30.96	**<0.001**	0.240	0.804	0.583	0.730	0.103	0.892
VH:CD	1.50	1.44	1.33	1.54	1.35	1.54	1.44	1.51	1.53	1.37	0.07	0.351	0.121	0.726	0.421	0.864	0.229	0.554

[1]values are least square means, n=6 per treatment. Bold *P* values are significant and underlined *P* values are considered as tendency.
[2] corrected standardized ileal digestible tryptophan:lysine%
[3] standard error of mean that applies to the statistical model
[4]Probability values of fixed effects (S-sanitation; T- dietary SID Trp:Lys; S × Trp –interaction effect between S and Trp; and linear (L) and quadratic (Q) effects for dietary SID Trp:Lys, %

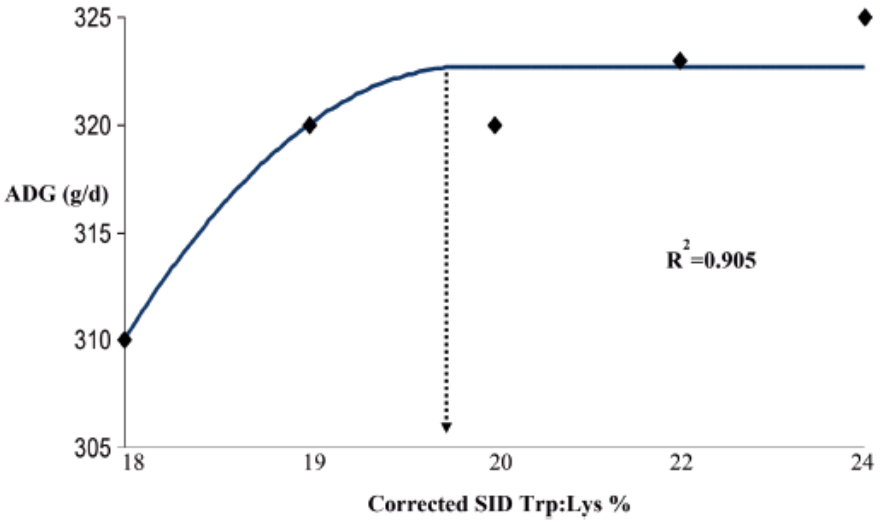

Figure 5.1. Optimal dietary standardized ileal digestible (SID) tryptophan (Trp): lysine (Lys) for average daily gain in weaned pigs reared under clean sanitary conditions determined using quadratic broken-line analysis was 19.7% (Y = 322.7-0.93 (19.70-x)^2) (SE = 0.35; R^2 = 0.905). Data points (▲) represent least square means of dietary treatments (n=6).

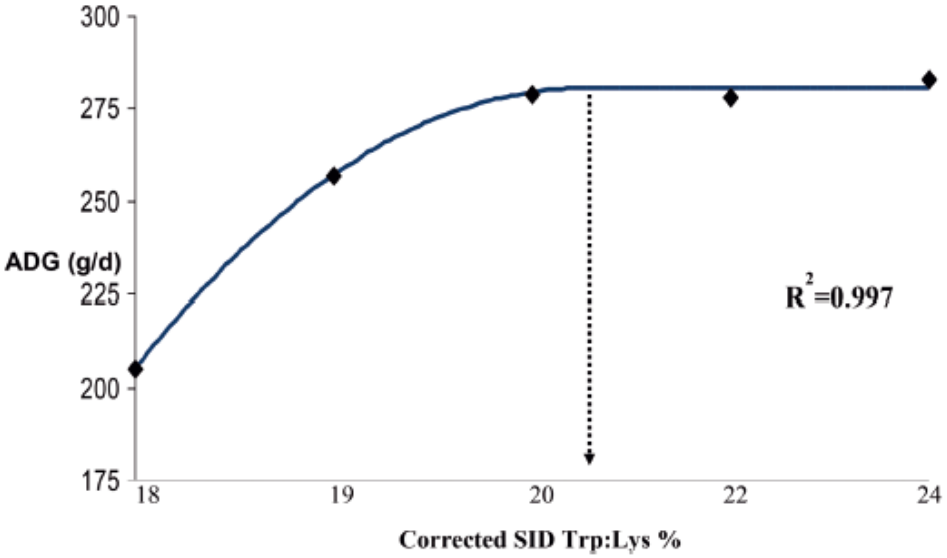

Figure 5.2. Optimal dietary standardized ileal digestible (SID) tryptophan (Trp): lysine (Lys) for average daily gain in weaned pigs reared under unclean sanitary conditions determined using quadratic broken-line analysis was 20.53% (Y = 280.4-3.67 (20.53 - x)^2) (SE = 0.38; R^2 = 0.99). Data points (▲) represent least square means of dietary treatments (n=6).

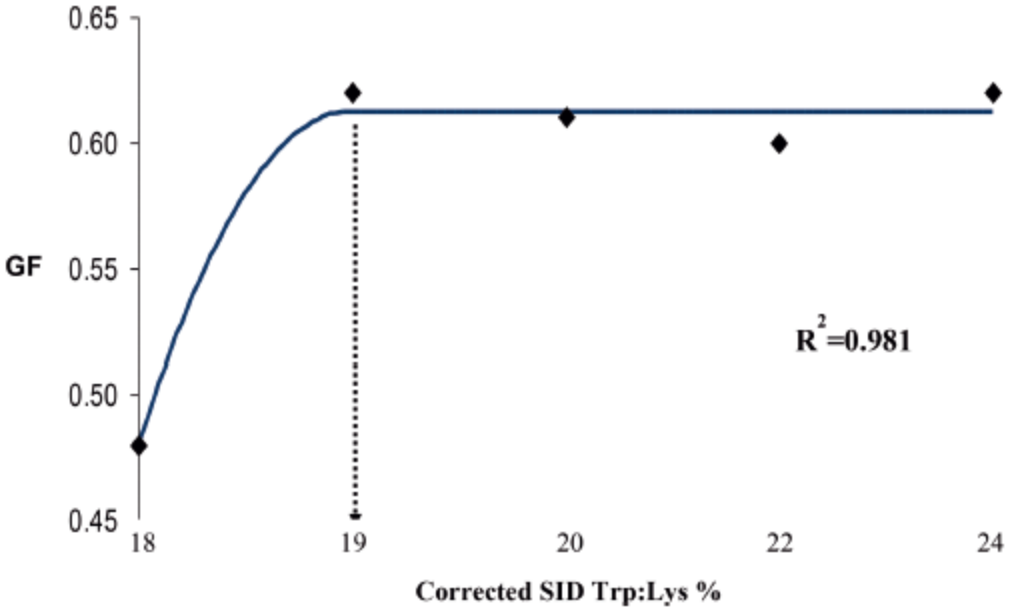

Figure 5.3. Optimal dietary standardized ileal digestible (SID) trytophan (Trp) : lysine (Lys) for gain: feed (G:F) in weaned pigs reared under unclean sanitary conditions determined by using quadratic broken-line analysis was 19% ($Y = 0.613\text{-}0.33\ (19.0\text{- } x)^2$) (SE = 0.37; $R^2 = 0.98$). Data points (▲) represent least square means of dietary treatments (n = 6).

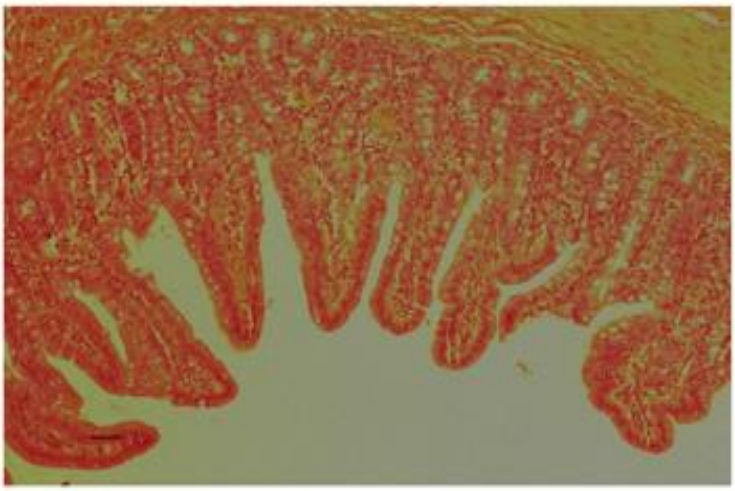

Figure A

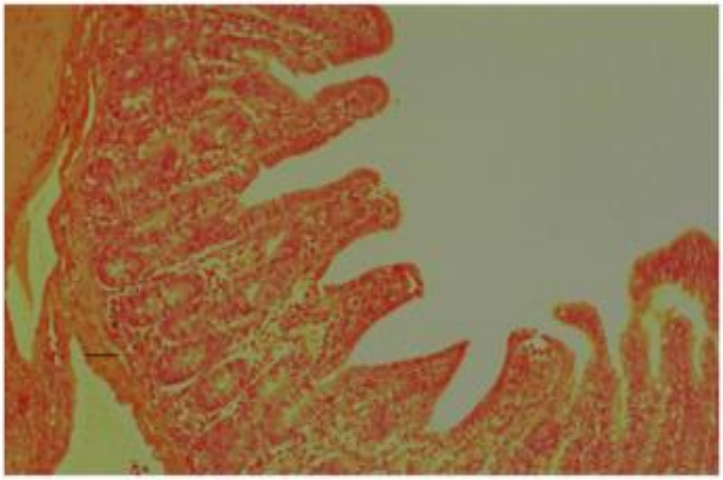

Figure B

Figure 5.4. Hematoxylin and eosin stained ileal tissue segments from piglets kept under clean (A) and unclean (B) sanitation conditions. Images were captured at 10x magnifications, and the bars shown above are 100μm in length.

higher villous height (VH) and deeper crypt depth (CD) than pigs under UCL conditions ($P < 0.01$). Dietary SID Trp:Lys did not affect ileal histomorphology in pigs.

5.5. DISCUSSION

The current study was carried out to determine the optimal standardized ileal digestible Trp:Lys ratio for weaned pigs reared under CL or UCL using growth performance and PUN as response criteria. In this study, diets were corn-wheat-soybean meal-based with a constant SID Lys content of 1.18% that was set to be 10% lower than the SID Lys requirement level that was established for pigs from the herd by Kahindi et al. (2014).

Subjecting weaned piglets to unclean sanitary conditions was used as a model of immune system stimulation (Le Floc'h et al., 2009), which was in turn expected to influence the partitioning of AA between lean tissue deposition and supporting the immune system (Williams et al. 1997). Results of this study showing that piglets reared under UCL had reduced growth performance compared with those raised under CL (Table 5.3) suggest that the sanitation model of immune challenge was effective. This observation is consistent with the findings of others (Lee et al., 2005; Le Floc'h et al., 2006; Kahindi et al., 2014) which showed reduced growth performance when piglets were subjected to poor sanitary conditions. Haptoglobin, an acute-phase protein is considered as a good bio-marker for the health status of pig and sanitary conditions of swine farms (Le Floc'h et al., 2009). In this study, pigs kept under UCL had a tendency to increase serum haptoglobin levels compared to CL, which could be due to stress induced by unclean conditions.

In commercial swine housing, NH_3 and H_2S are the most common gaseous emissions (Kim et al., 2008) and the maximum allowable level of NH_3 in the pig housing is 20 ppm (Donham, 2000). The concentration of NH_3 and H_2S beyond the threshold limit would result in

distress to breathing and diminish feed intake, leading to depressed growth performance in pigs (Ni et al., 2000). In this study, the unclean sanitary conditions increased aerial NH_3 and H_2S concentrations in the room. The deterioration of air quality could have caused discomfort, thus leading to the observed reduced growth performance of UCL pigs.

Under CL conditions, the greatest G:F was achieved at 19.0% and 24.0% dietary SID Trp:Lys. Based on ADG as response criteria, the estimated optimal SID Trp:Lys was 19.7%, which is in agreement with previous studies, estimating an optimal SID Trp:Lys in the range of 17.8% to 21% for weaned pigs using growth as the response criteria (Guzik et al., 2002; Guzik et al., 2005; Jansman et al., 2010).

Under UCL conditions, the overall ADG and feed efficiency improved with increasing dietary SID Trp:Lys, which suggests that Trp:Lys requirement might be higher when piglets are subjected to general immune challenge conditions. Using the BLQ model, the estimated optimal SID Trp:Lys for ADG and G:F was 20.5% (Fig. 5.2) and 19% (Fig. 5.3), respectively. The NRC (2012) recommends dietary SID Trp:Lys of 16% for pigs between 7 and 25 kg, which relates to healthy pigs under optimal growing conditions. Therefore, these levels might not be sufficient for piglets reared under commercial production conditions, whereby the chances of infections are high, and especially during the immediate post-weaning period. Factors such as dietary ingredient and composition, AA analysis, environmental conditions could account for the variation in Trp recommendations among different studies (Susenbeth, 2006).

Plasma urea nitrogen can be used as an indicator of protein utilization efficiency (Coma et al., 1995) as PUN is known to increase when there is an excess or deficiency of AA, because excess AA cannot be stored and therefore are degraded with the production of urea (Heo et al.,

2009; Waguespack et al., 2011). Under CL and UCL conditions, based on PUN as response criteria, the optimal SID Trp:Lys could not be estimated using regression analysis.

The growth and development of the intestine is critical for the optimal performance of weaned pigs (Heo et al., 2013). Higher VH has been considered as an indicator of an increased absorptive capacity of the small intestine and a healthy gut (Pluske et al., 1997; Nyachoti et al., 2006). In this study, pigs raised under CL had higher VH and deeper CD compared to those raised under UCL conditions, which could be the influence of sanitary conditions. Previous studies reported that weaned pigs subjected to *Escherichia coli* challenge had decreased VH (Rose et al. 1987; Yi et al. 2005). In this study, pigs under UCL conditions had reduced ileal villi height, which might have affected small intestinal digestion and absorption capacity for nutrients.

5.6. CONCLUSIONS

Weaned pigs raised under poor sanitation conditions had reduced growth performance compared to those pigs raised under clean conditions**.** Under clean sanitary conditions, the estimated optimal SID Trp:Lys based on ADG was 19.7%. Under poor sanitary conditions, the estimated optimal SID Trp: Lys for ADG and G:F were 20.5% and 19.0%, respectively, using the BLQ model.

CHAPTER SIX

MANUSCRIPT THREE

Pilot study to determine optimum culture conditions for enterotoxigenic *Escherichia coli* K88 based on fecal consistency score, performance, ileal histomorphology and immune responses in weaned piglets

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6.1. ABSTRACT

A pilot study was conducted to evaluate the efficacy of enterotoxigenic *Escherichia coli* (**ETEC)** K88 strain cultured either in Brain Heart infusion agar (**BHI**) or Luria-Bertani broth (**LB**) medium, and grown either under aerobic or anaerobic conditions, and inoculated orally without or with calcium carbonate solution in weaned piglets based on fecal consistency score, performance, ileal histomorphology and immune responses. Thirty two weaned piglets, with an average initial BW of 7.17 ± 0.05 kg were randomly assigned to 8 treatments in a 2 × 2 × 2 factorial arrangement for an 11-d study. The main factors were media ((LB) versus BHI); conditions (aerobic vs. anaerobic); orally inoculated in pigs with or without calcium carbonate **($CaCO_3$)**. All piglets were fed a common commercial swine starter diet throughout the experiment. On d 8, piglets were orally challenged with 6 ml of ETEC K88 (2×10^{11} colony forming units/mL (cfu/mL)). Fecal score was assessed during the pre-challenge and post-challenge period. There were no interactions ($P > 0.05$) on any of the treatment factors or significance in the performance parameters. During 24 h post-challenge, piglets inoculated with ETEC grown in BHI had significantly increased ($P < 0.05$) fecal consistency scores compared to those inoculated with ETEC grown in LB media. During post-challenge (6 h and 12 h), piglets inoculated with ETEC grown in BHI had higher ($P < 0.05$) rectal temperature than those inoculated with ETEC grown in LB agar. Serum concentrations of TNF-α and IL-6 increased ($P < 0.05$) in piglets challenged with ETEC K88. The growth performance of weaned piglets was not affected ($P > 0.10$) by different factors. Piglets inoculated with ETEC K88 cultured in BHI medium had lower villous height ($P < 0.05$) and deeper crypt depth ($P < 0.05$) than those inoculated with ETEC cultured in LB. The results from this pilot study indicated that ETEC K88

grown in BHI media under anaerobic conditions could be effective for oral challenge studies in weaned piglets.

Keywords: *Escherichia coli* K88, culture conditions, fecal score, weaned pigs

6.2. INTRODUCTION

In animal nutrition studies, disease challenge models have been used to determine the efficacy of nutrient or feed additives as replacements for antibiotics. Among the disease challenge models in weaned piglets, enterotoxigenic *Escherichia coli* **(ETEC)** K88 is one of the most commonly used (Bosi et al., 2004; Bhandari et al., 2008; Nyachoti et al., 2012; Khafipour et al., 2014; Trevisi et al., 2015). The most commonly used response criteria in ETEC K88 challenge model are growth performance, fecal consistency score, rectal temperature, plasma urea nitrogen, intestinal morphology, serum cytokine concentrations, enumeration of fecal and tissue bacteria, number of intestinal adherent bacteria and piglet mortality (Adewole et al., 2016). The success level of ETEC K88 challenge model depends on various factors such as age of pigs at the time of challenge, oral dosage and volume, buffer type, method of oral inoculation and adaptation period before challenge (Adewole et al., 2016).

An experiment was conducted to determine the optimal standardized ileal digestible (SID) threonine:lysine (Thr:Lys) ratio in piglets subjected to ETEC K88 challenge. Piglets (n=30) were orally challenged with 6 mL of *E. coli* K88 (2×10^9 cfu/mL). The inoculum was prepared as described by Khafipour et al. (2014). During the post-challenge period (d 8–14), pigs did not get sick which suggested that the *E. coli* challenge used in this experiment was not effective to activate immune system stimulation in weaned pigs (**Appendix 1**). Hence, there was a need to know the optimum culture medium and conditions for ETEC K88 in our lab.

In general, *E. coli* has a short generation time and a simple biology structure (Lenski et al., 1991). Although *E. coli* has been used in many studies, the choice of media and subculture intervals varied (Low et al., 2013). A previous study indicated that *E. coli*-12 cells cultured in Luria-Bertani broth **(LB)** had decreasing growth rate. The authors demonstrated that depletion of fermentable sugars resulted in decreased growth (Sezonov et al., 2007). Although Sezonov et al. (2007) had demonstrated that sugar depletion to be the main cause of reduced growth; this had not been demonstrated in other media. In addition to these findings, insufficient ETEC K88 challenge from our previous study (Appendix 1) prompted us to investigate the optimum culture conditions for *E. coli* K88 and its efficacy to induce an immune stimulation in weaned piglets. Therefore, the objective of this study was to assess the efficacy of *E. coli* K88 strain cultured in two different media (Luria Bertani broth and Brain heart infusion agar) under aerobic or anaerobic conditions.

6.3. Materials and Methods

6.3.1. Escherichia coli K88 in Brain Heart infusion medium or Luria Bertani broth culture conditions

The *E. coli* K88 strain was originally obtained from Veterinary Diagnostic Services of Manitoba, Winnipeg, Manitoba, Canada. From the frozen stock, *E. coli* K88 was streaked on brain heart infusion (BHI) agar or Luria Bertani (LB) broth and grown anaerobically at 37˚C overnight. Then a single colony was inoculated on two BHI plates (i.e. duplicate) or LB plates (duplicates) and incubated either aerobically or anaerobically at 37˚C overnight. Two tubes of 5 mL BHI broth (BD & Co., Franklin Lakes, New Jersey, USA) plus 2% casamino acids (Fisher Scientific, Waltham, MA, USA) or two tubes of 5 mL LB broth (BD & Co., Franklin Lakes,

New Jersey, USA) were inoculated from a single colony, respectively, and grown overnight at 37˚C with shaking (200 *rpm)*. The *E. coli* K88 identity was verified using an *E. coli* K88 fimbrex latex agglutination kit. Two flasks of 500 mL BHI broth plus 2% casamino acids or LB broth were inoculated with 2 ml *E. coli* K88+ from the 5 mL culture tube and then incubated anaerobically at 37˚C overnight with gentle shaking (200 *rpm*). The two 500 mL flasks were combined and thoroughly mixed. With serial dilution of the culture 10-fold in PBS, 10^6 to 10^9 dilutions were plated on BHI plates or LB plates to check that the culture was > 1×10^9. Incubation was done anaerobically at 37˚C overnight. The colonies on the dilution plates were counted the following day to determine concentration and 6 ml of 2×10^{11} cfu/mL per piglet was used for inoculation.

6.3.2. Animal experiment

The animal 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).

In this experiment, a total of 32 weaned piglets ((Duroc × [Yorkshire × Landrace], mixed sex) weighing 7.17 ± 0.05 kg (mean ± SEM) were obtained from Glenlea Research Station, MB. Piglets were randomly allocated to pens with 2 × 2 × 2 factorial arrangement (*E. coli* K88 grown in either BHI agar or LB medium × under either aerobic or anaerobic conditions, × piglets were inoculated *E. coli* K88 either without or with calcium carbonate (CaCO3) solution). All animals were fed a commercial starter diet ad libitum and had free access to water throughout the experiment. The experiment lasted for 11 d. Individual pig BW and pen feed disappearance were recorded during the pre-challenge and post-challenge periods to determine ADG, ADFI, and

G:F. On d 7, piglets were orally inoculated with 6 mL of *E. coli* K88 culture (2×10^{11} cfu/mL). Faecal consistency scoring (0=normal, 1=soft feces, 2=mild diarrhea, and 3=severe diarrhea) was performed by 2 trained individuals in a treatment-blinded manner as described by Marquardt et al. (1999). Rectal temperature was measured in piglets before (24 h) and after (6 h, 24 h, 48 h) after *E. coli* K88 challenge.

6.3.5 Ileal tissue collection and histomorphology

On d 13, all pigs were euthanized as described by Kiarie et al. (2007) and the abdominal cavity was opened from the sternum to pubis to expose the whole gastrointestinal tract. Ileum tissue collection and histomorphology procedure is as described in Chapter 5.

6.3.6 Serum cytokines

Serum concentrations of tumour necrosis factor (TNF-α) and interleukin-6 (IL-6) were determined using Quantikine ELISA kits (R & D Systems, Inc. Minneapolis, MN, USA).

6.3.7. Statistical analyses

Data were subjected to ANOVA using the General Linear Model procedure (SAS Inst. Cary. NC). The data were analyzed as a completely randomized design with a 2 × 2 × 2 factorial arrangement. The factors were culture media (LB or BHI), culture growth conditions (aerobic or anaerobic) and CaCO3 solution (without or with). For fecal consistency score, rectal temperature and serum TNF-α, a repeated measure analysis of variance was employed. Statistical significance was accepted at $P < 0.05$. Each pen was considered as an experimental unit.

6.4. RESULTS AND DISCUSSION

The purpose of this pilot study was to evaluate the efficacy of ETEC K88 strain cultured either in Brain Heart infusion agar (BHI) agar or Luria-Bertani broth (LB) medium, and grown either under aerobic or anaerobic conditions, and inoculated orally without or with CaCO3 solution in weaned piglets based on fecal consistency score, performance, ileal histomorphology and immune responses. In this study, the growth performance of weaned piglets was not affected ($P > 0.05$) by different factors (media, conditions, CaCO3) (**Table 6.1**).

Over the years, fecal consistency score has been used to quantify the severity of diarrhea in weaned piglets orally challenged with ETEC K88 (Owusu-Asiedu et al., 2002; Bhandari et al., 2010; Lee et al., 2012; Nyachoti et al., 2012; Khafipour et al., 2014). In this study, there were no interaction effects ($P > 0.10$) among the factors (media, condition and calcium carbonate) on fecal consistency scores at different time periods except during 12 h post-challenge (**Table 6.2**). During 6 h post-challenge, piglets inoculated with ETEC grown under anaerobic conditions tended ($P > 0.10$) to have higher fecal consistency score compared to those inoculated with ETEC grown under aerobic conditions. During 24 h post-challenge, piglets inoculated with ETEC grown in BHI media had greater ($P < 0.05$) fecal consistency scores compared to those inoculated with ETEC grown in LB media. Our results indicate that ETEC strain cultured in BHI agar was more efficient in increasing diarrhea score and elevating core body temperature (**Table 6.3**) in orally ETEC challenged piglets compared to those orally challenged with ETEC K88 grown in LB medium.Brain heart infusion medium might have favored the growth and virulence of enterotoxigenic *E. coli* K88.

For the maintenance of normal gut functions, the integrity of intestinal morphological structure is critical in weaned piglets (Pluske et al., 1997). Previous studies demonstrated that orally challenging piglets with ETEC K88 damage the intestinal morphology (Owusu-Asiedu,

Table 6.1. Effects of enterotoxigenic *Escherichia coli* K88 grown in two different media and culture conditions administered without and with calcium carbonate on growth performance of weaned piglets

Item			Initial BW	Final BW	d 0-7			D 8-11			Overall		
					ADG, g	ADFI, g	GF	ADG, g	ADFI, g	GF	ADG, g	ADFI, g	GF
Media	Oxygen	CaCO3											
LB			7.20	8.70	139	187	0.73	153	359	0.47	139	273	0.50
BHI			7.14	8.61	144	187	0.77	175	326	0.47	130	256	0.50
SEM			0.05	0.13	14.2	8.30	0.05	27.1	14.9	0.06	12.5	7.80	0.04
	Aerobic		7.18	8.72	144	195	0.77	176	354	0.49	130	274	0.51
	Anaerobic		7.16	8.59	139	179	0.73	151	330	0.44	140	268	0.50
	SEM		0.05	0.13	14.2	8.30	0.05	27.1	14.9	0.06	12.5	7.80	0.04
		With	7.17	8.64	136	180	0.75	154	323	0.47	129	278	0.51
		Without	7.17	8.68	147	193	0.75	173	362	0.46	141	251	0.50
		SEM	0.05	0.13	14.2	8.30	0.05	27.1	14.9	0.06	12.5	7.80	0.04
Source of variations													
Media			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Oxygen			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
CaCO3			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Media × Oxygen × CaCO3			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

BHI- Brain Heart infusion agar; CaCO3- calcium carbonate; LB- Luria Bertani broth; NS- non-significant; SEM-standard error of mean

Table 6.2. Effects of enterotoxigenic *Escherichia coli* K88 grown in two different media and culture conditions administered without and with calcium carbonate on fecal score of weaned piglets

Item			Pre-challenge	6 h post-challenge	12 h post-challenge	24 h post-challenge	48 h post-challenge
Media	Oxygen	CaCO3[1]					
BHI[2]			1.19	1.38	2.19	2.94	2.06
LB[3]			1.19	1.13	1.75	2.03	1.62
SEM[4]			0.14	0.14	0.19	0.17	0.24
	Aerobic		1.31	1.06	1.94	2.44	1.81
	Anaerobic		1.03	1.44	2.00	2.63	1.88
	SEM		0.14	0.14	0.19	0.17	0.24
		With	1.13	1.13	1.82	2.56	2.00
		Without	1.25	1.37	2.13	2.50	1.68
		SEM	0.14	0.14	0.19	0.17	0.24
Source of variations							
Media			NS[5]	NS	NS	**0.010**	NS
Oxygen			NS	0.094	NS	NS	NS
CaCO3			NS	NS	NS	NS	NS
Media × Oxygen × CaCO3			NS	NS	0.023	NS	NS

[1]CaCO3- calcium carbonate
[2]BHI- Brain Heart infusion agar.
[3]LB- Luria Bertani broth
[4]SEM-standard error of mean
[5]NS- non-significant

Table 6.3. Effects of enterotoxigenic *Escherichia coli* K88 grown in two different media and culture conditions administered without and with calcium carbonate on rectal temperature of weaned piglets

Item			Pre-challenge	6 h post-challenge	12 h post-challenge	24 h post-challenge	48 h post-challenge
Media	Oxygen	CaCO3[1]					
BHI[2]			39.6	40.0	39.8	39.4	38.9
LB[3]			39.6	39.4	39.3	39.3	38.9
SEM[4]			0.06	0.15	0.04	0.05	0.11
	Aerobic		39.6	39.8	39.4	39.3	39.0
	Anaerobic		39.6	39.7	39.5	39.4	38.8
	SEM		0.06	0.15	0.04	0.05	0.11
		With	39.6	39.8	39.5	39.4	39.0
		Without	39.5	39.7	39.4	39.3	38.9
		SEM	0.06	0.15	0.05	0.05	0.11
Source of variations							
Media			NS[5]	**0.037**	**0.024**	NS	NS
Oxygen			NS	NS	NS	NS	NS
CaCO3			NS	NS	NS	NS	NS
Media × Oxygen × CaCO3			NS	NS	0.093	NS	NS

[1]CaCO3- calcium carbonate
[2]BHI- Brain Heart infusion agar.
[3]LB- Luria Bertani broth
[4]SEM-standard error of mean
[5]NS- non-significant

Table 6.4. Effects of enterotoxigenic *Escherichia coli* K88 grown in two different media and culture conditions administered without and with calcium carbonate on ileal histomorphology of weaned piglets

Item			Villous height (VH) (µm)	Crypt depth (CD) (µm)	VH:CD
Media	Oxygen	CaCO3[1]			
BHI[2]			385	252	1.54
LB[3]			453	281	1.65
SEM[4]			13.4	13.2	0.09
	Aerobic		443	279	1.58
	Anaerobic		395	254	1.61
	SEM		13.4	13.2	0.09
		With	400	240	1.67
		Without	438	294	1.52
		SEM	13.4	13.2	0.09
Source of variations					
Media			**0.007**	**0.016**	NS[5]
Oxygen			0.037	NS	NS
CaCO3			0.080	0.021	NS
Media × Oxygen × CaCO3			NS	NS	NS

[1]CaCO3- calcium carbonate
[2]BHI- Brain Heart infusion agar.
[3]LB- Luria Bertani broth
[4]SEM-standard error of mean
[5]NS- non-significant

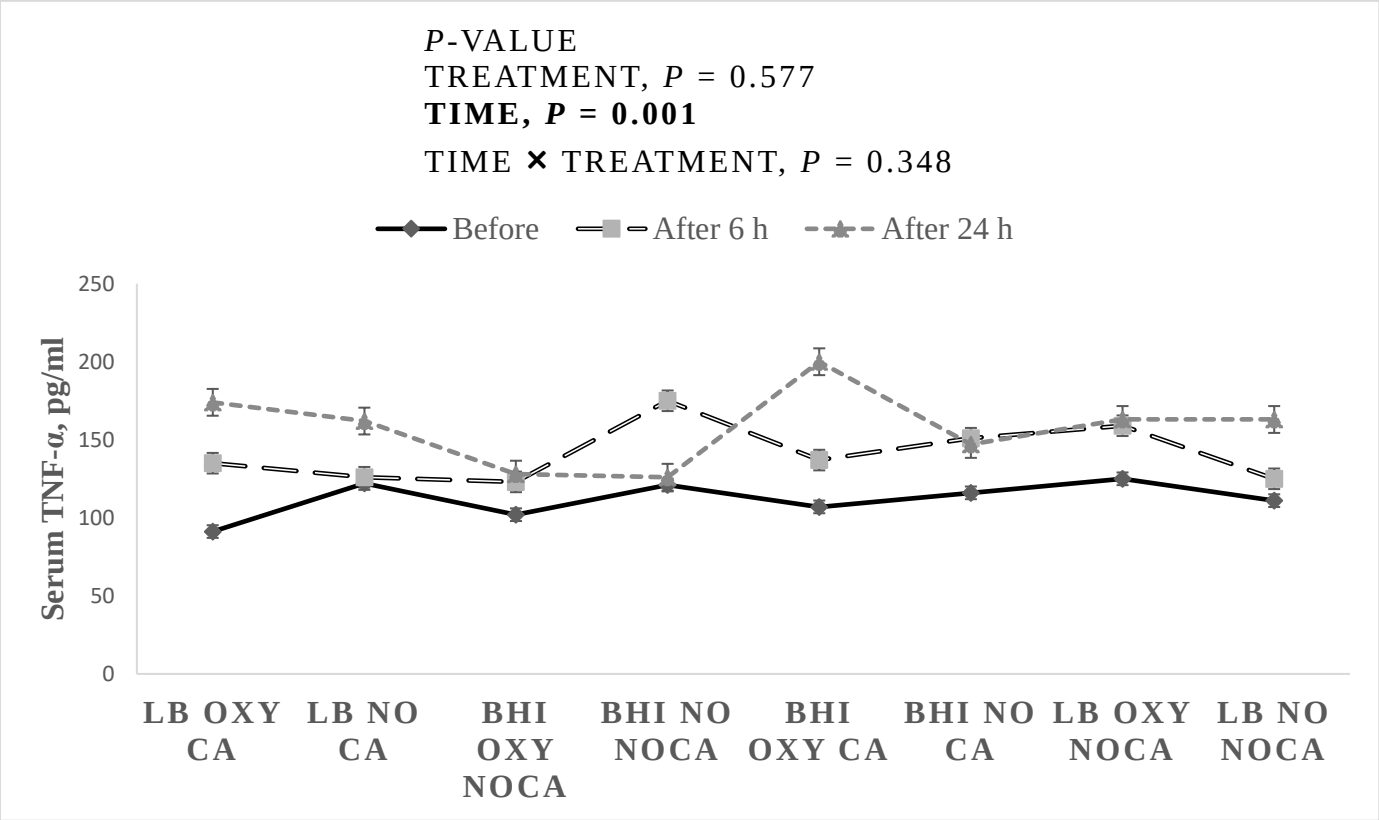

Figure 6.1. Effect of ETEC K88 grown in different media, conditions and calcium carbonate on serum tumor necrosis factor alpha concentration of weaned pigs.

BHI- Brain Heart infusion agar; LB- Luria Bertani broth; NoCa- no Calcium carbonate; NS- non-significant; SEM-standard error of mean. Values are means ± SEM, n=2. Pigs were challenged with 6 mL of ETEC by gavage on d 8 after weaning. Serum TNF-α is represented by 24 h before ETEC challenge (before), after 6 h and 24 h after ETEC challenge

2002; Bhandari et al., 2008; Opapeju et al., 2009). In the current study, piglets inoculated with ETEC cultured in BHI medium had lower VH ($P < 0.01$) than those inoculated with ETEC cultured in LB medium (**Table 6.4**). There were no interactions ($P > 0.10$) among culture media, conditions and $CaCO_3$ on any parameters measured.

The major pro-inflammatory cytokines such as TNF-α, IL-6 and interferon-γ play a critical role in response to infections (Adewole et al., 2016). In the present study, serum concentrations of TNF-α increased in piglets challenged with ETEC K88 (**Fig 6.1**), which is consistent with previous studies (Lee et al., 2012; Nyachoti et al., 2012; Kahindi et al., 2014).

6.5. CONCLUSIONS

Based on the fecal consistency score, core body temperature and gut morphology, ETEC K88 grown in BHI media under anaerobic conditions was more effective than that grown in LB media to induce challenge in weaned piglets. In conclusion, ETEC K88 strain grown in BHI medium under anaerobic conditions could be effective for oral challenge study in weaned pigs.

CHAPTER SEVEN

MANUSCRIPT FOUR

Effects of dietary standardized ileal digestible tryptophan:lysine ratio on performance, plasma urea nitrogen, ileal histomorphology and immune responses in weaned pigs challenged with *Escherichia coli* K88

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7.1. ABSTRACT

A study was conducted to determine the optimal standardized ileal digestible (SID) tryptophan:lysine (Trp: Lys) in piglets challenged with *Escherichia coli* K88 (*E.coli* K88) and fed antibiotic-free diets. Thirty individually housed mixed-sex pigs (Duroc × [Yorkshire × Landrace]) with an initial body weight (BW) of 6.4 ± 0.18 kg and weaned at 21 ± 1 day (d) were randomly assigned to 5 dietary treatments each with 6 pig replicates. Dietary treatments consisted of increasing levels of SID Trp: Lys ratios (16.1, 18.6, 20.3, 22.9, and 24.6%). Diets were corn-wheat-soybean meal-based with a constant SID Lys of 1.18% that was set to be the second limiting amino acid (AA) but adequate in other AA. Pigs were fed *ad libitum* and had free access to water for 13 d. After feeding the experimental diets for 6 d, pigs were orally challenged with 6 mL of *E. coli* K88 (2×10^{11} cfu/mL) on d 7. Body weights and pen feed disappearance were recorded weekly to determine ADG, ADFI and G:F. On d 13, all pigs were euthanized and ileal tissue samples were collected to quantify mRNA expression of gene encoding of tumor necrosis factor-α (TNF- α), interleukin-10 (IL-10) and interferon-γ (IFN-γ) using qRT-PCR. During the pre-challenge period (d 0 to 6), increasing dietary SID Trp:Lys increased (linear, $P < 0.05$) ADG (157, 162, 173, 179 and 201 g/d) and G:F (0.71, 0.73, 0.74, 0.81 and 0.84). During the post-challenge period, increasing SID Trp:Lys ratios tended (linear, $P = 0.076$) to increase ADG (177, 180, 208, 210 and 213 g/d), whereas there was no effect on G:F (0.58, 0.63, 0.67, 0.66, 0.65) ($P > 0.10$). The optimal SID Trp:Lys determined using the broken-line regression analysis for ADG and G:F in piglets subjected to *E.coli* K88 challenge was 21.7% and 20.1%, respectively. The expression of TNF-α mRNA tended to linearly decrease ($P = 0.10$) with increasing SID Trp:Lys ratio. The expression of IL-10 mRNA increased (linear and quadratic, $P < 0.01$) with increasing SID Trp:Lys ratio. In conclusion, in antibiotic-free starter

diets, an average optimal SID Trp:Lys of 21% optimized performance of piglets under an *E. coli* challenge. Under an immune challenge, the optimal SID Trp:Lys could be higher than reported values in NRC (2012).

Keywords: *Escherichia coli* K88; tryptophan; immunity; lysine; weaned piglets

7.2 INTRODUCTION

Tryptophan (Trp) is considered as the second or third limiting amino acid (AA) in cereal-based diets for pigs (Guzik et al., 2005). The importance of Trp is not only for protein synthesis, but also for the control of immune response and health maintenance (Le Floc'h and Sève, 2007). The role of Trp in the control of immune response is related to indoleamine 2, 3 dioxygenase (IDO) pathway, an enzyme involved in the catabolism of Trp into kynurenine, further leading to the production of xanthurenic acid and anthranilic acid (**Fig 2.4**). Under inflammation or immune challenge conditions, there is an increase in Trp catabolism to kynurenine (Melchior et al., 2004; Le Floc'h et al., 2008), consequently results in reduced plasma Trp concentrations (LeFloc'h and Se`ve, 2007). During immune challenge conditions, pro-inflammatory cytokines, especially interferon-γ (IFN- γ) induce IDO pathway which would limit Trp availability demonstrating the essential role of Trp in immune and inflammatory responses (Takikawa et al., 1986; Le Floc'h and Se`ve, 2007). This suggests that providing sufficient Trp is beneficial for pigs under immune challenge conditions.

Standardized ileal digestible (SID) Trp:Lys requirements for young piglets (7 and 25 kg BW) is 16% (NRC, 2012). The Trp:Lys requirement for piglets might vary when clinical or subclinical infections occur, specifically, when piglets are exposed to immunological challenge as is often the case under *Escherichia coli* K88 (*E. coli* K88) infection. Furthermore, *E. coli* K88

is one of the most common causes of diarrhoea in weaned piglets (Trevisi et al., 2009) characterized by watery faeces discharge during the post-weaning period and accompanied with the growth of *E. coli* K88 in the gut mucosa (Fairbrother et al., 2005).

Previous studies have shown that metabolism and requirement of Trp are modified in pigs during immune challenge conditions (Le Floc'h and Sève, 2007; Le Floc'h et al., 2009). Trevisi et al. (2009) reported that piglets fed diets with SID Trp:Lys ranging from 20 to 28% and challenged with *E. coli* K88 had increased feed intake and maintained growth in *E. coli* K88 susceptible pigs, thereby partially compensating for immune system stimulation caused by *E. coli* K88 infection. Information about the Trp:Lys requirement for piglets fed AGP-free diets and subjected to an immunological challenge is limited. We hypothesized that in weaned pigs subjected to immune challenge, dietary Trp:Lys requirement is higher than current recommendations (NRC, 2012). Thus, the aim of this experiment was to determine the optimal SID Trp:Lys ratio for weaned piglets subjected to an *E. coli* K88 challenge.

7.3. MATERIALS AND METHODS

The animal 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).

7.3.1. Animals and experimental design

Thirty piglets ((Duroc × [Yorkshire × Landrace], mixed sex) weighing 6.41 ± 0.18 kg (Mean ± SD) were obtained from Glenlea Research Station, MB at weaning (21 ± 1 d of age). Diets were corn, wheat and soybean meal (SBM)-based with a constant SID Lys of 1.18% that was set to be the second limiting AA (**Table 7.1**). Ingredients contributing AA (corn, wheat,

Table 7.1. The composition and calculated nutrient contents of experimental diets

Items	Standardized ileal digestible tryptophan:lysine, %				
Ingredient, g/kg	16.1	18.6	20.3	22.9	24.6
Corn	434.00	434.00	434.00	434.00	434.00
Wheat	210.00	210.00	210.00	210.00	210.00
Soybean meal	274.00	274.00	274.00	274.00	274.00
Vegetable oil	29.20	29.20	29.20	29.20	29.20
Corn starch	5.00	4.80	4.60	4.30	4.00
Limestone	10.90	10.90	10.90	10.90	10.90
Dicalcium phosphate	15.60	15.60	15.60	15.60	15.60
Iodized salt	3.00	3.00	3.00	3.00	3.00
Vitamin-mineral premix[a]	10.00	10.00	10.00	10.00	10.00
L-Lysine HCl	4.40	4.40	4.40	4.40	4.40
DL-Methionine	1.70	1.70	1.70	1.70	1.70
L-Threonine	1.90	1.90	1.90	1.90	1.90
L-Tryptophan	0.00	0.24	0.48	0.72	0.96
L-Valine	0.60	0.60	0.60	0.60	0.60
Calculated net energy and nutrient content					
NE (MJ kg^{-1})	14.00	14.00	14.00	14.00	14.00
CP	200	200	200	200	200
Ether extract	29.3	29.3	29.3	29.3	29.3
NDF	94.4	94.4	94.4	94.4	94.4
ADF	34.7	34.7	34.7	34.7	34.7
Total calcium	9.5	9.5	9.5	9.5	9.5
Total phosphorus	7.1	7.1	7.1	7.1	7.1
Available phosphorus	3.8	3.8	3.8	3.8	3.8
SID[b] Lys	11.8	11.8	11.8	11.8	11.8
SID Met	4.3	4.3	4.3	4.3	4.3
SID Met + Cys	7.1	7.1	7.1	7.1	7.1
SID Thr	7.6	7.6	7.6	7.6	7.6
SID Trp	1.9	2.2	2.4	2.7	2.9
SID Ile	6.6	6.6	6.6	6.6	6.6
SID Leu	13.7	13.7	13.7	13.7	13.7

SID Val	8.0	8.0	8.0	8.0	8.0
SID His	4.4	4.4	4.4	4.4	4.4
SID Arg	10.4	10.4	10.4	10.4	10.4
SID Phe	7.9	7.9	7.9	7.9	7.9
Analyzed amino acid composition (g kg^{-1})					
CP	197.0	196.0	192.0	191.0	190.0
Lys	13.8	13.4	13.2	13.0	13.3
Trp	2.6	2.7	2.8	3.0	3.3
Met	4.5	4.8	4.9	4.9	4.8
Met + Cys	8.1	8.2	8.1	8.1	8.0
Thr	8.9	8.3	8.1	8.8	8.5
His	4.8	4.4	4.5	4.8	4.5
Ile	7.8	7.2	7.4	7.6	7.2
Leu	15.1	14.2	14.5	14.9	14.2
Phe	9.3	8.5	8.8	8.9	8.6
Val	9.5	9.1	9.1	9.2	9.1

[a]Supplied the following per kilogram of diet:8250 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 µg 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

[b]SID = standardized ileal digestible.

SBM) were analyzed for AA contents and these values in combination with the respective SID of ingredients were used in diet formulation. The diets contained increasing levels of SID Trp:Lys (16.1, 18.6, 20.3, 22.9 and 24.6%) (Table 7.1). All other nutrients were provided in quantities meeting or exceeding the NRC (2012) requirements for 6 to 10 kg pigs. Pigs were fed *ad libitum* and had free access to water. The experiment lasted for 13 d. Individual pig BW and pen feed disappearance were recorded during the pre-challenge and post-challenge periods to determine ADG, ADFI and G:F. On d 7, all pigs were orally inoculated with 6 mL of *E. coli* K88 blinded manner as described by Marquardt et al. (1999). Rectal temperature was measured in piglets before (24 h) and after (6 h, 24 h, 48 h) *E. coli* K88 challenge. Faecal consistency scoring (0 = normal, 1 = soft faeces, 2 = mild diarrhoea, and 3 = severe diarrhoea) was performed by 2 trained individuals in a treatment.

7.3.2 Escherichia coli K88 and culture condition

The procedure for *E. coli* K88 culture has been described in **Chapter 6.**

7.3.3. Chemical analysis of feed ingredients and diets

Prior to lab analyses, ingredient and diet samples were ground through a 1-mm mesh screen. Dry matter content was determined according to AOAC (2000) whereas dietary N content was determined with a gas combustion method using a Leco FP-2000 Nitrogen Analyzer (Leco Corp., St. Joseph, MI). Amino acid analyses for the feed ingredients and diets were carried by Evonik Nutrition & Care GmbH, (Hanau-Wolfgang, Germany) using the method described by Llames and Fontaine (1994).

7.3.4 Plasma urea nitrogen and serum tumour necrosis factor alpha analysis

Blood collection (for serum and plasma) and plasma urea nitrogen (PUN) analysis were performed as described in **Chapter 5**. Serum concentrations of tumour necrosis factor (TNF-α) was determined using Quantikine ELISA kits (R & D Systems, Inc. Minneapolis, MN, USA).

7.3.5 Ileal tissue collection and histomorphology

On d 13, all pigs were euthanized as described by Kiarie et al. (2009) and the abdominal cavity was opened from the sternum to pubis to expose the whole gastrointestinal tract. Ileal tissue collection and histomorphology procedure are described in **Chapter 5.**

7.3.6. Extraction of total RNA from ileal mucosa and reverse transcription

Distal ileum sections (5 cm) from all piglets were flushed with 1 × Phosphate buffer saline (1xPBS) and snap-frozen in liquid nitrogen and stored at -80˚C, until RNA isolation. Total RNA was extracted from ileal mucosa using Trizol extraction method (Ivitrogen Canada Inc., Burlington, ON, Canada). RNA concentration and purity was determined using Nanodrop spectroscopy (Thermo Scientific, Boston, MA, USA). cDNA was manufactured using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA),.

7.3.7 Quantitative real time PCR

Primer sequences for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), tumor necrosis factor (TNF-α), interleukin-10 (IL-10) and interferon-gamma (IFN-γ) were designed using Gene bank database sequences from National Center for Biotechnology Information (Bethesda, MD, USA). Quantitative real-time PCR was performed in duplicate reactions

including nuclease free water, the forward and reverse primers of each gene, template cDNA and SYBR Green using a CFX Connect ™ Real-Time PCR Detection System (Life Science Research, Bio-Rad, Canada). Data were generated using the ΔΔCt method by normalizing the expression of the target genes to a housekeeping gene (GAPDH), and the values were reported as fold changes of the expression of the target genes in SID Trp:Lys ratios 18.6, 20.3, 22.9, 24.6 compared with the 16.1% SID Trp:Lys group. Means were declared significant at $P < 0.05$. Pairs of primers used for Qualitative real-time PCR (qRT-PCR) assay and their sequences are presented in **Table 7.2.**

7.3.8. Statistical analyses

Growth performance, ileal histomorphology and cytokine gene expression data were subjected to ANOVA using the General Linear Model procedure (SAS Inst. Cary. NC). The data were analyzed as a completely randomized design. Each experimental unit was a pen of 1 piglet and there were 6 experimental units per treatment and thus 30 experimental units in total. Orthogonal polynomial contrasts were used to determine linear and quadratic effects of increasing SID Trp:Lys ratio. For fecal consistency score, rectal temperature and serum TNF-α, a repeated measure analysis of variance was employed. All means are reported as least square means and statistical significance was accepted at $P < 0.05$ and $0.05 < P < 0.10$ was considered a trend. The model for the response criteria is $Y_{ij} = \mu + Trp_i + E_{ij}$, where,

Y_{ij} is the $_j$th observation of the $_i$th treatment,

μ is the population mean,

Trp_i is the treatment effect of the ith treatment, and

E_{ij} is the random error.

Table 7.2. List of genes and sequences of the primers used for real-time PCR

mRNA target	Primers → (5' 3')[2]	Product size (base pairs)	Annealing temperature
GADPH	GGTGAAGGTCGGAGTGAACG (forward) GGGATCTCGCTCCTGGAAGA (reverse)	238	58˚C
TNF-α	CACTGACCACCACCAAGAATTGGA(forward) CATTCCAGATGTCCCAGGTTGCAT (reverse)	94	60˚C
IL-10	GATATCAAGGAGCACGTGAACTC (forward) GAGCTTGCTAAAGGCACTCTTC (reverse)	137	60˚C
IFN-γ	GTTTTTCTGGCTCTTAACTGC(forward) CTTCCGCTTTCTTAGGTTAG (reverse)	125	58˚C

GAPDH: glyceraldehyde 3-phosphate dehydrogenase, TNF-α: tumor necrosis factor, IL-10: interleukin-10, IFN-γ: interferon-gamma

To determine the optimal SID Trp:Lys ratio, growth performance data were subjected to a broken-line analysis (Robbins et al., 2006) using the Proc NLIN of SAS (SAS Inst. Inc. Cary, NC). The estimated SID Trp:Lys in this study was compared with the current recommendations (NRC, 2012) using pooled t-test.

7.4. RESULTS

The analyzed AA and crude protein contents of the experimental diets are close to calculated values (**Table 7.1**), and thus, the calculated SID Trp:Lys ratios were used for the regression analysis.

7.4.1. Faecal score and rectal temperature

Faecal consistency (watery faeces) increased during the post-challenge (6 h, 24 h and 48 h) and then declined at 72 h period (**Fig 7.1**). Rectal temperature was not affected ($P > 0.10$) by *E. coli* K88 challenge (**Fig 7.2**). No mortalities were observed during the experiment.

7.4.2 Growth performance and plasma urea nitrogen

The growth performance data is presented in **Table 7.3**. During the pre-challenge period, pigs fed the diet containing SID Trp:Lys ratio of 24.6% had a higher ($P < 0.05$) ADG compared to those fed other diets, whereas feed intake was not affected by different dietary SID Trp:Lys. Increasing dietary SID Trp:Lys increased (linear, $P < 0.01$) G:F. During post-challenge period, ADG tended to increase ($P = 0.07$), whereas ADFI increased (linear; $P < 0.05$) with increasing dietary SID Trp:Lys. Based on ADG (**Fig. 7.3**) and G:F (**Fig.7.4**) and using the linear broken-line model, the optimal SID Trp:Lys were determined to be 21.7% ($P < 0.05$; $R^2 = 0.87$) and 20.1% ($P < 0.05$; $R^2 = 0.96$), respectively. On d 7, PUN (**Table 7.3**) was affected (quadratic; $P < 0.05$) by different dietary treatments, with the highest and lowest PUN concentrations observed

Table 7.3. Effect of increasing levels of dietary standardized ileal digestible tryptophan:lysine ratio on the performance and plasma urea nitrogen of weaned piglets during the pre- and post-challenge periods

Items	Standardized ileal digestible tryptophan:lysine, %						*P*-value*2*	
	16.1	18.6	20.3	22.9	24.6	SEM[1]	TrpL[3]	TrpQ[4]
Pre-challenge (d 0 to 7)								
ADG (g/d)	157	162	173	179	201	12.2	0.016	0.525
ADFI (g/d)	221	223	231	221	239	11.2	0.328	0.751
G:F	0.71	0.73	0.74	0.81	0.84	0.03	**0.004**	0.479
Post-challenge (d8 to 13)								
ADG (g/d)	177	180	208	210	213	17.9	0.076	0.665
ADFI (g/d)	302	287	308	341	329	11.6	**0.006**	0.702
G:F	0.58	0.63	0.67	0.66	0.65	0.04	0.283	0.271
Whole experimental period (d 0 to 13)								
ADG (g/d)	170	173	192	195	205	10.8	**0.012**	0.921
ADFI (g/d)	263	255	271	281	282	7.04	**0.009**	0.641
G:F	0.64	0.68	0.71	0.72	0.73	0.03	**0.043**	0.440
Initial BW (kg)	6.37	6.34	6.47	6.50	6.37	0.18	-	-
Final BW (kg)	8.36	8.54	8.68	8.82	8.68	0.28	0.276	0.516
Plasma urea nitrogen (mmol/L)								
Day 7	2.64	3.08	3.42	3.23	2.47	0.29	0.822	**0.012**
Day 13	4.33	3.27	3.09	3.38	2.95	0.27	**0.004**	0.138
Pooled value	3.44	3.20	3.33	3.52	2.63	0.23	0.084	0.163

[1]Standard error of the mean. Means are presented as least square means (n = 6).

[2]considered significant when $P < 0.05$ and considered tendency when $0.05 < P \leq 0.10$.

[3]TrpL - Linear contrast for dietary Trp:Lys effect.

[4]TrpQ - Quadratic contrast for dietary Trp:Lys effect.

Table 7.4. Effects of increasing levels of dietary standardized ileal digestible tryptophan:lysine ratio on ileal histomorphology of weaned piglets subjected to *E.coli* K88[1]

Items	Dietary SID Trp:Lys (%)						*P* value[3]	
	16.1	18.6	20.3	22.9	24.6	SEM[2]	TrpL[4]	TrpQ[5]
Villous height (VH), µm	464	476	437	476	453	33.2	0.827	0.949
Crypt depth (CD), µm	301	298	254	290	290	21.8	0.664	0.289
VH:CD[c]	1.58	1.62	1.74	1.64	1.56	0.09	0.945	0.215

[1]Ileal tissue samples collected 7 d after challenge. n = 6/dietary treatment.

[2]Standard error of the mean. Means are presented as least square means.

[3]considered significant when $P < 0.05$ and considered tendency when $0.05 < P \leq 0.10$.

[4]TrpL - Linear contrast for dietary Trp:Lys effect.

[5]TrpQ - Quadratic contrast for dietary Trp:Lys effect.

Table 7.5. Effects of increasing levels of dietary standardized ileal digestible tryptophan:lysine ratio on on tumour necrosis factor alpha (TNF-α), interleukin-10 (IL-10) and interferon gamma (IFN-G) gene expression in ileal mucosa[a] of weaned pigs challenged with *E. coli* K88.

Items	Dietary SID Trp:Lys (%)						*P* –value[3]	
	16.1	18.6	20.3	22.9	24.6	SEM[2]	TrpL[4]	TrpQ[5]
TNF- α	0.71	0.81	0.70	0.62	0.54	0.12	0.102	0.325
IL-10	0.84	0.80	1.54	2.10	0.86	0.16	**0.011**	**<0.001**
IFN-G	0.73	0.66	0.66	0.75	0.86	0.17	0.480	0.451

[1]Ileal tissue samples collected 6 days after challenge. n = 6/dietary treatment.

[2]Standard error of the mean.

[3]considered significant when $P < 0.05$ and considered tendency when $0.05 < P \leq 0.10$.

[4]TrpL - Linear contrast for dietary Trp:Lys effect.

[5]TrpQ - Quadratic contrast for dietary Trp:Lys effect.

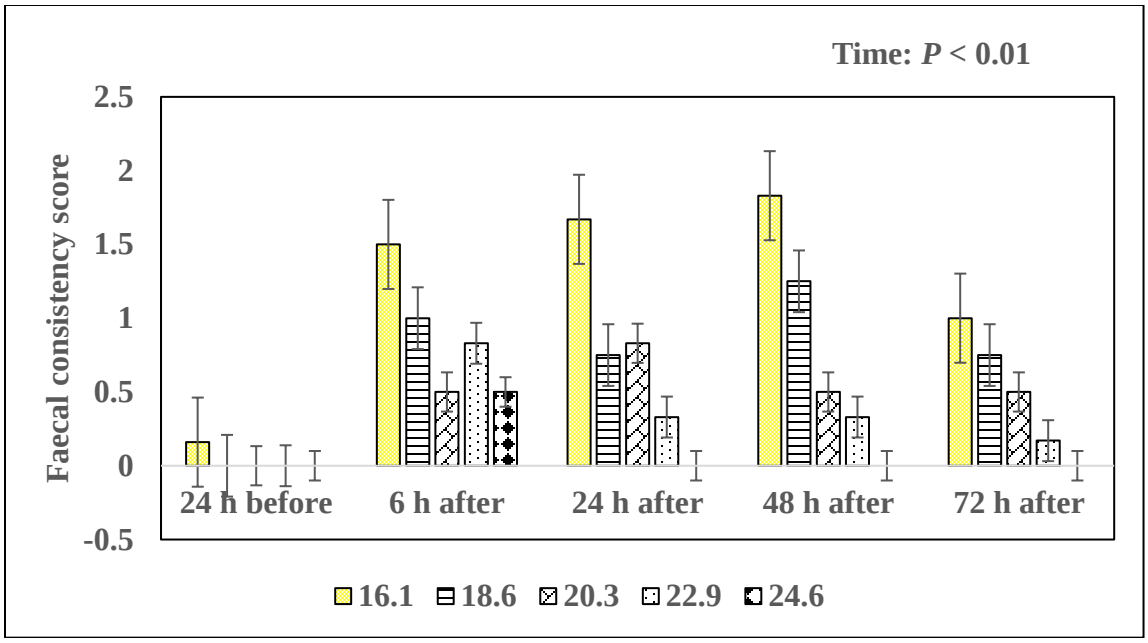

Fig 7.1. Effect of different time period on fecal consistency scores of weaned piglets challenged with *E. coli* K88.
SID Trp:Lys ratio – standardized ileal digestible tryptophan:lysine ratio, Time indicates before (24 h), and after (6 h and 24 h) *E. coli* K88 challenge

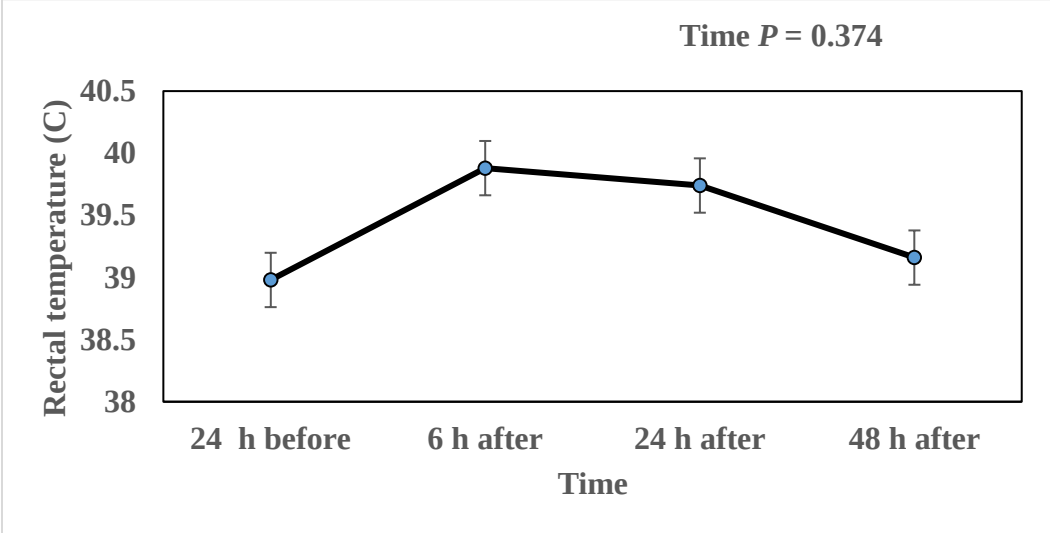

Fig 7.2. Average rectal temperature (˚C) of piglets challenged with *E. coli* K88.
Time indicates before (24 h), and after (6 h, 24 h and 48 h) *Escherichia coli* K88 challenge in pigs.

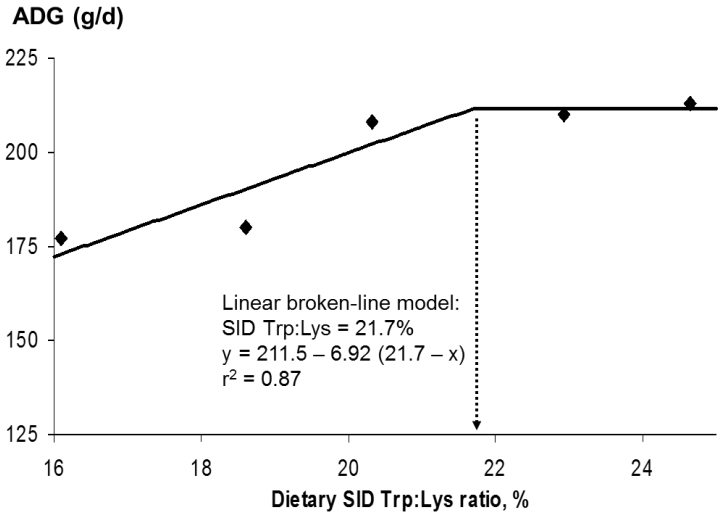

Fig. 7.3. The optimal dietary standardized ileal digestible tryptophan: lysine (SID Trp:Lys) for average daily gain (ADG) in weaned pigs subjected to *E. coli* K88 challenge determined using a linear broken-line regression. (SE = 0.41; R^2 = 0.87)

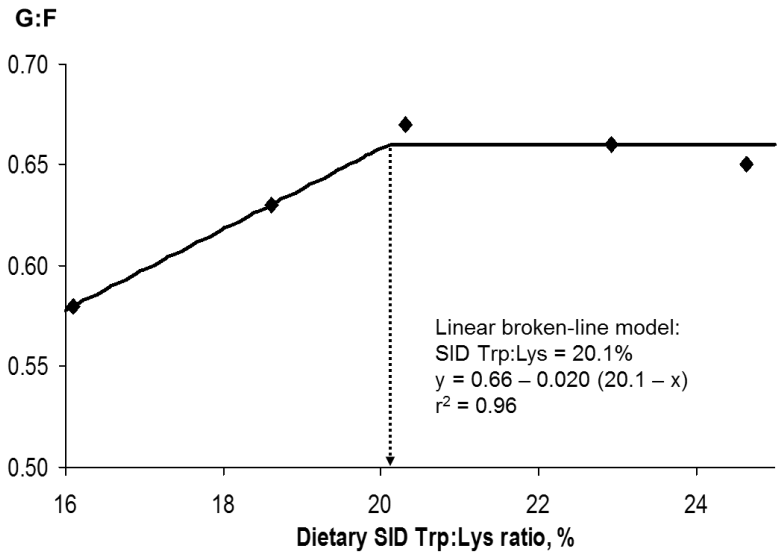

Fig.7.4. The optimal dietary standardized ileal digestible tryptophan: lysine (SID Trp:Lys) for gain:feed (G:F) in weaned pigs subjected to *E. coli* K88 challenge determined using a linear broken-line regression. (SE = 0.39; R^2 = 0.96)

for the diets with 20.3% and 24.6% SID Trp:Lys, respectively. On d 13, PUN declined (linear, $P = 0.01$) with increasing dietary SID Trp:Lys. The pooled PUN tended to decrease (linear; $P = 0.084$) with increasing dietary SID Trp:Lys.

7.4.3. Serum TNF-α and ileal mRNA gene expression of cytokines and ileal histomorphology

Serum TNF-α determined at 6 hr post-challenge was higher ($P < 0.05$) than at 24 h before challenge (Fig. 7.1). Dietary treatment did not affect ($P > 0.10$) serum TNF-α (Fig 7.5). There was no difference in mRNA expression of gene encoding of TNF-α or IFN-γ among the dietary treatments. Dietary treatments increased the expression of gene encoding of IL-10. Dietary treatment did not affect ileal histomorphology (**Table 7.4**).

7.5. DISCUSSION

This study was carried out to determine the optimal SID Trp:Lys for weaned pigs subjected to *E. coli* K88 challenge using performance and PUN as response criteria. Diets were corn-wheat-soybean meal-based with a constant SID Lys of 1.18% that was set to be 10% lower than the SID Lys requirement for this BW range of pig from the same herd previously determined by Kahindi et al. (2016). To induce the immune system in weaned pigs, the ETEC challenge model (Owusu-Asiedu et al., 2002) was used. In the present study, oral inoculation with *E. coli* K88 was sufficient in inducing an immune system stimulation in weaned pigs, as evidenced by increased faecal consistency scores and serum TNF-α levels.

During the pre-challenge period, the ADG and G:F were improved due to increasing dietary levels of SID Trp:Lys, which agrees with the results of Trevisi et al. (2010). In a recent meta-analysis, Simongiovanni et al. (2012) indicated that there was an 8% improvement in ADG with increasing SID Trp:Lys from 17 to 22%.

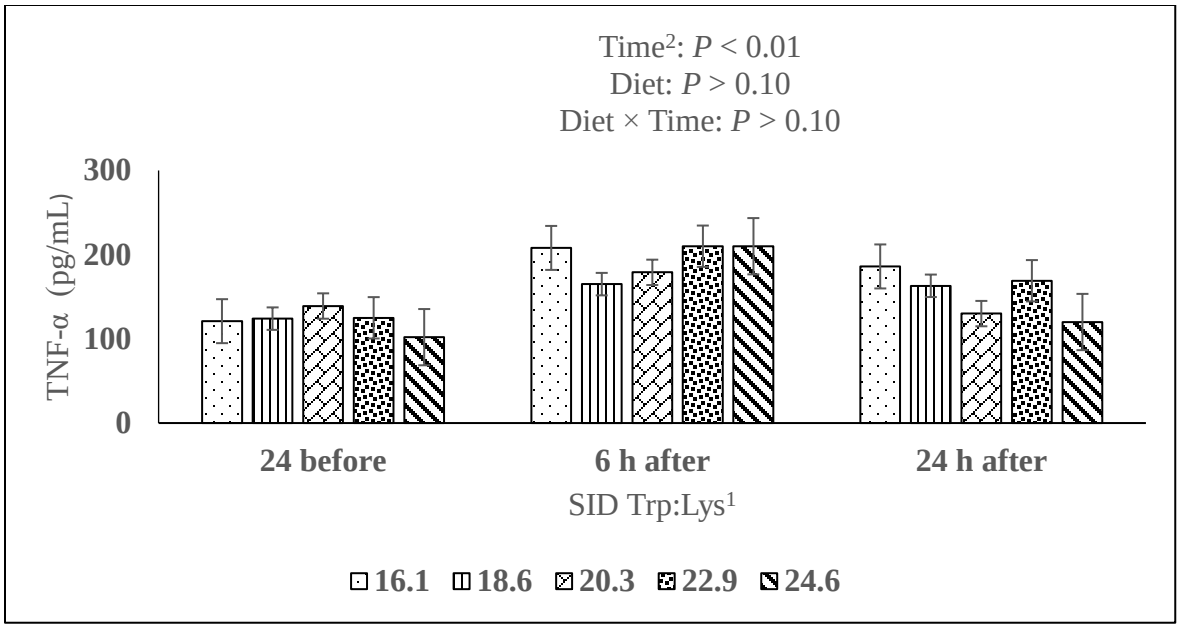

Fig.7.5. Effect of different time and SID Trp:Lys on serum concentrations of TNF-α in weaned piglets challenged with *E. coli* K88.

[1]SID Trp:Lys- standardized ileal digestible tryptophan:lysine ratio

[2]Time indicates before (24 h), and after (6 h and 24 h) *E. coli* K88 challenge in pigs.

During the post-challenge period, increasing SID Trp:Lys increased feed intake, which might have caused the tendency towards increased ADG. During the post-challenge period, the estimated optimal SID Trp:Lys based on ADG and G:F were 21.7 and 20.1% respectively, which are in agreement with the results of Capozzalo et al. (2015) showing that a dietary SID Trp:Lys of 24% improved feed conversion ratio for weaned pigs challenged with *E. coli* K88. Similarly, Trevisi et al. (2010) reported that a SID Trp:Lys of 22% was needed to maintain post-weaning BW in pigs susceptible to *E. coli* K88. During an infection, Trp could be utilized for synthesis of acute-phase proteins (Le Floc'h and Sève, 2007) and an increased Trp catabolism occurs in kynurenine pathway (de Ridder et al., 2012), further might reduce the availability of Trp for growth. Piglets subjected to *E. coli* K88 challenge needed higher levels of SID Trp:Lys to maintain feed intake and growth rate, which could be due to the fact that Trp is the major AA controlling feed intake (Sève et al., 1991; Le Floc'h and Sève, 2007). Moreover, during an immune challenge (natural or induced), there is an increased catabolism of Trp (Le Floc'h et al., 2009) through the activation of indoleamine 2, 3 dioxygenase (IDO). Thus, during infection, Trp need might be increased for optimal growth performance and health status.

Plasma urea nitrogen is used as an indicator of protein utilization efficiency because it is known to increase when there is an imbalance of dietary AA (Coma et al., 1996). In this study, increasing dietary SID Trp:Lys linearly decreased PUN, possibly attributed to an increase in nitrogen use efficiency or a decrease in protein breakdown (Shen et al., 2012). This observation is in agreement with previous work (de Ridder et al., 2012), in which increasing dietary Trp reduced PUN in piglets subjected to *E. coli* lipopolysacharide challenge, indicating that efficiency of Trp utilization was improved with increasing dietary Trp content. In the current study, the highest ADG was observed at dietary SID Trp:Lys of 24.6%, which coincided with the

lowest PUN concentration in the pigs fed the diet with a SID Trp:Lys of 24.6%, suggesting improved AA utilization efficiency.

Tumour necrosis factor-alpha (TNF-α) is an important pro-inflammatory cytokine, which regulates the host immune response to pathogenic infection. During inflammation, TNF-α is produced by macrophages, which in turn stimulates the hypothalamus causing fever and stimulate the liver to produce acute phase proteins (van Reeth and Nauwynck, 2000). Serum concentrations of TNF-α have been used as an indicator of the degree of inflammation in the circulation of pigs (Liu et al., 2010). In this study, serum TNF-α concentration determined at 6 h post-*E. coli* K88 challenge was higher than pre-challenge (24 h before) which indicates the acute stimulation of immune system.

Attachment to and colonization of the ileal mucosa by *E. coli* K88 are associated with the stimulation of the host's innate immune system. Cytokines are the main mediators in the regulation of the immune and inflammatory responses, which are expressed by the intestinal epithelium and might play a role in the basal influx of immune cells into the mucosa, in epithelial cell growth, and in homeostasis (Pie et al., 2004). Some cytokines, such as TNF-α, IL-8 and Il-1β are significantly expressed in response to pathogenic infection (Pie et al., 2004). In the present study, a lower expression of TNF-α was observed in ileal mucosa during 6 d post-challenge. This could possibly explain that piglets can rapidly overcome the *E. coli* K88 challenge, in terms of expression of TNF-α, as excessive inflammatory response could result in tissue damage. Increased expression of TNF-α may lead to the development of diarrhea (Pie et al., 2004) as it could be involved in the stimulus of chloride ion secretion in the ileum (Kandli et al., 1994). In the current study, the incidence of diarrhea was reduced in piglets at 72 h post-challenge which is related with the reduced expression of TNF-α mRNA. On the other hand, a significant

upregulation of IL-10 mRNA expression was observed, whereas mRNA expression of IFN-γ was not affected by dietary treatments. Interleukin 10 is an important anti-inflammatory cytokine, which is capable of inhibiting host immune responses (Abbas et al., 2000). In the present study, the mRNA expression of IL-10 might have inhibited the production of TNF-α. This is in agreement with Van Reeth and Nauwynck (2000) who reported that pigs subjected to porcine reproductive and respiratory syndrome virus challenge, had lower TNF-α concentration, which could be due to inhibition by anti-inflammatory cytokines.

Intestinal morphology can be used as an indicator of stress in pigs (Pluske et al., 1997). During stressful conditions, such as colibacillosis, weaned pigs have shortened villi and deeper crypts (Pluske et al., 1997; Owusu-Asiedu et al., 2002). Shortened villi is expected to cause impairment of the digestive capability of the small intestine, consequently prompting malabsorption and diarrhea in weaned pigs (Tsukahara et al., 2016). In the present study, ileal histomorphology was not affected by different dietary SID Trp:Lys which could be due to an increased availability of Trp in pigs challenged *with E. coli* K88, that might have had a compensatory effect in increasing villi height (Bosi et al., 2004; Trevisi et al., 2009). Pro-inflammatory cytokine TNF-α may participate in maintenance of tissue integrity (Williams, 2001) through involvement in cell renewal and the morphological changes observed in the intestinal epithelium in piglets (Pie et al., 2004). In this study, downregulation of TNF-α at 6 d post-challenge implies that it may have been involved in the maintenance of ileal tissue integrity.

7.4. Conclusions

Based on ADG and G:F, the estimated optimal SID Trp:Lys for weaned pigs subjected to *E. coli* K88 challenge was 21.7% and 20.1%, respectively. There was upregulation of the

expression of IL-10 in ileal mucosa of piglets and was highest at 22% SID Trp:Lys. Under an immune challenge (*E. coli* K88), dietary Trp:Lys requirements for weaned piglets could be higher than reported values in NRC (2012).

CHAPTER EIGHT

MANUSCRIPT FIVE

Effects of dietary standardized ileal digestible threonine:lysine ratio on performance, ileal histomorphology, mucin gene expression and immune responses in weaned pigs challenged with *Escherichia coli* K88 and fed antibiotic-free diets[1]

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8.1. ABSTRACT

A study was conducted to determine the optimal standardized ileal digestible (SID) threonine: lysine ratio (Thr:Lys) in piglets fed antibiotic-free diets and not challenged without (Exp 1) or challenged with *Escherichia coli* K88 (*E. coli* K88; Exp 2). In Exp 1, thirty individually housed mixed-sex pigs (Duroc × [Yorkshire × Landrace]) with an initial BW of 6.47 ± 0.13 kg (mean ± SE) and weaned at 21 ± 1 d were randomly assigned to 5 dietary treatments each with 6 replicates. In Exp 2, thirty individually housed mixed-sex pigs (Duroc × [Yorkshire × Landrace]) with an initial BW of 6.64 ± 0.19 kg and weaned at 21 ± 1 d were randomly assigned to 5 dietary treatments each with 6 replicates. In Exp 1 and 2, dietary treatments consisted of 4 graded levels of SID Thr:Lys (53, 59, 65, and 71%) and a control diet containing 59% SID Thr:Lys supplemented with an antimicrobial growth promoter (Aureomycin 220 G). Diets were corn-wheat-soybean meal-based with a constant SID Lys of 1.18% that was set to be the second limiting amino acid (AA) but adequate in other AA. Pigs had *ad libitum* access to feed and water for 14 d. Body weights and pen feed disappearance were recorded weekly to determine ADG, ADFI and G:F. In Exp 2, all piglets were orally challenged with 6 mL of *E. coli* K88 (10^{11} cfu/mL) on d 8. All pigs in both experiments were euthanized on d 14 to obtain ileal tissue samples to measure histomorphology parameters. In Exp 2, ileal mucin (MUC 1, 4 and 20) mRNA expression and ileal cytokine (tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)) mRNA expression were measured using quantitative real-time PCR (qRT-PCR). In Exp 1, the optimal SID Thr:Lys based on ADG as response criteria was 59%. In Exp 2, dietary SID Thr:Lys did not affect ($P > 0.10$) growth performance. Increasing dietary SID Thr:Lys linearly decreased ($P < 0.05$) PUN on d 14. Increasing dietary SID Thr:Lys tended to increase (quadratic; $P = 0.073$) VH:CD. A SID Thr:Lys of 65.6% optimized ileal VH:CD. In Exp 2, increasing

Thr:Lys tended to increase (linear; $P < 0.05$) the mRNA expression of MUC 20, whereas dietary SIDThr:Lys ratio did not affect ($P > 0.10$) mRNA expression of MUC 1 and MUC 4. Increasing dietary SID Thr:Lys tended to increase ($P < 0.05$) the mRNA expression of IL-1 β, but did not affect ($P > 0.10$) mRNA expression of TNF-α. In conclusion, SID Thr:Lys of 59% optimized feed efficiency in weaned piglets fed AGP-free diets. The optimal SID Thr:Lys based on ileal histomorphology in weaned pigs challenged with *E. coli* K88 was 65.6%. Under an immune challenge, the optimal SID Thr:Lys could be higher than reported values in NRC (2012) for weaned piglets.

Keywords: *E. coli* K88, piglets, standardized ileal digestible, threonine:lysine

8.2. INTRODUCTION

In swine nutrition, threonine (**Thr**) is considered as the second or third limiting amino acid (AA) in cereal-based diets (Mao et al., 2011). The importance of Thr is not only for protein synthesis, but also for the maintenance of intestinal barrier function, and control of immune response in pigs (Li et al., 1999; Wang et al., 2006; Hamard et al., 2007; Mao et al., 2014). Threonine is one of the most important AA for mucin synthesis in the intestinal mucosa, and aid in the formation of mucus gel, supporting lubrication and protection from pathogenic bacterial attachment to the mucosal surface (Trevisi et al., 2015).

In young pigs (10 and 25 kg), the requirements for Thr:Lys is reported to be 65% and 59% by BSAS (2003) and NRC (2012), respectively. Dietary Thr:Lys requirement for piglets might vary when clinical or subclinical infections occur, specifically when piglets are exposed to immunological challenge, as is often the case under *Escherichia coli* K88 (*E. coli* K88) infection.

Post-weaning diarrhea (PWD) caused by *E. coli* K88 is one of the most important causes of mortality in young piglets characterized by watery fecal discharge and accompanied with the attachment of *E. coli* K88 to the gut mucosa (Fairbrother et al., 2005). Besides affecting gut integrity and function, the toxins secreted by *E. coli* K88 also impairs the immune system in weaned pigs (Owusu-Asiedu et al., 2002; Opapeju et al., 2009). Antimicrobial growth promoters **(AGP)** have been used in starter diets for reduction of pathogenic bacteria and also for promoting growth performance of weaned pigs. Usage of AGP in swine diets could develop antibiotic resistance to animals and also for humans which is one of the most important global concern. Removal of AGP from swine diets could increase disease challenge and antibiotic treatments for animals (Diana et al., 2017). Thus, there is a mounting attention in the nutritional management of pigs to develop nutritional programs without in-feed AGP (de Lange et al., 2010; Heo et al., 2013). It is expected that this development may alter the dietary Thr:Lys requirement for optimal performance, especially when piglets are exposed to an immunological challenge.

Information about the SID Thr:Lys ratio for piglets fed AGP-free diets and subjected to an immunological challenge is limited. The hypothesis was that the Thr:Lys requirement is in piglets subjected to immune challenge conditions is higher than reported values by NRC (2012) for weaned pigs. It was also hypothesized that piglets challenged with *E. coli* K88 need higher SID Thr:Lys requirement to enhance the mucin and cytokine gene expression. Thus, the first objective of was to determine the optimum SID Thr:Lys ratio for weaned pigs fed AGP-free diets and subjected to *E. coli* K88 challenge. The second objective was to quantify the mucin and cytokine mRNA expression in piglets challenged with *E. coli* K88.

8.3. MATERIALS AND METHODS

The experimental 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). The experiment was conducted at T. K. Cheung Centre for Animal Science Research, University of Manitoba, Winnipeg, MB, Canada.

8.3.1. Animals and Experimental design

8.3.1.1. Experiment 1

Thirty individually housed mixed-sex pigs (Duroc × [Yorkshire × Landrace]) with an initial BW of 6.47 ± 0.13 kg and weaned at 21 ± 1 d were randomly assigned to 5 dietary treatments each with 6 replicates. Diets were corn, wheat and soybean meal-based with a constant SID Lys of 1.18% that was set to be the second limiting AA (**Table 8.1**). Ingredients contributing AA (corn, wheat, SBM) were analyzed for AA composition and these values were used in diet formulation. The diets contained 4 increasing levels of SID Thr:Lys (53, 59, 65 and 71%) and a control diet containing 59% SID Thr:Lys with an AGP (Aureomycin 220 G). All other nutrients were provided in quantities meeting or exceeding NRC (2012) requirements for a 6 to 10 kg pig. Pigs had *ad libitum* access to feed and water. The experiment lasted for 14 d.

8.3.1.2. Experiment 2

In Exp 2, thirty individually housed mixed-sex pigs (Duroc × [Yorkshire × Landrace]) with an initial BW of 6.64 ± 0.19 kg and weaned at 21 ± 1 d were randomly assigned to 5 dietary treatments each with 6 replicates. Diets contained 4 increasing levels of SID Thr:Lys (53, 59, 65 and 71%) and a diet containing 59% SID Thr:Lys with an AGP (Aureomycin 220 G). Individual

Table 8.1. Ingredient and nutrient composition of experimental diets (as-is-basis, g kg^{-1})

Items	Standardized ileal digestible threonine:lysine, %				
Ingredient composition	53	59	65	71	59 + AGP
Corn	435	435	435	435	435
Wheat	193	193	193	193	193
Soybean meal	280	280	280	280	280
Vegetable oil	40	40	40	40	40
Corn starch	5.50	4.80	4.10	3.40	4.80
Limestone	10.90	10.90	10.90	10.90	10.90
Dicalcium phosphate	15.60	15.60	15.60	15.60	15.60
Iodized salt	3.00	3.00	3.00	3.00	3.00
Vitamin-mineral premix[a]	10.00	10.00	10.00	10.00	10.00
L-Lysine	4.40	4.40	4.40	4.40	4.40
DL-Methionine	1.70	1.70	1.70	1.70	1.70
Threonine	0.00	7.0	14.0	22.0	7.0
L-Tryptophan	0.24	0.24	0.24	0.24	0.24
Valine	0.60	0.60	0.60	0.60	0.60
Total	1000	1000	1000	1000	1000
Calculated net energy and nutrient content (g kg^{-1})					
NE (MJ kg^{-1})	14	14	14	14	14
CP	199	200	201	201	200
Ether extract	29.2	29.2	29.2	29.2	29.2
Crude fiber	39.4	39.4	39.4	39.4	39.4
NDF	92.7	92.7	92.7	92.7	92.7
ADF	34.4	34.4	34.4	34.4	34.4
Total calcium	9.6	9.6	9.6	9.6	9.6
Total phosphorus	7.1	7.1	7.1	7.1	7.1
Available phosphorus	3.8	3.8	3.8	3.8	3.8
Lys	11.8	11.8	11.8	11.8	11.8
Met	4.3	4.3	4.3	4.3	4.3
Cys	2.8	2.8	2.8	2.8	2.8
Thr	7.3	7.9	8.6	9.3	7.9
Trp	2.7	2.7	2.7	2.7	2.7

Ile	6.6	6.6	6.6	6.6	6.6
Leu	13.7	13.7	13.7	13.7	13.7
Val	8.0	8.0	8.0	8.0	8.0
His	4.4	4.4	4.4	4.4	4.4
Arg	10.4	10.4	10.4	10.4	10.4
Phe	7.9	7.9	7.9	7.9	7.9
Analyzed CP and AA contents (*(g kg^{-1})*					
CP	19.7	19.5	19.2	19.1	19.4
Lys	12.4	11.8	11.5	12.1	11.4
Trp	0.28	0.25	0.24	0.27	0.25
Met	0.42	0.41	0.45	0.42	0.43
Cys	3.6	3.4	3.2	3.2	3.2
Met + Cys	8.1	8.2	8.1	8.1	8.0
Thr	6.5	7.0	7.6	8.6	6.8
His	4.8	4.4	4.5	4.8	4.5
Ile	7.8	7.2	7.4	7.6	7.2
Leu	15.1	14.2	14.5	14.9	14.2
Phe	9.3	8.5	8.8	8.9	8.6
Val	9.5	9.1	9.1	9.2	9.1

[a]Supplied the following per kilogram of diet: 8250 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 µg 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

pig BW and pen feed disappearance were recorded during the pre-challenge and post-challenge periods to determine ADG, ADFI and G:F. On d 8, all pigs were orally inoculated with 6 mL of *E. coli* K88 culture (2×10^{11} cfu/mL). Faceal consistency scoring (0 = normal, 1 = soft faeces, 2 = mild diarrhoea, and 3 = severe diarrhoea; Marquardt et al., 1999) was performed by 2 trained individuals in a treatment-blinded manner. Rectal temperature was measured in piglets before (24 h) and after (6 h, 24 h, 48 h) *E. coli* K88 challenge.

8.3.2. Escherichia coli K88 and culture condition

The procedure has been described in Chapter 6.

8.3.3. Chemical analysis of feed ingredients and diets

Prior to lab analyses, ingredient and diet samples were ground through a 1-mm mesh screen. Dry matter content was determined according to AOAC (2000) whereas dietary N content was determined with a gas combustion method using a Leco FP-2000 Nitrogen Analyzer (Leco Corp., St. Joseph, MI). Amino acid analyses for the feed ingredients and diets were carried out at the lab of Evonik Industries AG, Hanau-Wolfgang, Germany, using the method described by Llames and Fontaine (1994).

8.3.4. Plasma urea nitrogen and serum tumour necrosis factor alpha

On d 7 and d 14, a 10-mL blood sample was collected from each pig via jugular venipuncture into heparinized vacutainer tube (Becton Dickinson, Rutherford, US) and stored on ice for 20 min before being centrifuged at 2000 × *g* for 10 min at 4˚C to recover plasma. Plasma samples were stored at -20˚C until used for further analysis. Plasma samples were thawed and

then analyzed for PUN using a Nova Stat Profile M blood gas and electrolyte analyzer (Nova Biomedical Corporation, Waltham, MA, USA). Procedure for serum sample collection has been described in Chapter 5. Serum concentrations of tumour necrosis factor (TNF-α) was determined using Quantikine ELISA kits (R & D Systems, Inc. Minneapolis, MN, USA).

8.3.5. Ileal tissue collection and histomorphology

In Exp 1 and 2, on d 14, all pigs were euthanized for ileum tissue collection for histomorphology. The procedure is described in Chapter 5.3.7.

8.3.6. Extraction of total RNA from ileal mucosa and reverse transcription

Distal ileum sections (5 cm) from all piglets were flushed with 10-fold Phosphate buffer saline and snap-frozen in liquid N and stored at -80˚C until used. Ileal mucosa was scraped on a sterile glass surface, placed in a sterile tube and frozen immediately in liquid nitrogen. Total RNA was extracted from ileal mucosa samples using Trizol extraction method (Ivitrogen Canada Inc., Burlington, ON, Canada). To measure the quantity and purity of RNA, Nanodrop spectroscopy (Thermo Scientific, Boston, MA, USA) was used with the ratio of absorbance at 260 nm and 280 nm. Reverse transcription was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA).

8.3.7. Quantitative real time PCR

Primer sequences for GADPH, tumor necrosis factor (TNF-α), interleukin-10 (IL-10), interleukin-1β (IL-1β), MUC 1, MUC 4, and MUC 20 were designed using Gene bank database sequences from National Center for Biotechnology Information (Bethesda, MD, USA) (**Table**

8.2). Quantitative real-time (qRT) PCR was performed using the Step one thermocycler (Applied Biosystems, Mississauga, ON, Canada) in a 96-well plate with 25 µL of total reaction volume as described by Pfaffle and Hageleit (2001). The iTaq universal SYBR Green super mix was used as qRT-PCR master mix and each reaction was done in duplicate. The PCR cycling protocol included an initial denaturation step at 95˚C, followed by amplification for 40 cycles at 95˚C in 10 s, annealing step at a temperature described in Table 8.2 for each primer and extension at 72˚C for 10 s.

8.3.8. Statistical analyses

Growth performance, PUN, ileal histomorphology, cytokine and relative mRNA expression of mucin gene data were subjected to ANOVA using the general linear model procedure (SAS Inst. Cary. NC). The data were analyzed as a completely randomized design. Each pen containing 1 piglet was considered as the experimental unit and there were 6 experimental units per treatment. Orthogonal polynomial contrasts were used to determine linear and quadratic effects of increasing levels of SID Thr:Lys ratio. For fecal consistency score, rectal temperature and serum TNF-α, a repeated measure analysis of variance was employed. All means are reported as least square means and statistical significance was accepted at $P < 0.05$ and $0.05 < P < 0.10$ was considered a trend.

The model for the response criteria was $Y_{ij} = \mu + Thr_i + E_{ij}$, where,

Y_{ij} is the $_j$th observation of the

$_i$th treatment, µ is the population mean,

Thr_i is the treatment effect of the ith treatment, and

E_{ij} is the random error.

Table 8.2. List of genes and sequences of the primers used for real-time PCR

mRNA target	Primers → (5' 3')[2]	Product size (base pairs)	Annealing temperature
GADPH[1]	CTCTTCCAGCCCTCCTTCCT (forward)	104	60˚C
	GCGTAGAGGTCCTTCCTGATGT (reverse)		
TNF-α[2]	CACTGACCACCACCAAGAATTGGA(forward)	94	60˚C
	CATTCCAGATGTCCCAGGTTGCAT (reverse)		
IL-10	GATATCAAGGAGCACGTGAACTC (forward)	137	60˚C
	GAGCTTGCTAAAGGCACTCTTC (reverse)		
IFN-γ	GTTTTTCTGGCTCTTAACTGC(forward)	125	58˚C
	CTTCCGCTTTCTTAGGTTAG (reverse)		

[1]GDPH –glyceraldehyde 3-phosphate dehydrogenase

[2]TNF-α – tumor necrosis factor-alpha

[3]IL-10- interleukin-10

[4]IFN-γ – interferon-gamma

To determine the optimal SID Thr:Lys level, growth performance data were subjected to regression analysis (Kaps and Lamberson, 2004) using the Proc NLIN of SAS (SAS Inst. Inc. Cary, NC).

8.4. RESULTS

Experiment 1

The growth performance data are presented in **Table 8.3**. The overall ADG and G:F was different ($P < 0.05$) among the dietary treatments. There was a linear increase ($P < 0.05$) in overall G:F, as the dietary SID Thr:Lys increased. Dietary treatments did not affect ($P > 0.10$) the feed intake throughout the experiment. Pigs fed the diet containing 59% SID Thr:Lys and AGP had higher ($P < 0.05$) ADG and G:F than those fed the diet with 59% SID Thr:Lys without AGP. The final BW in pigs fed the diet with 59% SID Thr:Lys and AGP tended (linear; $P = 0.053$) to be higher compared to those fed with 59% SID Thr:Lys diet without AGP. Based on G:F, the estimated optimal SID Thr:Lys was 59% (**Table 8.4**). Dietary SID Thr:Lys did not affect ($P > 0.10$) PUN (**Table 8.5**).

Ileal histomorphology data are presesented in **Table 8.6**. Increasing dietary SID Thr:Lys increased ileal VH (quadratic; $P < 0.05$), however, other ileal morphology parameters (CD, VH: CD, VW and VSA) were not affected ($P > 0.10$). Piglets fed the 59% SID Thr: Lys diet without AGP had greater ($P < 0.05$) VH compared to those fed the 59% SID Thr:Lys diet with AGP. Based on VH, the estimated optimal SID Thr:Lys was 59.7%.

Experiment 2

Fecal score increased during the post-challenge (24 h, 48 h, and 72 h) and then declined at 96 h time point (**Fig 8.1**). Rectal temperature was not affected ($P > 0.10$) by *E. coli* K88 challenge (**Fig 8.2**). No mortalities were observed during the experiment.

Table 8.3. Effects of increasing standardized ileal digestible threonine: lysine ratio on growth performance of weaned pigs subjected to *E. coli* K88[1]

Items	Dietary SID Thr:Lys (%)							Contrasts		
	53	59	65	71	59 + AGP	SEM[2]	*P* value[3]	TrpL[4]	TrpQ[5]	59 vs 59 + AGP[6]
Exp 1 (*Piglets raised under unchallenge conditions*)										
Initial BW, kg	6.54	6.49	6.49	6.47	6.47	0.13	0.996	-	-	-
Final BW, kg	9.35	9.26	9.75	10.01	10.40	0.40	0.258	0.174	0.661	0.053
Day 0 to 7										
ADG, g	153	102	144	158	200	22.0	**0.038**	0.542	0.125	**0.002**
ADFI, g	225	173	187	217	223	29.6	0.566	0.934	0.152	0.201
G:F	0.63	0.59	0.69	0.69	0.82	0.05	0.066	0.293	0.759	**0.001**
Day 8-14										
ADG, g	234	274	341	334	427	38.0	**0.011**	**0.033**	0.502	**0.005**
ADFI, g	503	526	569	488	563	45.0	0.592	0.992	0.226	0.540
G:F	0.50	0.55	0.60	0.69	0.72	0.06	0.115	**0.040**	0.662	0.063
Overall experimental period										
ADG, g	201	198	233	238	314	25.0	**0.019**	0.184	0.871	**0.002**
ADFI, g	364	350	378	353	393	31.1	0.812	0.963	0.855	0.292
G:F	0.55	0.56	0.62	0.68	0.75	0.04	**0.012**	**0.016**	0.480	**0.003**
Exp 2 (*Piglets challenged with E. coli K88*)										
Initial BW, kg	6.64	6.65	6.65	6.64	6.49	0.19	0.960	-	-	-
Final BW, kg	9.27	9.31	9.14	9.46	9.23	0.41	0.985	0.816	0.729	0.859

Pre-challenge period (d 0-7)										
ADG, g	121	167	142	124	125	28.3	0.676	0.871	0.204	0.285
ADFI, g	266	292	272	271	251	20.4	0.667	0.951	0.466	0.147
G:F	0.46	0.56	0.52	0.44	0.51	0.08	0.755	0.786	0.207	0.635
Post-challenge period (d 8-13)										
ADG, g	272	273	234	296	284	45.0	0.843	0.850	0.434	0.854
ADFI, g	442	517	511	513	508	35.7	0.363	0.128	0.243	0.849
G:F	0.62	0.52	0.46	0.58	0.59	0.09	0.688	0.616	0.203	0.611
Overall experimental period										
ADG, g	202	224	191	217	211	31.3	0.931	0.933	0.950	0.756
ADFI, g	354	406	392	393	379	21.8	0.384	0.217	0.199	0.394
G:F	0.57	0.55	0.49	0.55	0.58	0.08	0.909	0.682	0.539	0.800

[1]n=6

[2]Standard error of mean

[3]Considered significant when $P < 0.05$ and a tendency when $0.05 < P \leq 0.10$.

[4]Probability values of linear effects for dietary SID Thr:Lys

[5]Probability values of quadratic effects for dietary SID Thr:Lys

[6]Contrast: control diet (59% SID Thr:Lys) vs. control diet +AGP

Table 8.4. Estimated optimal standardized ileal threonine:lysine of weaned piglets fed AGP-free diets

Item	Estimate	SE	Equation	R^2
Exp 1				
GF[1]	59.0	1.2	Y = 0.46 + 0.002 GF – 0.0008	0.83
VH[2]	59.7	3.4	Y = -287 + 13.8 VH – 21.8	0.99
Exp 2				
VH:CD[3]	65.6	2.6	Y = 0.26 + 0.02 VH:CD – 0.06	0.80

[1]gain:feed ratio

[2]VH-villous height

[3]VH:CD-villous height:crypt depth

Table 8.5. Effects of increasing standardized ileal digestible threonine: lysine ratio on plasma urea nitrogen (mmol/L) in weaned pigs subjected to *E. coli* K88[1]

Items	Dietary SID Thr:Lys (%)							Contrasts	
	53	59	65	71	59 + AGP	SEM[2]	*P*-value[3]	TrpL[4]	TrpQ[5]	59 vs 59 + AGP[6]
Unchallenge conditions										
d 7	4.05	3.47	3.96	3.78	3.62	0.43	0.830	0.863	0.616	0.788
d 14	3.58	3.70	3.12	3.75	3.65	0.53	0.914	0.971	0.611	0.943
Pooled	3.82	3.58	3.54	3.77	3.63	0.35	0.978	0.903	0.528	0.921
Challenge conditions										
d 7	3.67	2.48	3.72	3.65	3.00	0.40	0.144	0.511	0.171	0.366
d 14	5.0	3.57	3.22	3.17	3.7	0.47	0.068	0.010	0.155	0.843
Pooled	4.33	3.17	3.47	3.41	3.35	0.41	0.253	0.153	0.162	0.748

[1] Ileal tissue samples collected 6 d post-challenge, n=6

[2]Standard error of mean

[3]Considered significant when $P < 0.05$ and a tendency when $0.05 < P \leq 0.10$.

[4]Linear contrast for dietary threonine:lysine effect

[5]Quadratic contrast for dietary threonine:lysine effect

[6] Contrast: control diet (59% SID Thr:Lys) vs. control diet +AGP

Table 8.6. Effects of increasing standardized ileal digestible threonine: lysine ratio on ileal histomorphogy of weaned pigs subjected to *E. coli* K88[1]

Items	Dietary SID Thr:Lys (%)							Contrasts		
	53	59	65	71	59 + AGP	SEM[2]	*P*-value[3]	TrpL[4]	TrpQ[5]	59 vs 59 + AGP[6]
Unchallenge conditions										
Villous height, µm	446	529	497	449	449	31.2	0.250	0.854	**0.046**	0.092
Crypt depth, µm	329	340	324	281	305	26.0	0.534	0.183	0.303	0.343
VH:CD[c]	1.36	1.60	1.58	1.68	1.51	0.15	0.631	0.168	0.653	0.683
VW[8]	165	164	149	161	160	9.7	0.806	0.570	0.521	0.801
Challenge conditions										
Villous height, µm	402	450	388	423	419	21.1	0.323	0.993	0.764	0.307
Crypt depth, µm	330	317	308	348	326	26.8	0.862	0.712	0.326	0.804
VH:CD[7]	1.23	1.45	1.33	1.23	1.31	0.09	0.362	0.713	**0.073**	0.237
VW, µm	151	156	155	162	149	8.4	0.850	0.406	0.937	0.578

[1]Ileal tissue samples collected 6 d post-challenge, n=6

[2]Standard error of mean

[3]Considered significant when $P < 0.05$ and a tendency when $0.05 < P \leq 0.10$.

[4]Linear contrast for dietary threonine:lysine effect

[5]ThrQ- Quadratic contrast for dietary threonine:lysine effect

[6]Contrast: control diet (59% SID Thr:Lys) vs. control diet +AGP

[7]VH:CD – villous height : crypt depth ratio

[8]Villous width

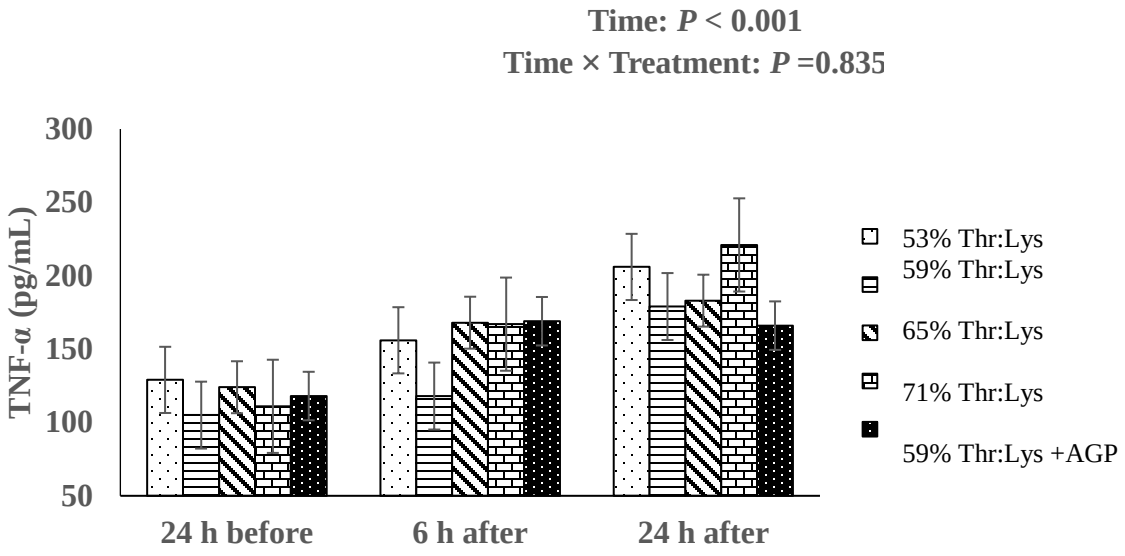

Fig 8.1. Effects of dietary SID Thr:Lys on serum concentrations of TNF-α at different sampling times in weaned piglets challenged with *E. coli* K88.

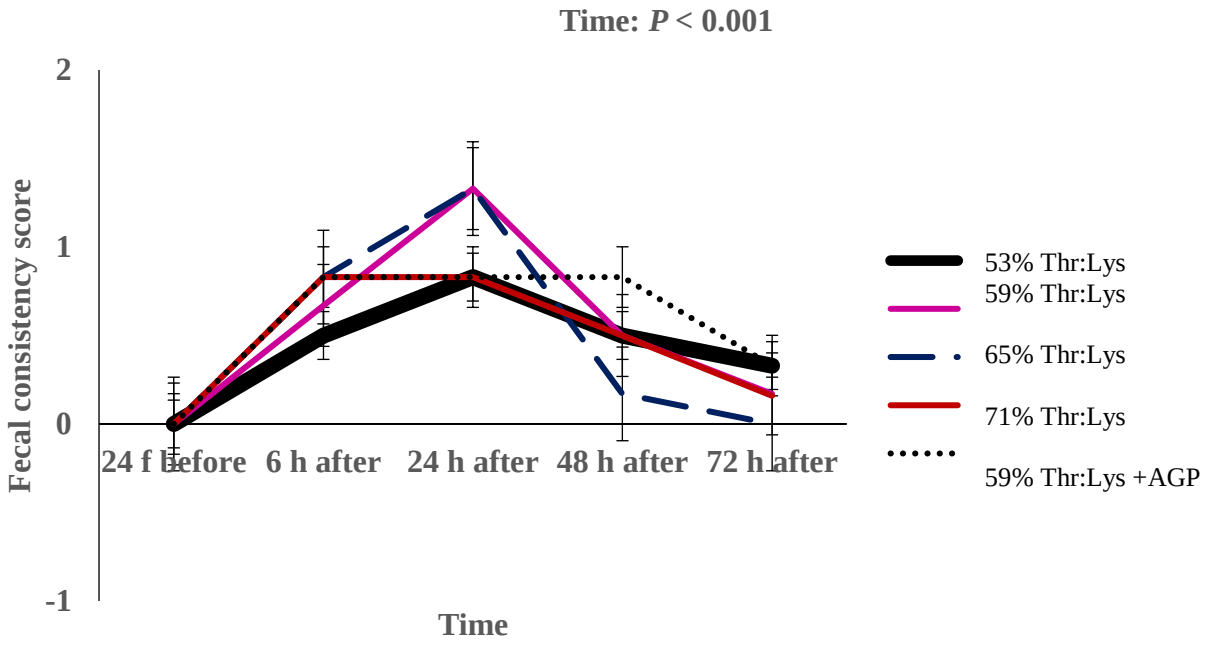

Fig 8.2. Effects of dietary SID Thr:Lys on fecal consistency score at different time points in weaned piglets challenged with *E. coli* K88

The growth performance and final BW did not differ ($P > 0.10$) among dietary treatments (Table 8.3). On d 7, feeding diets with increasing SID Thr:Lys linearly ($P < 0.05$) decreased PUN concentration (Table 8.5). The optimal SID Thr:Lys could not be determined using growth as response criteria. Increasing dietary SID Thr:Lys tended to increase (quadratic; $P = 0.073$) VH:CD ratio in ileum but other ileal morphology parameters were not affected ($P > 0.10$) in piglets (**Table 8.6**). Based on VH:CD as response criteria, the estimated optimal Thr:Lys was 65.6% (Table 8.4).

Data on ileal mucin and cytokine gene expression are presented in **Table 8.7**. Increasing SID Thr:Lys tended to increase (linear; $P < 0.05$) the mRNA expression of MUC 20, whereas mRNA expression of MUC 1 and MUC 4 were not affected ($P > 0.10$). Increasing dietary Thr:Lys tended to increase ($P < 0.05$) the mRNA expression of IL-1 β, but did not affect ($P > 0.10$) mRNA expression of TNF-α.

8.5. DISCUSSION

This study was carried out to determine the optimal SID Thr:Lys requirement for pigs unchallenged (Exp 1) or challenged with *E. coli* K88 (Exp 2). In both experiments, diets were corn-wheat-soybean meal-based with a constant SID Lys of 1.18% that was set to be 10% lower than the SID Lys requirement level that was established for pigs from the same herd established by Kahindi et al. (2016).

Estimated optimal Thr:Lys requirement for piglets unchallenged or challenged with E.coli K88

Under unchallenge conditions, increasing dietary SID Thr:Lys improved overall feed efficiency in weaned piglets which is in agreement with Mathai et al. (2016). An SID Thr:Lys of

Table 8.7. Effects of dietary standardized ileal digestible threonine to lysine ratio on mRNA expression of mucin 1 (MUC1), mucin 20 (MUC20), mucin 4 (MUC4), tumour necrosis factor (TNF-α), and interleukin-1 (IL-1) in ileal mucosa of weaned pigs challenged with *E. coli* K88[1].

Items	Dietary SID Thr:Lys (%)						*P* value[3]		59 vs 59 + AGP[6]
	53	59	65	71	59 + AGP	SEM[2]	Lin[4]	Quad[5]	
MUC1	0.57	0.56	0.62	0.58	0.77	0.12	0.840	0.909	0.245
MUC20	0.68^{b}	1.05^{ab}	0.89^{ab}	1.37^{a}	1.22^{ab}	0.19	**0.034**	0.769	0.527
MUC4	0.79	0.93	0.68	0.66	0.73	0.12	0.257	0.516	0.258
TNF-α	1.09	0.72	1.18	0.96	0.92	0.17	0.909	0.646	0.361
IL-1β	0.62^{b}	0.78^{b}	1.35^{a}	0.73^{b}	0.92^{ab}	0.20	0.242	**0.046**	0.613

[1]Ileal tissue samples collected 6 d post-challenge, n=6. Least square means with a different superscript letters indicate a significant between treatments.

[2]Standard error of mean

[3]Considered significant when $P < 0.05$ and considered tendency when $0.05 < P \leq 0.10$.

[4]Linear contrast for dietary threonine:lysine effect

[5]Quadratic contrast for dietary threonine:lysine effect

[6] Contrast: control diet (59% SID Thr:Lys) vs. control diet +AGP

59% optimized ADG which coincides with NRC (2012). In the previous experiment (Chapter 4), the estimated Thr:Lys for piglets raised under clean condition was 65%. It was anticipated that Thr:Lys requirement for piglets fed AGP-free diets could be higher than NRC (2012) (59%), however, the estimated optimal SID Thr:Lys was the same as NRC (2012).

In newly weaned pigs, the morphology and function of the small intestine is severely impaired, which consequently decreases small intestinal nutrient digestion and absorption capacity of nutrients (Wijtten et al., 2011). Intestinal villous atrophy could be due to deficiency of dietary Thr (Wang et al., 2010). In the present study, the VH quadratically increased due to increasing dietary SID Thr:Lys, which is in agreement with Ren et al. (2014).

Plasma urea N has been used as an indicator of AA utilization efficiency, because it is known to increase when there is an imbalance of dietary AA (Coma et al., 1996). In ETEC challenge conditions, increasing SID Thr:Lys linearly decrease PUN on d 7. This obervation is in agreement with Mao et al. (2011), in which increasing dietary Thr reduced PUN in piglets subjected to challenge induced by swine Pseudorabies live vaccine, indicating that efficiency of Thr utilization was improved with increasing dietary Thr content.

In the present study, increasing SID Thr:Lys tended to increase the VH:CD ratio of the ileum in *E. coli* challenged piglets. This observation is in agreement with Ren et al. (2014) demonstrating that *E. coli* K88 challenged piglets fed 7.5 or 11.1 g/kg SID Thr diets had increased villi heights and VH:CD compared to those fed 3.5 g/kg. The estimated optimal SID Thr:Lys based on VH:CD as response criterion was 65.6%. It is not conventional to use gut morphology as a response criterion in AA requirement studies, however, the optimum AA requirement for gut morphology would give information on maintenance of gut structural

integrity. The results from the current study and previous studies indicate that the increase in dietary Thr levels could have beneficial effects in maintaining intestinal structural integrity.

Mucin gene expression

Previous studies indicated that the availability of Thr is important for protein and mucin synthesis in neonatal pigs and rats (Faure et al., 2006; Law et al., 2007; Nichols and Bertolo, 2008; Puiman et al., 2011). Dietary factors such as fibre, protein and anti-nutritional factors are known to directly influence the synthesis and secretion of mucin from goblet cells and the recovery of mucin in digesta. The direct impact of dietary Thr was examined by looking at the mRNA expression of key mucin genes. In the present study, piglets fed diets with increasing dietary SID Thr:Lys had higher expression (linear, P = 0.034) of MUC 20 in ileal mucosa during 7 d post-challenge. This finding is in agreement with Wang et al. (2010) demonstrating that weaned piglets fed 0.89% true ileal digestible (TID) Thr had higher mucin mRNA levels in intestinal mucosa compared to 0.37 and 1.11% TID Thr. Upregulated MUC 20 gene expression in the current study was inferred as being beneficial for the weaned piglets challenged with ETEC because it may assist to improve mucin turnover, thus maintaining and enhancing the protective mucosal layer in the gastrointestinal tract of these animals.

Escherichia coli K88 attachment and colonization in ileal mucosa are associated with the stimulation of the piglet's immune system. The main mediators in the regulation of the immune and inflammatory responses are cytokines, which are expressed by the intestinal epithelium and may play a role in the basal influx of immune cells into the mucosa, and in epithelial cell growth, and homeostasis (Pie et al., 2004). In mammals, cytokines such as TNF-α, IL-8, and IL-1β are significantly expressed in response to pathogenic infection (Pie et al., 2004). In the present study,

increasing SID Thr:Lys tended to increase the mRNA expression of IL-1β, whereas the mRNA expression of TNF-α was not affected by dietary treatments.

Effects of AGP on growth performance

In pig starter diets, AGP have been used to minimize PWD and improve growth performance (Bikker et al., 2006). In Exp 1, piglets fed control diet supplemented with AGP had improved overall growth performance compared to pigs fed control diet. This is in agreement with Samuel et al. (2016) indicating the beneficial effect of AGP supplementation in young piglets. However, in Exp 2, the growth performance and other response criteria were similar among piglets supplemented with or without AGP.

8.6. Conclusions

An estimate of SID Thr:Lys of 59% optimized ADG in weaned piglets fed AGP-free diets. The optimal Thr:Lys requirement could not be determined based on growth response criteria in piglets under *E. coli* K88 challenge conditions. The growth performance of piglets subjected to immune challenge and fed AGP was similar to those fed without AGP. The optimal SID Thr:Lys based on VH:CD as response criteria in weaned piglets challenged with *E. coli* K88 was 65.6%. In summary, under immune challenge conditions (*E. coli* K88 infections), the Thr:Lys requirement could be higher than reported value by NRC (2012). It is suggested that five or more levels of increasing Thr:Lys ratio could be used in future studies to determine the optimal SID Thr:Lys ratio in piglets.

CHAPTER NINE

GENERAL DISCUSSION

Weaning is an abrupt and stressful period in a pig's life (Pluske et al., 1997). Post-weaning growth-check is one of the most important constraints in swine production, which is characterized by reduced feed intake, followed by post-weaning diarrhea, finally leading to poor growth performance (Heo et al., 2013). Post-weaning diarrhea in piglets is often characterised by discharge of watery feces during the first 2 weeks and is considered as a major economic loss due to mortality and morbidity (Fairbrother et al., 2005). To minimize the effects of weaning and its consequences, antimicrobial growth promoters (AGP) have been used in swine starter diets (Stein, 2002). Prolonged use of AGP causes antibiotic resistance in livestock species and also antibiotic residues in meat. Hence, a current interest in animal production is to reduce or eliminate the use of in-feed AGP. Under AGP-free diets, amino acid (AA) requirements might be increased for weaned pigs.

Threonine (Thr) is the second or third limiting AA after lysine and methionine in cereal-based swine diets (Mao et al., 2011). Besides protein synthesis, Thr is also essential for maintenance of gut integrity and immunity (Wang et al., 2006; Mao et al., 2011). In young pigs (10 and 25 kg), the requirements for Thr:Lys are reported to be 65% and 59%, BSAS (2003) and NRC (2012), respectively. However, under immune-challenge conditions, requirement for Thr:Lys could be increased (Trevisi et al., 2015). Hence, there is a need to determine the requirements of Thr:Lys in weaned pigs fed AGP-free diets and subjected to immune challenge conditions.

Tryptophan (Trp) is the third or fourth limiting AA in cereal-based swine starter diets (Guzik et al., 2005). In addition to protein accretion, Trp regulates feed intake and acts as a

modulator for controlling immune response and maintaining health in challenged pigs (Le Floc'h et al., 2009). Therefore, the requirements of SID Trp:Lys need to be determined in weaned pigs fed AGP-free diets and subjected to immune challenged conditions. Thus, we hypothesized that dietary Thr:Lys and Trp:Lys requirements for weaned pigs fed AGP-free diets would be increased under immunological challenge conditions.

A series of experiments were conducted with the primary objective of determining the SID Thr:Lys and SID Trp:Lys requirements in weaned piglets fed AGP-free starter diets and subjected to immune-challenge conditions. In this study, sanitation and enterotoxigenic *Escherichia coli* K88 (ETEC K88) challenge models were used to elicit an immune challenge in weaned pigs.

The first study (**Manuscript 1**) was conducted to determine the SID Thr:Lys requirements for weaned piglets raised under clean (CL) or unclean (UCL) sanitary conditions and fed AGP-free starter diets. The five dietary SID Thr:Lys were 55, 59, 63, 67 and 71%, and diets were corn-soybean meal-based with a constant SID Lys content of 1.18% that was set to be 10% lower than the SID Lys requirement level that was established for pigs from same herd determined by Kahindi et al. (2016). In this study, piglets reared under UCL had reduced body weight gain and feed intake compared to those raised under CL conditions (**Fig 9.1**). The optimal SID Thr:Lys based on feed efficiency as a response criterion was 65% and 67% for weaned pigs under CL and UCL conditions, respectively. The estimated SID Thr:Lys requirement for the weaned pigs under CL conditions was numerically higher (11%) than NRC (2012) requirement (59% SID Thr:Lys). Under both CL and UCL conditions, increasing levels of dietary Thr increased serum-free Thr, which is consistent with previous studies (Li et al., 1999; Wang et al., 2006).

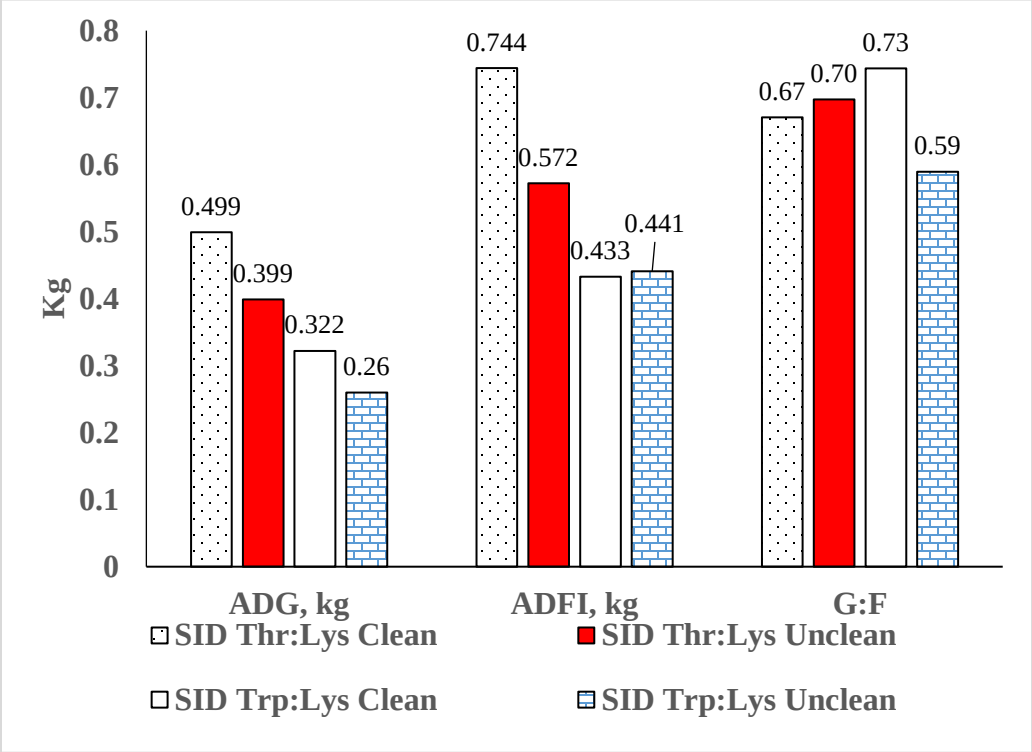

Fig 9.1. Effects of sanitation conditions on growth performance in weaned piglets.

Values are least square means from study 1 and study 2.

Clean: piglets subjected to clean sanitary conditions were housed in a cleaned and disinfected rooms and fed antibiotic-free diets.

Unclean: piglets subjected to unclean sanitary conditions were housed in a room where cleaning and disinfection were not done, moreover, manure from swine herd was added to all the pens on d 0 and d 7 of the experiment.

SID Thr:Lys: standardized ileal digestible threonine:lysine

SID Trp:Lys: standardized ileal digestible tryptophan:lysine

To determine the optimal SID Trp:Lys for weaned piglets under CL or UCL conditions and fed AGP-free starter diets, a study (**Manuscript 2**) was conducted. In this study, the five dietary SID Trp:Lys levels were 18, 19, 20, 22 and 24%, and diets were corn-soybean meal-based with a constant SID Lys content of 1.18% that was set to be 10% lower than the SID Lys requirement level that was established for pigs from same herd determined by Kahindi et al. (2016). Similar to Study 1, under UCL conditions, piglets had reduced ADG and G:F compared to CL condition (**Fig 9.1**). The deterioration of air quality due to UCL conditions could have caused discomfort leading to the reduced growth. Based on ADG as response criteria, the estimated optimal SID Trp:Lys under CL conditions was 19.7%, which is in agreement with previous studies (Guzik et al., 2002; Guzik et al., 2005; Jansman et al., 2010). Under UCL conditions, the estimated SID Trp:Lys based on ADG and G:F was 20.5% and 19.0%, respectively. Gut morphology in piglets reared under CL conditions was characterized by higher villous height and deeper crypt depth compared to those raised under UCL conditions, which further indicated the influence of the sanitary conditions. Reduced ileal VH observed in piglets under UCL might have affected small intestinal digestion and absorption capacity for nutrients.

In the third experiment, the objective was to determine the optimal SID Thr:Lys of weaned piglets challenged with ETEC K88 (**Appendix** 1). We used Luria-Bertani agar (LB agar) for culturing ETEC under aerobic conditions. During the post-challenge period (d 8 to 14), there were no effects of increasing SID Thr:Lys on growth performance and PUN response criteria which suggested that ETEC challenge used in this experiment was insufficiently effective to activate immune system in weaned pigs. It was understood that LB medium may not favor the *E. coli* K88. Therefore, a pilot study (**Manuscript 3**) was conducted to evaluate the efficacy of *E. coli* K88 strain cultured in two different media (LB agar or Brain heart infusion agar (BHI))

under aerobic or anaerobic conditions in weaned pigs inoculated without or with calcium carbonate solution. Results showed that piglets inoculated with ETEC grown in BHI media had significantly increased fecal consistency scores compared to those inoculated with ETEC grown in LB media. Our results indicated that ETEC strain cultured in BHI agar was more efficient in increasing diarrhea score and elevated rectal temperature in orally ETEC challenged piglets compared to those orally challenged with ETEC K88 grown in LB medium. Piglets inoculated with ETEC cultured in BHI medium had lower VH and deeper CD than those inoculated with ETEC cultured in LB medium. Thus, it was concluded that ETEC cultured in BHI agar under anaerobic conditions would be optimum for challenge studies in piglets.

The objective of study 4 (**Manuscript 4**) was to determine the optimal SID Trp:Lys for weaned piglets fed AGP-free starter diets and challenged with *E. coli* K88. To further understand the effect of different SID Trp:Lys on immune responses in weaned pigs challenged with ETEC K88, mRNA expression of ileal cytokines was determined. The dietary treatments consisted of 5 SID Trp:Lys levels (16.1, 18.6, 20.3, 22.9 and 24.6%). Increasing dietary SID Trp:Lys significantly upregulated the expression of IL-10 in ileal mucosa of piglets and was highest at 22% SID Trp:Lys. Based on ADG and G:F, the optimal SID Trp:Lys for weaned piglets subjected to *E. coli* K88 challenge were 21.7 and 20.1%, respectively (**Table 9.1**), which are in agreement with the results of Capozzalo et al. (2015) showing that a dietary SID Trp:Lys of 24% improved feed conversion for weaned pigs challenged with *E. coli* K88. Under an immune challenge (*E. coli* K88), dietary Trp:Lys requirements for weaned piglets could be higher than reported values by NRC (2012).

Table 9.1. Estimated optimal SID threonine:lysine and tryptophan:lysine ratios in weaned pigs fed antibiotic-free starter diets

Items	Response criteria	Estimated Optimal AA:lysine (%)	NRC (2012)
SID Thr:Lys[1]			
Clean[2]	G:F	65	59
Unclean[3]	G:F	67	
SID Trp:Lys[4]			
Clean	ADG	19.7	16
Unclean	ADG	20.5	
SID Trp:Lys			
E. coli challenge	ADG	21.7	16
	G:F	20.1	
SID Thr:Lys			
E. coli challenge	VH:CD	65.6	

[1] standardized ileal digestible threonine:lysine

[2] piglets subjected to clean sanitary conditions were housed in a cleaned and disinfected room and fed antibiotic-free diets.

[3] piglets subjected to unclean sanitary conditions were housed in a room where cleaning and disinfection was not done, moreover, manure from swine herd was added to all the pens on d 0 and d 7 of the experiment.

[4] standardized ileal digestible tryptophan:lysine

In study 5 (**Manuscript 5**), the primary objective was to determine the optimal SID Thr:Lys in weaned piglets fed AGP-free diets and challenged with or without *E. coli* K88. The dietary treatments consisted of 4 graded levels of SID Thr:Lys (53, 59, 65 and 71%) and a control diet containing 59% SID Thr:Lys supplemented with AGP. The estimated optimum Thr:Lys requirements for unchallenged pigs was 59%. Based on performance as response criteria, the estimated SID Thr:Lys requirement could not be determined in piglets challenged with ETEC. In ETEC challenged group, the estimated VH:CD-based optimum Thr:Lys requirement was 65.6%. Piglets fed increasing dietary SID Thr:Lys had higher expression of MUC 20 in ileal mucosa on 7 d post-challenge. This finding is in agreement with Wang et al. (2010) demonstrating that weaned piglets fed 0.89% true ileal digestible (TID) Thr had higher mucin mRNA levels in intestinal mucosa compared to 0.37 and 1.11% TID Thr. An upregulated MUC 20 gene expression in the current study was inferred as being beneficial for the weaned piglets challenged with ETEC because it may assist to improve mucin turnover, thus maintaining and enhancing the protective mucosal layer in the gastrointestinal tract of these animals.

In general, under commercial conditions, pigs are routinely exposed to pathogens, which would stimulate the immune system and redirect nutrients from supporting growth toward activating an immune response (Williams et al., 1997; de Ridder et al., 2012; Rakshandeh et al., 2012). The results from Studies 1 and 2, indicated that growth performance was significantly affected in piglets raised under unclean conditions compared to those raised under clean sanitary conditions. In Study 2, piglets kept under UCL had reduced VH:CD, which indicates that sanitation has an impact on gut morphology. During immune stimulation, enhanced Trp catabolism occurs (Le Floc'h et al., 2009), and moreover, the concentrations of Trp in acute phase proteins is also high (de Ridder et al., 2012), which indicates a clear link between Trp

catabolism and immune response in disease-challenged conditions (Moffett and Namboodiri, 2003). Amino acids required to support growth could have been directed to stimulate an immune response, which might have resulted in higher AA requirement for weaned piglets. To support this notion, recent studies demonstrated that pigs subjected to LPS challenge increased AA requirements (de Ridder et al., 2012; Rakshandeh and de Lange, 2012).

Summary

An SID Thr:Lys of 65% and 67% optimized growth performance in piglets raised under clean and unclean sanitary conditions, respectively. An average SID Trp:Lys of 19.7% and 20.5% optimized growth performance in weaned piglets raised under clean and unclean sanitary conditions. The SID Thr:Lys and Trp:Lys requirements were similar for weaned piglets raised under clean or unclean sanitation conditions. Under an immune challenge (*E. coli* K88), the SID Trp:Lys requirements could be higher than reported values by NRC (2012) for weaned piglets. Ensuring an adequate amino acids in starter diets allows to maintain good performance under immune-challenged conditions.

CHAPTER TEN

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

The following conclusions can be drawn from the present studies:

1. Weaned piglets raised under clean sanitary conditions had better growth performance compared to those raised under unclean sanitary conditions. These results indicate that the sanitation challenge model was effective in weaned piglets.

2. The optimal SID Thr:Lys of 65% and 67% could be used to optimize feed efficiency for weaned pigs reared under clean and unclean sanitary conditions, respectively.

3. The estimated optimal SID Trp:Lys based on ADG for weaned piglets reared under clean and unclean sanitary conditions were 19.7% and 20.5%, respectively.

4. *Escherichia coli* K88 grown in Brain Heart Infusion agar under anaerobic conditions could be effective for showing clinical signs in weaned piglets after oral challenge.

5. Based on ADG and feed efficiency, the optimal SID Trp:Lys for weaned pigs subjected to *E. coli* K88 challenge was 21.7% and 20.1%, respectively.

6. Increasing dietary SID Thr:Lys improved gut morphology in weaned pigs challenged with *E. coli* K88 and fed AGP-free diets.

7. Estimated VH:CD-based optimum Thr:Lys requirement for piglets challenged with *E. coli* K88 was 65.6%.

8. Piglets fed increasing dietary Thr:Lys had higher expression of MUC 20 in ileal mucosa during 7 d post-challenge. Upregulated MUC 20 gene expression in the current study was inferred as being beneficial for the weaned piglets challenged with ETEC because it may assist to improve

mucin turnover, thus maintaining and enhancing the protective mucosal layer in the gastrointestinal tract of these animals.

9. Overall, the AA (Thr and Trp) requirements could be increased for weaned piglets fed AGP-free diets and subjected to immune challenge conditions (*E.coli* K88 in our study).

FUTURE DIRECTIONS

1. Studies need to be conducted in a commercial farm to determine the optimum level of Trp and Thr in the diet for maximum production in weaner pigs fed AGP-free diets.
2. Additional research should be conducted to determine whether Thr and Trp requirements for piglets under AGP-free diets would be sufficient with the use of AGP alternatives such as probiotics, prebiotics and organic acid.
3. It is necessary to establish whether the AA requirement obtained under AGP-free feeding would be sufficient or be in excess with use of AGP alternatives such as probiotic, prebiotic, and acidifiers in piglet diets.
4. Studies should be conducted to explore potential antioxidative effects of Trp in weaned piglets fed AGP-free diets and challenged with *E. coli* K88.
5. Future studies should evaluate the potential roles of dietary Thr supplementation on intestinal functions in weaned pigs fed AGP-free diets and challenged with *E. coli* lipopolysaccharide.

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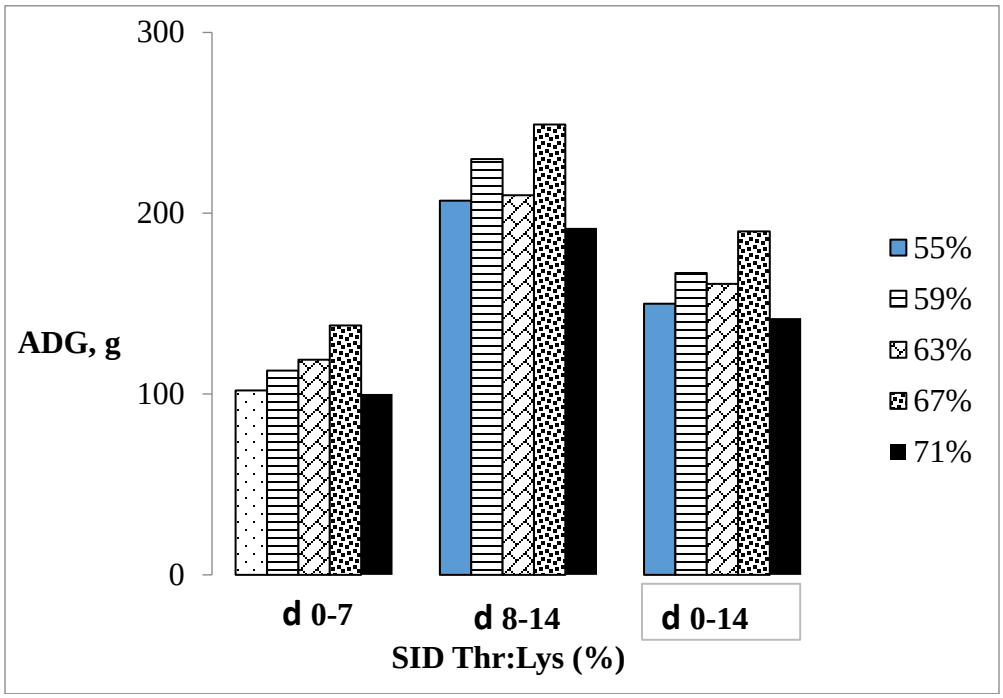
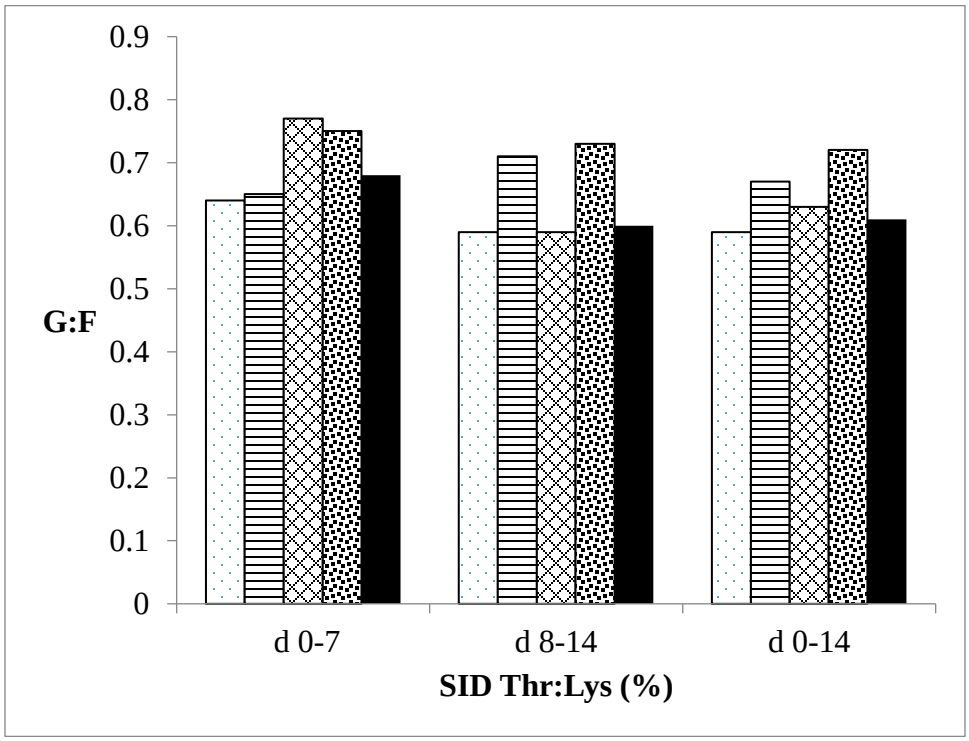
Appendix

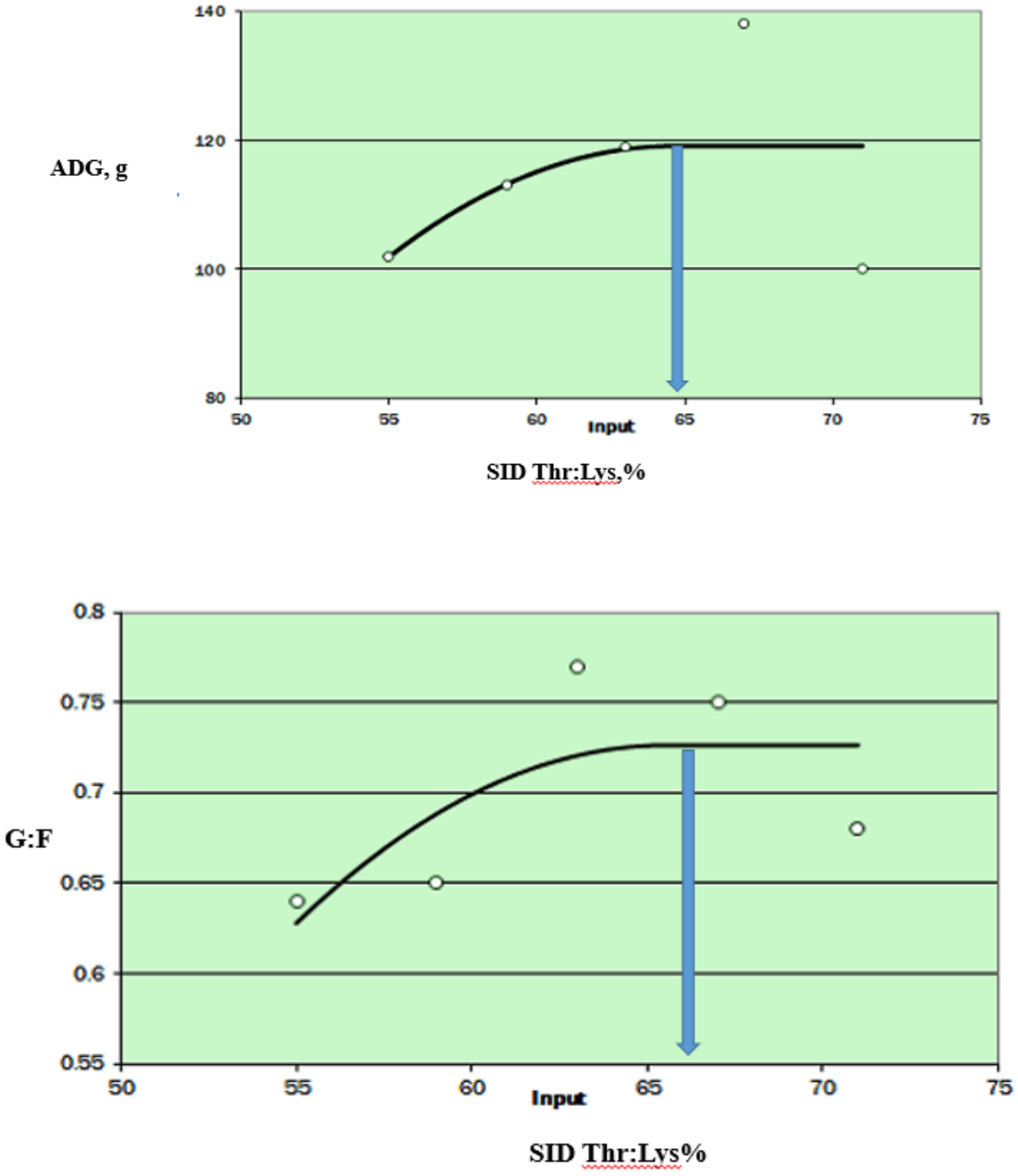
Table 1 Ingredient and nutrient composition of experimental diets

Items	**SID Thr:Lys %**				
	55	**59**	**63**	**67**	**71**
Wheat	15.10	15.10	15.10	15.10	15.10
Corn	43.20	43.20	43.20	43.20	43.20
Soybean meal	33.00	33.00	33.00	33.00	33.00
Vegetable oil	3.85	3.85	3.85	3.85	3.85
Corn starch	0.50	0.45	0.39	0.34	0.28
Monocalcium Phosphate	1.50	1.50	1.50	1.50	1.50
Limestone	1.08	1.08	1.08	1.08	1.08
Salt	0.30	0.30	0.30	0.30	0.30
Mineral-vitamin premix	1.00	1.00	1.00	1.00	1.00
L-Lys	0.28	0.28	0.28	0.28	0.28
L-Trp	0.05	0.05	0.05	0.05	0.05
DL-Met	0.15	0.15	0.15	0.15	0.15
L-Thr	0.00	0.06	0.11	0.17	0.22
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Calculated NE (MJ/kg)	10.4	10.4	10.4	10.4	10.4
Analyzed CP (%)	20.8	20.3	20.9	21.3	20.8
Analyzed amino acid composition (%)					
Met	0.46	0.49	0.48	0.48	0.48
Cys	0.34	0.33	0.35	0.34	0.34
Met + Cys	0.80	0.82	0.83	0.82	0.81
Trp	0.31	0.30	0.31	0.32	0.31
Lys	1.32	1.29	1.34	1.35	1.32
Thr	0.76	0.79	0.88	0.95	1.00
Thr:Lys %	**58**	**61**	**66**	**70**	**75**





Appendix Fig 1. Optimal standardized ileal digestible threonine:lysine for weaned piglets SID Thr:Lys. On d 8, piglets were orally challenged with *E. coli* K88.



Appendix Fig 2 Optimal standardized ileal digestible threonine:lysine for weaned piglets. SID Thr:Lys: standardized ileal digestible threonine:lysine ratio; On d 8, piglets were orally challenged with *E. coli* K88