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Growth performance and expression of genes encoding enzymes involved in methionine and cysteine metabolism in piglets fed increasing sulphur amino acid to lysine ratio during enterotoxigenic *Escherichia coli* challenge

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ABSTRACT

A study was conducted to examine the effect of standardised ileal digestible (SID) sulphur amino acids:lysine ratio (SAA:Lys) on performance and expression methionine adenosyl transferase 1 and 2-alpha (MAT1 α and MAT2 α), and cystathionine γ -lyase (CTH) in piglets challenged with an enterotoxigenic *Escherichia coli* (ETEC). Thirty five [Duroc x (Yorkshire x Landrace)] piglets (6.9 ± 0.5 kg) were randomly assigned to 5 dietary treatments. The diets were antibiotic free with SID SAA:Lys of 48, 54, 60, 66, and 72%. Pigs were orally challenged with 6 and 15 mL of ciprofloxacin-resistant ETEC K88⁺ on d 7 and 10. Blood samples were collected before (BC) and 6, 24, and 48 h after challenge (AC). BWG & FI were collected on d 0, 6, & 12 to determine average daily gain (ADG). Gain to feed ratio (G:F) was calculated by dividing ADG by ADFI. On d 13, all pigs were euthanized to collect liver and ileal samples to analyse gene expression using real-time PCR. Pigs fed the diet containing SAA:Lys of 66 had the highest ADG, ADFI and G:F BC. However, ADG, ADFI and G:F were similar across all ratios AC. Serum TNF α concentration at 6 h AC was higher ($P < 0.05$) than BC and was improved with increasing SAA:Lys. Increasing SAA:Lys quadratically increased ($P < 0.01$) CTH and MAT1 α expression. Ileal expression of CTH and MAT2 α were quadratically increased ($P < 0.05$) with increasing SAA:Lys. In conclusion, SAA:Lys of 60 is suggested to be optimum for piglets to tolerate ETEC pathogenic challenge.

Key words: pig, *E.coli* K88, MAT1 α and MAT2 α , CTH, SAA:Lys ratio

Abbreviations: BW, body weight; CTH, cystathionine γ -lyase; ETEC, enterotoxigenic *Escherichia coli*; MAT1 α and 2 α , methionine adenosyltransferase 1 and 2 alpha, TNF α , tumour necrosis alpha.

INTRODUCTION

Post-weaning piglet loss due to diarrhoea is responsible for economic loss in swine industry because in commercial production piglets are weaned at the very early stage and face numerous challenges. Unfortunately, this period coincides with the period when the gastrointestinal barrier function is not well developed. In the past, in-feed antibiotic growth promoters (AGPs) have been used to minimise this problem. However, due to an increasing public concern about the development of antibiotic resistant bacterial strains and drug residue in meat, the use of in-feed AGPs has been banned in many jurisdictions (Gaggia et al. 2010; Vondruskova et al. 2010; Vander Fels-Klerx et al. 2011). In the past two decades, an intensive amount of research has focused on the development of alternatives to antibiotics to maintain animal health and performance. However, the majority of these compounds produce inconsistent results and rarely equal antibiotics in their effectiveness. One of the methods to curb this problem is to devise feeding strategies that aim at boosting the immunological status of preweaning piglets. Formulating diets that have an optimal essential amino acids (AA) ratio is a possible way to achieve this goal. According to Grimble (2006), methionine (Met) and cysteine (Cys) are known as sulphur AA (SAA) and are essential not only for protein accretion but also for production of cytokines, glutathione (GSH) and acute phase proteins (APP). The dietary SAA requirement expressed as SAA:Lys is 55 for healthy 7 to 11 kg pigs (NRC 2012). It is a ratio considered to be optimum for muscle protein accretion. However, a negative whole body protein balance resulting from muscle catabolism to release AA for energy and synthesis of cytokines and APP may occur when an animal is under pathogenic challenge (Grimble and Grimble, 1998; Le Floc'h et al. 2004; Le Floc'h and Se`ve, 2007; Melchior et al. 2004).

The SAA belong to the AA utilized for APP synthesis (Grimble and Grimble, 2004) and constitute up to 56% of their structure (Grimble, 2006). Moreover, a dietary methionine deficiency impairs the immune response by affecting the development of lymphoid organs and by reducing production of antibodies and T cell proliferation in pigs (Kim et al., 2012; Jahoor et al., 1995) and chickens (Maroufyan et al., 2010). Thus, higher SAA supplementation can minimize the negative effects of immunological challenges and help the host maintaining health and reduce muscle loss (Rakhshandeh et al. 2014).

Methionine is metabolized in the liver and small intestine through three major pathways; transmethylation, remethylation and transsulfuration. Ileum is a site of choice for these pathways in immune compromised piglets because besides its function in AA absorption, the small intestine contains gut-associated lymphoid tissues and plays an active role in gut health (Ruth and Field, 2013). Methionine adenosyl transferase (MAT) is a product of transmethylation of methionine, which in turn, regulates remethylation and trans-sulfuration of Met depending on the need for Cys and dietary SAA content (Stipanuk 2004). Up-regulation of the trans-sulfuration pathway was observed in septic rats demonstrating the need for increased Cys during an immune challenge (Stipanuk 2004). Results of recent research in our laboratory (Kahindi et al. 2014) has indicated that 1.18% SID Lys is a second limiting AA for piglets and SID Lys requirement for 7 to 16 kg piglets fed AGP-free diet was 1.32%.

The need for SAA to support the immune system as opposed to accretion of lean tissue calls for a response criteria besides performance to determine its requirements in immune compromised animals. Therefore, the objectives of this study were to 1. Determine the effects of increasing standardised ileal digestible (SID) SAA:Lys ratios on the performance of piglets subjected to an

enterotoxigenic *Escherichia coli* (ETEC) immunological challenge. 2. Analyse the mRNA expression of enzymes involved in methionine and cysteine metabolism.

MATERIALS AND METHODS

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

EXPERIMENTAL DIETS and FEEDING REGIMEN

Five corn-wheat-soybean-meal based antimicrobial growth promoter (AGP) free diets with graded SID SAA:Lys ratios (48, 54, 60, 66, and 72) and a constant 1.18% SID Lys were used (Table 1). The graded levels of SID SAA:Lys ratios were attained by replacing corn starch with DL-Met. The concentrations of essential AA were similar for all diets and diets were balanced to meet the ideal AA ratio for protein accretion (NRC 1998) (Table 2). The diets were offered at 4% of BW to ensure consistency in the content of SAA intake and pigs were fed twice daily at 0800 and 1600 h. Daily and average feed intake (ADFI) was calculated. Piglets had unlimited access to water via a low-pressure water drinking nipple.

ANIMALS and HOUSING

Thirty five [Duroc x (Yorkshire x Landrace)] male and female piglets, weaned at 21 ± 1 d with an initial average BW of 6.9 ± 0.5 kg were assigned to dietary treatments in a completely randomized design for a 12-d study. The experiment was conducted in T. K. Cheung Centre for Animal Science Research and the piglets were obtained from Glenlea Swine Research Unit, University of Manitoba. Piglets were kept in individual pens in a temperature-controlled room. Body weighed gain and feed intake were collected on d 0, 6, and 12 to determine average daily

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

BACTERIAL CHALLENGE

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

FAECAL CONSISTENCY SCORES and RECTAL TEMPERATURE

Faecal consistency scores and incidence of diarrhoea were determined before and after ETEC inoculation. The faecal consistency was scored according to the method of Marquardt et al. (1999)⁽¹⁹⁾, where, 1 = normal; 2 = soft faeces; 3 = mild diarrhoea; 4 = severe diarrhoea. Rectal temperature was measured a day before and every day after the ETEC inoculation.

SAMPLE COLLECTION and ANALYSIS

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

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

TISSUE COLLECTION, RNA ISOLATION and REAL TIME-PCR

On d 13, all piglets were anesthetized by an intramuscular injection of ketamine:xylazine (20:2 mg/kg; Bimeda-MTC Animal Health Inc., Cambridge Ontario, Canada). This was followed by euthanization through intravenous administration of sodium pentobarbital (50 mg/kg of BW; Bimeda-MTC Animal Health Inc.) to collect liver and ileum samples for RNA isolation. Samples were immediately snap frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from liver or ileum mucosal scrapping using TRIzol reagent (Invitrogen, Canada) according to the manufacturer's instruction. The isolated total RNA was reverse transcribed using a high capacity reverse transcription kit (Applied Biosystems, Burlington, ON). Pairs of primers for each gene were designed using NCBI gene data base. Cystathionine β -synthase was substituted with CTH because of the absence of a complete sequence of the former in swine genome data base. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in duplicate reactions including nuclease free water, the forward and reverse primers of each gene, cDNA and SYBR Green as a detector using CFX ConnectTM Real-Time PCR Detection System (Life Science Research, Bio-Rad). Data were generated using $\Delta\Delta C_t$ method by normalizing the expression of the target gene to an internal control gene, GAPDH. Pairs of primers used for real-time qPCR assay were shown in Table 3.

DIET and INGREDIENTS ANALYSES

Dietary AA concentrations in ingredients and diets 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 *N* HCL for 24 h at 110°C and quantified with the internal standard by measuring the absorption of reaction products with ninhydrin at 570 nm. Tryptophan was determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm), after alkaline hydrolysis with barium hydroxide octahydrate for 20 h at 110°C (Commission Directive 2000).

DATA ANALYSES

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

RESULTS

Piglets were healthy in the pre-challenge period and although their feed intake differed, the faecal consistency score was close to 1, meaning that they had normal faeces (Table 4). The faecal consistency score increased after challenge due to increased incidence of diarrhoea to 17% (data not shown), with a mean faecal consistency score of 1.33. Increasing dietary SAA:Lys had no effect on faecal consistency after challenge. The diets were offered at 4% BW to ensure consistency in the amount of SAA intakes per treatment; however, some piglets did not eat all the feed allocated to them. Increasing SAA:Lys ratio quadratically increased ($P < 0.05$) ADG, ADFI and G:F before challenge (Table 5). In particular, pigs fed the diet containing SAA:Lys

ratio of 66 had the highest ($P < 0.05$) ADG, ADFI and G:F. However, ADG, ADFI and G:F were not different across all SAA:Lys ratios after challenge.

The total faecal coliform populations were not different among treatments. Increasing the SAA:Lys linearly increased ($P = 0.014$) and tended to quadratically decrease ($P = 0.065$) ciprofloxacin ETEC population on d 3 and 5, respectively, after the primary challenge with ETEC. Serum TNF- α concentration at 6 h after primary inoculation was higher ($P < 0.05$) than before challenge concentration irrespective of dietary treatments (Table 4). However, serum TNF- α concentration 24 h after the secondary inoculation was similar to before inoculation concentration. Increasing SAA:Lys quadratically increased the serum TNF- α concentration after the secondary inoculation (Table 4). The diet with SAA:Lys ratio of 48 had the lowest TNF- α concentration after both inoculations.

Hepatic mRNA expression of CTH and MAT1A were linearly and quadratically increased with SAA:Lys ratio ($P < 0.01$) (Table 6). In the ileum, increasing SAA:Lys ratio quadratically increased CTH and MAT1A expression (Table 6).

DISCUSSION

Escherichia coli caused diarrhoea is a multifactorial condition which usually occurs during the post-weaning and is responsible for economic losses in swine production. One approach, to evaluate the suitability of a given alternative to in-feed AGPs in controlling post weaning diarrhea is to use an appropriate disease model. The model should be capable to induce diarrhoea and the shedding of the pathogen should be evident. In this study, the presence of ETEC in the piglets' intestine was confirmed by ETEC shedding on d 3 and 5 after inoculation and the faecal consistency score was more than 1 for all treatments, indicating the ETEC inoculation caused mild diarrhoea. Inoculation with ETEC caused an inflammation in piglets as reflected by the

slight increase in rectal temperature both at primary and secondary inoculations. Furthermore, TNF α concentration 6 h after the primary inoculation was higher compared to before inoculation. Tumour necrosis factor- α is produced by macrophages during inflammation and stimulates the hypothalamus to trigger APP production in the liver (Gruys et al. 2005). Production of TNF α in the current study was dependent on the dietary SAA:Lys ratio. Hence, with the exception of the diet with a 72 SAA:Lys ratio, higher SAA:Lys ratio resulted in an increased TNF α concentration in the blood. This indicates that SAA is required for both pro- and anti-inflammatory responses and high dietary SAA contents enable the host to exert strong inflammatory responses. In a study by Litvak et al. (2013) piglets fed a diet with a 50% reduction of the SAA content had lower plasma albumin concentration as compared to those on 100 % SAA diet. The drop in plasma albumin concentration in that study was attributed to the lack of nutrients for APP synthesis. Because APP synthesis is triggered by cytokines like TNF α , IL1 β and IL6, the concentration of these cytokines will positively influence APP concentration in the blood. Interestingly, exposing piglets to a secondary immune challenge did not increase serum TNF α when compared to the concentrations before challenge. The response could be due to the fact that secondary immune response is rapid and less severe because the body have already synthesized the necessary antibodies and memory cells as a result of the primary challenge (Abbas et al. 2012).

During the pre-challenge period the highest ADG and ADFI were achieved at SAA:Lys ratio of 66%. Feeding SAA:Lys ratio of 48 and 72 dropped ADG by 54 and 62% and ADFI by 27 and 46%, respectively, compared to SAA:Lys ratio of 66. Hence, growth depression in piglets fed diets with SAA:Lys ratio of 48 and 72 was not only because of reduction in feed intake but also due to reduction of dietary SAA content (Table 5). Consequently, the drop in SAA intake by piglets on 72 SAA:Lys treatment compromised their immune response as indicated by the low 6

h TNF α concentration (Table 4). However, this was not accompanied by the responses in performance parameters as the post-challenge ADFI and ADG were not different across dietary treatments. These results were comparable to Capozzalo et al. (2012) who did not observe difference in ADG and ADFI in piglets orally inoculated with *E.coli* and fed diets with graded Trp levels. This is likely because during the immune challenge, growth is of secondary importance and nutrients are partitioned towards support of an immune response and the body is undergoing muscle catabolism to release AA that are required for gluconeogenesis and APP synthesis.

We hypothesized that Cys requirement for GSH, mucin and APP production would increase during an enteric infection and that the mRNA expression of enzymes that are involved in methionine transmethylation, remethylation and trans-sulfuration pathways would increase.

Methionine metabolism may occur through transmethylation reactions and by conversion of methionine to cysteine. Transmethylation is the transfer of methyl group (CH₃) from active methionine to an acceptor. Methionine has to be activated to S-adenosylmethionine (SAM) or active methionine to donate the methyl group and this reaction is catalysed by methionine S-adenosyltransferases (MAT) of which MAT1 α and MAT2 α are members. The quadratic increase in the expression of MAT1 α (liver) and MAT2 α (ileum) in the present study as the SAA:Lys ratio increased indicates there is an optimum ratio of SAA:Lys where the synthesis of these enzymes take place.

The trans-sulfuration pathway is a metabolic pathway involving the inter-conversion of cysteine and homocysteine. This pathway is expected to be the most dominant because of the ETEC challenge that increases the demand for Met metabolites such as Cys and GSH. Cystathionine gamma-lyase (CTH), also known as cystathioninase, is an enzyme catalysing the hydrolysis of

cystathionine into cysteine and alpha-ketobuturate. The enzyme catalyses the last step in the trans-sulfuration pathway from methionine to cysteine. Beside protein synthesis, cysteine is used for mucin and GSH production and supports the innate immunity through maintenance of gut barrier function and redox status. A study by Malmezat et al (2000) reported 80% increase in liver trans-sulfuration in rats injected with live *E.coli* and consequently a 45% increase in the liver GSH. The mRNA expression of gene encoding CTH was also quadratically increased in the liver as the SAA:Lys ratio increases showing this role.

It is expected that the remethylation pathway would be dominant over trans-sulfuration when dietary SAA content is low, as explained by Grimble (2006) and that increasing dietary Met intake favours the trans-sulfuration pathway over remethylation. A study by Riedijk et al. (2007) demonstrated that there is not only Met transmethylation but also trans-sulfuration in the pig intestine. Therefore, low CTH mRNA expression in the ileum does not indicate the absence of trans-sulfuration but only this pathway was not affected by dietary SAA:Lys ratio. Additionally, the expression of CTH mRNA confirms that the bulk of the trans-sulfuration is done by the liver due to low activity of CTH in the intestine (Stipanuk 2004). Hence, although there were no data to run the pathway analysis, it appears that trans-sulfuration pathway was favoured over remethylation with increasing SAA:Lys ratio in the current study. In healthy neonates, Met deficiency is associated with up regulation of intestinal SAM, methionine synthase and cystathionine β -synthase but down regulation of SAM and up regulation of cystathionine β -synthase in the liver (Bauchart-Thevret et al. 2009) implying that remethylation is dominant in the liver whereas all other pathways were equally active in the intestine. Therefore, the changes in metabolites with varying dietary SAA content in the current study were similar to those observed in study by Bauchart-Thevert et al. (2009).

The SAA:Lys ratio of 60% is higher than the recommended 55 for protein accretion (NRC 1998; 2012). These findings are contrary to the study by Kim et al. (2012) where SAA:Lys ratio of 75% was suggested to be optimum to support animal health in LPS-injected grower pigs. The variability in optimal SAA:Lys between the two studies may be explained by differences in the models used. Injecting LPS will induce sepsis that stimulates inflammation within 60 min of inoculation (Thorgersen et al. 2010) unlike enteric infection where diarrhoea can take a minimum of 6 h to set, hence it is possible that piglets would require a lower dietary SAA:Lys ratio to boost their immune system during an enteric infection than systemic infection.

Transmethylation, remethylation and trans-sulfuration pathways are the most important pathways through which methionine and cysteine are metabolised. In this study, we measured the mRNA expression of enzymes that are involved in methionine and cysteine metabolism. However, metabolomics analysis and methylation studies would have provided us a better picture for the metabolic profile and the methylation status of these AA. Additionally, determining the point where remethylation is more dominant than trans-sulfuration would indicate the optimum SAA requirement for metabolic need during an ETEC challenge.

The meaningful conclusion that can be drawn from any pathogenic challenge experiment is that the animals that were fed a given diet should be capable to tolerate the pathogenic challenge and grow very well. Therefore, we conclude that SAA:Lys ratio of 60 is the optimum level for piglets exposed to ETEC challenge as feed efficiency was the highest in piglets fed this diet.

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CONFLICT OF INTEREST

The authors disclose no conflict of interest.

REFERENCES

- Abbas, A., Lichtman, A. H. and Pillai, S. 2012. Basic Immunology: Functions and disorders of the immune system. 4th rev. ed. Philadelphia, PA.
- Bauchart-Thevret, C., Stoll, B, Chacko, S. and Burrin, D. G. 2009. Sulphur amino acid deficiency upregulates intestinal methionine cycle activity and suppresses epithelial growth in neonatal pigs. *Am J Physiol Endoc Met.* **296**: E1239-E1250.
- Capozzalo, M. M., Kim, J. C., Htoo J. K., de Lange, C. F., Mullan, B. P., Hansen, C. F., Resink, J. W., Stumbles, P. A., Hampson, D. J. and Pluske, J. R. 2012. An increased ratio of dietary tryptophan to lysine improves feed efficiency and elevates plasma tryptophan and kynurenine in the absence of antimicrobials and regardless of infection with enterotoxigenic *Escherichia coli* in weaned pigs. *J Anim Sci* **90**: 191-193.
- Canadian Council on Animal Care. 2009. Guidelines on the care and use of experimental animals in research, teaching and testing. Ottawa, ON, Canada.
- Commission Directive. 1998. Establishing community methods for the determination of amino acids, crude oils and fats, and olan-quinox in feeding stuff and amending Directive 71/393/EEC, annex part A. Determination of Amino Acids. *Offic J L257*: 14-23.
- Commission Directive. 2000. Establishing community methods for the determination of vitamin A, vitamin E and tryptophan, annex part C. Determination of Tryptophan. *Offic. J. L174*: 45-50.

- 302 Gaggia, F., Mattarelli, P and B. Biavati. 2010. Probiotics and prebiotics in animal feeding for
303 safe food production. *Int J Food Microbiol. Suppl 1*: S15-28.
- 304 Grimble, R. F. and Grimble, G. K. 1998. Immunonutrition: Role of sulphur amino acids, related
305 amino acids, and polyamines. *J Nutr.* **14**: 605-610.
- 306 Grimble, R. F. 2006. The effects of sulphur amino acid intake on immune function in humans. *J*
307 *Nutr.* **136**: 1660S-1665S.
- 308 Gruys, E., Toussaint, M. J., Niewold, T. A. and S.J. Koopmans, S. J. 2005. Acute phase reaction
309 and acute phase proteins. *J Zhejiang Univ Sci. B.* **6**: 1045-1056.
- 310 Jahoor, F., Wykes, L. J., Reeds, P. J., del Rosario, M. P. and Frazer, M. E. 1995. Protein-
311 deficient pigs cannot maintain reduced glutathione homeostasis when subjected to the stress of
312 inflammation. *J Nutr.* **125**: 1462-1472.
- 313 Kahindi, R. K., Htoo, J. K. and Nyachoti, C. M. 2014. The standardised ileal digestible lysine
314 requirement of 7 to 16 kg weaned pigs fed antibiotic-free diet. *Proc. University of Nottingham,*
315 *Jubilee Campus, Wollaton Road, Nottingham, UK.*
- 316 Kim, J. C., Mullan, B. P., Frey, B. Payne, H. G. and Pluske, J. R. 2012. Whole body protein
317 deposition and plasma amino acid profiles in growing and/or finishing pigs fed increasing levels
318 of sulphur amino acids with and without *Escherichia coli* lipopolysaccharide challenge. *J Anim*
319 *Sci.* **90**: 362-365.
- 320 Le Floc'h, N. and Se`ve, B. 2007. Biological roles of tryptophan and its metabolism: potential
321 implications for pig feeding. *Livest Sci.* **112**: 23-32.
- 322 Le Floc'h, N., Melchior, D. and Obled, C. 2004. Modifications of protein and amino acid
323 metabolism during inflammation and immune system activation. *Livest Prod Sci.* **87**: 37-45.

- 324 Litvak, N., Htoo, J. K. and de Lange, C. F. 2013. Restricting sulphur amino acid intake in
325 growing pigs challenged with lipopolysaccharides decreases plasma protein and albumin
326 synthesis. *Can. J Anim Sci.* **93**: 505-515.
- 327 Llames, C. R. and Fontaine, J. 1994. Determination of amino acids in feeds: Collaborative study.
328 *Assoc Offic Anal Chem.* **77**: 1362-1402.
- 329 Malmezat, T., Breuille, D., Capitan, P., Buffière C., Denis, P., Mirand, P. P. and Obled, C. 2000.
330 Methionine transsulfuration is increased during sepsis in rats. *Am J Physiol Endocrinol Metab.*
331 **279**: E1391-1397.
- 332 Maroufyan, E., Kasim, A., Hashemi, S. R., Loh, T. C., Bejo, M. H. and Davoodi, H. 2010. The
333 effect of methionine and threonine supplementations on immune responses of broiler chickens
334 challenged with infectious bursal disease. *Am J Applied Sci.* **7**: 44-50.
- 335 Marquardt, R., Jin, L. Z., Kim, J.W., Fang, L., Frohlich, A. A. and Baidoo, S. K. 1999. Passive
336 protective effect of egg-yolk antibodies against enterotoxigenic *Escherichia coli* K88+ infection
337 in neonatal and early-weaned piglets. *FEMS. Immunol Med Microbiol.* **23**: 283-288.
- 338 Melchior, D., Se`ve, B. and Le Floc'h, N. 2004. Chronic lung inflammation affects plasma
339 amino acid concentrations in pigs. *J Anim Sci.* **82**: 1091-1099.
- 340 National Research Council. 1998. Nutrient requirements of swine. 10th rev. ed. National
341 Academy Press, Washington, DC. P 211.
- 342 National Research Council. 2012. Nutrient requirements of swine. 11th rev. ed. National
343 AcadmyPress, Washington, DC. P 420.
- 344 Opapeju, F. O., Krause, D. O., Payne, R. L. Rademacher, M., Nyachoti, C. M. 2009. Effect of
345 dietary protein level on growth performance, indicators of enteric health and gastrointestinal

- 346 microbial ecology of weaned pigs induced with post-weaning colibacillosis. *J Anim Sci.* **87**:
347 2635-2643.
- 348 Rakhshandeh, A., Htoo, J. K., Karrow, N., Miller, S. P. and de Lange, C. F. 2014. Impact of
349 immune system stimulation on the ileal nutrient digestibility and utilisation of methionine plus
350 cysteine intake for whole-body protein deposition in growing pigs. *Br J Nutr.* **111**: 101-110.
- 351 Riedijk, M. A., Stoll, B., Chacko, S., Schierbeek, H., Sunehag, A. L., van Goudoever, J. B. and
352 Douglas G. Burrin, D. G. 2007. Methionine transmethylation and transsulfuration in the piglet
353 gastrointestinal tract. *PNAS* **104**: 3408-3413.
- 354 Ruth, M. R. and Field, C. J. 2013. The immune modifying effects of amino acids on gut-
355 associated lymphoid tissue. *J Anim Sci Biotechnol.* **4**: 27.
- 356 SAS Institute, Inc. 2010. SAS User's guide: Statistics version. Second ed. SAS Institute, Inc.,
357 Cary, NC. 959 pp.
- 358 Setia, A., Bhandari, S. K., House, J. D., Nyachoti, C. M. and Krause, D. O. 2009. Development
359 and in vitro evaluation of an *Escherichia coli* probiotic able to inhibit the growth of pathogenic
360 *E. coli* K88. *J Anim Sci.* **87**: 2005-2012.
- 361 Stipanuk, M. H. 2004. Sulphur amino acid metabolism: pathways for production and removal of
362 homocysteine and cysteine. *Annu Rev Nutr.* **24**: 539-577.
- 363 Thorgersen, E. B., Hellerud, B. C., Nielsen, E. W., Barratt-Due, A., Fure, H., Lindstad, J. K.,
364 Pharo, A., Fosse, E., Tønnessen, T. I., Johansen, H. T., Castellheim, A. and Mollnes, T. E. 2010.
365 CD14 inhibition efficiently attenuates early inflammatory and hemostatic responses *Escherichia*
366 *coli* sepsis in pigs. *FASEB J.* **24**: 712-722.
- 367 Van der Fels-Klerx, H. J., Puister-Jansen, L. F., Van Asselt, E. D., Burgers, S. L. 2011. Farm
368 factors associated with the use of antibiotics in pig production. *J Anim Sci.* **89**: 1922-1929.

369 Vondruskova, H., Slamova, R., Trckova, M., Zrally, Z. and Pavli I. 2010. Alternatives to
370 antibiotic growth promoters in prevention of diarrhea in weaned piglets: a review Vet Med.
371 55:199-224.

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For Review Only

375 Table 1. Ingredient composition (%) of diets.

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

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378 Table 2. Calculated and analysed nutrient composition of experimental diets.

Item	SID SAA:Lys ratio				
	48	54	60	66	72
Calculated nutrient content					
ME, MJ kg ⁻¹	13.94	13.94	13.94	13.94	13.93
NE, MJ kg ⁻¹	10.25	10.25	10.25	10.25	10.25
CP, %	19.52	19.52	19.52	19.52	19.52
Total Lys, %	1.29	1.29	1.29	1.29	1.29
SID Lys, %	1.18	1.18	1.18	1.18	1.18
SID Met, %	0.26	0.33	0.40	0.49	0.54
SID Cys, %	0.30	0.30	0.30	0.30	0.30
SID Met+cys, %	0.56	0.63	0.70	0.79	0.84
SID Thr %	0.75	0.75	0.75	0.75	0.75
SID Trp %	0.28	0.28	0.28	0.28	0.28
SID Arg, %	1.11	1.11	1.11	1.11	1.11
SID Ile %	0.71	0.71	0.71	0.71	0.71
SID Val %	0.80	0.80	0.80	0.80	0.80
SID Leu, %	1.33	1.33	1.33	1.33	1.33
SID His, %	0.43	0.43	0.43	0.43	0.43
SID Phe, %	0.86	0.86	0.86	0.86	0.86
SID SAA:Lys ratio	0.48	0.54	0.60	0.66	0.72
SID Met:SAA ratio	0.48	0.50	0.57	0.62	0.64
Analysed nutrient content, %					

CP	19.43	20.85	20.56	21.23	20.53
Total Met	0.27	0.39	0.43	0.48	0.60
Total Cys	0.35	0.36	0.35	0.38	0.36
Total Met+Cys	0.62	0.75	0.79	0.86	0.96
SID Met+Cys	0.54	0.66	0.71	0.78	0.87
Total Lys	1.41	1.32	1.35	1.35	1.34
SID Lys	1.28	1.20	1.23	1.23	1.22
Total Thr	0.85	0.84	0.84	0.86	0.85
Total Trp	0.29	0.29	0.29	0.30	0.29
Total Arg	1.17	1.25	1.23	1.27	1.21
Total Ile	0.76	0.84	0.82	0.84	0.80
Total Leu	1.37	1.49	1.45	1.52	1.44
Total Val	0.89	0.96	0.95	0.96	0.91
Total His	0.47	0.50	0.49	0.51	0.49
Total Phe	0.93	1.01	0.98	1.03	0.98
SID SAA:Lys ratio	0.42	0.55	0.58	0.63	0.71

380 Table 3. Primers and their sequences used for real-time PCR assay.

Gene Name	Primer-Forward	Primer-Reverse	Annealing Tem. (°C)
MAT1 α	GGCTTGCGGACCATCTCT	AAGTCAAAGCCACAGGCCAC	60
MAT2 α	GCTCGTTGGGTGGCAAAATC	GCTGTCCCTACCAAAGTGGC	60
CTH	TATCCTGGGTTGCCCTCTCA	AATGGGTCATGATTGCCGGA	58
GAPDH	GGTGAAGGTCGGAGTGAACG	GGGATCTCGCTCCTGGAAGA	58

381 MAT1 α = methionine adenosyltransferase 1 alpha, MAT2 α = methionine adenosyltransferase 2 alpha, MTR = 5-
 382 methyltetrahydrofolate-homocysteine methyltransferase, CTH = cystathionine gamma-lyase, GAPDH = glyceraldehyde-3-phosphate
 383 dehydrogenase.

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Table 4. Faecal consistency score, rectal temperature and serum TNF α concentration of ETEC challenged pigs fed diets with graded SAA:Lys ratios.

Item	SAA:Lys ratio					SEM	P-value		
	48	54	60	66	72		Model	Linear	Quadratic
Diarrhoea score									
Pre-challenge	1.00	1.00	1.03	1.06	1.14	0.04	0.167	0.023	0.316
Post-challenge	1.30	1.43	1.38	1.30	1.23	0.27	0.986	0.760	0.673
Rectal temperature ¹									
Before	38.79	38.74	39.09	39.12	38.53	0.26	0.482	0.865	0.158
24h after	39.04	39.11	39.43	39.24	39.14	0.18	0.628	0.578	0.232
Microbial ² CFU									
Coliforms	6.19	6.42	5.65	5.60	5.73	0.48	0.674	0.255	0.765
ETEC d3	2.35	2.67	2.67	4.41	5.46	0.95	0.123	0.014	0.382
ETEC d5	4.85	2.86	3.23	2.77	5.28	1.12	0.378	0.834	0.065
TNF- α ³									
Before	33.83	50.21	49.69	59.04	37.32	14.94	0.980	0.741	0.685

6h after	36.50c	104.24b	109.85b	239.59a	52.05b	36.81	0.014	0.120	0.011
24h after	35.84b	73.89a	46.63b	71.56a	42.81b	9.69	0.026	0.707	0.042

¹Rectal temperature: before challenge and 24 h after primary challenge; ²Microbial: coliforms before challenge, Ciproflaxin resistant ETEC 3 and 5 days after challenge; ³TNF- α before challenge, 6 h after primary challenge and 24 h after secondary challenge. N = 6

Means with in columns followed by different superscripts are significantly different ($P < 0.05$)

Table 5. Performance of pigs fed diets with graded SAA:Lys levels before and after ETEC challenge.

	SAA:Lys ratio					SEM	P-value		
	48	54	60	66	72		Model	Linear	Quadratic
Before challenge									
ADG, g	73c	126ab	109b	157a	60c	22.02	0.031	0.945	0.009
ADFI, g	150b	176b	172ab	205a	110c	17.67	0.019	0.655	0.012
G:F, g kg ⁻¹	480	710	630	760	540	87.92	0.113	0.798	0.031
SAA intake, g d ⁻¹	0.81	0.99	1.22	1.60	0.52		ND ¹	ND	ND
After challenge									
ADG, g	178	175	195	182	157	31.68	0.945	0.737	0.525
ADFI, g	269	250	289	278	248	21.17	0.573	0.835	0.375
G:F, g kg ⁻¹	660	700	784	650	630	80.80	0.754	0.336	0.435
SAA intake, g d ⁻¹	1.45	1.65	2.05	2.17	2.16	-	ND	ND	ND

¹ND = Not determined; N = 6

Means with in columns followed by different superscripts are significantly different (P < 0.05)

Table 6. Effect of SAA:Lys ratio on liver and ileal gene¹ expression in fold change

	SAA:Lys						P value		
	48	54	60	66	72	SEM	Model	Linear	Quadratic
Liver									
CTH	0.78c	1.01bc	1.31a	1.21ab	1.07b	0.056	<0.001	<.001	<0.001
MAT1A	1.06bc	1.00bc	1.12b	1.27a	0.96c	0.047	<0.001	0.673	0.001
Ileum									
CTH	0.71b	1.03a	0.54c	0.72b	0.77b	0.038	<0.001	0.102	0.038
MAT2A	0.95b	1.03b	1.92a	1.64a	1.21b	0.166	0.001	0.040	0.002

¹MAT1A and MAT2A = methionine adenosyltransferase 1 alpha and 2 alpha, CTH = cystathionine gamma-lyase (n = 6)

Means with in columns followed by different superscripts are significantly different (P < 0.05)