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# Effects of dietary lysine levels on jejunal expression of amino acids transporters and hindgut microflora in weaned pigs

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**ABSTRACT.** The objective of the study was to evaluate the effects of increasing dietary standardized ileal digestible (SID) lysine (Lys) level on the expression of jejunal amino acids (AAs) transporters and the microflora in the hindgut of weaned pigs. One hundred and twenty weanling pigs weighing 8.10 ± 0.48 kg were randomly assigned according to body weight and sex to 5 treatments with 6 replicates per treatment and 4 pigs per replicate. Pigs were fed diets with 0.98, 1.11, 1.23, 1.35 or 1.48% of SID Lys for 28 days. The mRNA expression of cationic amino acids transporter 1 (*CAT1*) in jejunum was higher in groups fed with 1.23, 1.35 and 1.48% SID Lys addition ($P < 0.05$). There was stated a linear increase in the mRNA expressions of *CAT1*, excitatory amino acids carrier 1 (*EAAC1*) and peptide transporter T1 (*PEPT1*) (linear, $P < 0.05$). In the caecum, the populations of bacteria and the content of butyric acid were significantly influenced ($P < 0.05$) by dietary SID Lys. In animals fed diet with 1.35% SID Lys a content of butyric acid was the highest ($P < 0.05$). Moreover, the populations of *Lactobacillus* and *Bifidobacterium* in caecum and colon increased (linear, $P < 0.05$; quadratic, $P < 0.05$) as dietary SID Lys level increased. The obtained results showed that dietary SID Lys level may influence AAs absorption and promote the hindgut health. The suggested supplementation of SID Lys to the weaned pigs' diet is 1.35%.

## Introduction

Amino acids (AAs), important substrates for the synthesis of proteins and other nitrogenous compounds, are also key regulators of major metabolic pathways essential for organism maintenance, growth, immunity and reproduction (Jobgen et al., 2006; Suenaga et al., 2008). It is well known that the intestinal nutrients absorption is mediated by transporter proteins expressed on enterocytes. AAs are transported into the cell by several specific transporters (Verrey et al., 2004) or as di- and tri-peptides by the peptide transporter (Leibach and Ganapathy, 1996). For example, transporters have been characterized as specific for basic amino acids (e.g., cationic amino acids transporter 1, CAT1), neutral amino acids (e.g., system B0 neutral amino acids transporter, B0AT1) and acidic amino acids (e.g., excitatory amino acids carrier 1, EAAC1) (Kanai and Hediger, 2003). The AAs transporters: B0AT1, CAT1, EAAC1 and peptide transporter T1 (PEPT1) are closely associated with AAs absorption capacity in the small intestinal epithelium (Regnault et al., 2002; Hu et al., 2008). In swine diets, Lys is

typically the first limiting AA (Gatrell et al., 2013). A deficiency or excess of Lys can reduce or increase nitrogen retention and whole-body protein turnover (Ren et al., 2007), and affect growth performance, carcass characteristics (Apple et al., 2004), serum parameters (Cameron et al., 2003) and nutrient digestibility (Kim et al., 2011). It has been reported that changes in nutrient content can affect the levels of AAs transporters in the small intestine, skeletal muscle and mammary tissue (Laspiur et al. 2009). However, only few studies on the regulation of AAs transporters mRNA expression by dietary standardized ileal digestible (SID) Lys levels in porcine jejunum have been reported.

The intestinal microflora built of huge amounts of a vast variety of microorganisms plays a key role in keeping individual health. It has been described as an integral part of the host, constituting a so-called 'metaorganism' (Qin et al., 2010). The importance of intestinal microflora to health has gained extensive researchers attention in the last years. It is proven that dietary composition is one of the major factors that can influence the microbial population in the gastrointestinal tract (Zhang et al., 2010). However, it is still unknown whether dietary SID Lys can modulate the microbial population in hindgut of weaned pigs.

Identifying different SID Lys levels that can regulate AAs transporters and the hindgut microflora in weaned pigs is of enormous nutritional importance. Therefore, the aim of the present study was to determine the effects of different dietary SID Lys levels on the expression of AAs transporters in jejunum and changes in microbial population and metabolites in caecum and colon of weaned pigs.

## Material and methods

The experimental design and procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University (Ya'an, China). The experiment was conducted at the swine research facility of the Institute of Animal Nutrition, Sichuan Agricultural University (Ya'an, China).

### Animals, housing and dietary treatments

In total 120 crossbred [Duroc × (Landrace × Yorkshire)] pigs weaned at 21 ± 1 day of age were obtained from a commercial farm (New Hope Group, Sichuan, China). The experiment lasted 33 days (5 days of adaptation and 28 days of feeding experimental diets). Pigs with initial body weight (BW) of 8.10 ± 0.48 kg were assigned to 1 of 5 dietary treatments depending on the BW and sex. Each treatment had 6 replicate pens with 4 pigs per pen. Each pen (2.5 × 1.8 m) was equipped with a 4-hole self-feeder and a single nipple-waterer, allowing pigs to have *ad libitum* access to feed and water. Room temperature for the first week was initially set at 28 °C and was gradually decreased to 25 °C by the end of the trial.

The experimental diets were based on maize, soybean meal and crystalline AAs. The compositions of these diets are given in Tables 1 and 2, respectively. The 5 diets were formulated to contain 0.98, 1.11, 1.23, 1.35 and 1.48% SID Lys. L-Lys · HCl was supplemented to achieve the target SID Lys contents. Crystalline AAs (L-Threonine, DL-Methionine, L-Tryptophan, L-Isoleucine, L-Valine, L-Leucine, L-Histidine, and L-Phenylala-nine; Shanghai Immense Chemical Co., Ltd., Shanghai, China) were also supplemented to maintain the established ideal ratios (AMINO Pig® 1.0, Evonik Industries, Hanau-Wolfgang, Germany). Diets were formulated based on values of AAs content and SID values in ingredients. All diets were formulated to contain similar net energy (NE) content (2510 kcal/kg), and the NE values of ingredients were based on the recommendations of the National Research Council (NRC, 2012).

**Table 1.** Compositions of the experimental diets, as-fed basis

| Ingredients, % | SID Lys[1], % | | | | |
|---|---|---|---|---|---|
| | 0.98 | 1.11 | 1.23 | 1.35 | 1.48 |
| Maize | 62.69 | 63.25 | 64.04 | 65.15 | 66.68 |
| Soybean meal | 22.56 | 21.82 | 20.79 | 19.39 | 17.46 |
| Whey powder | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 |
| Spray-dried plasma | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Soybean oil | 2.41 | 2.22 | 1.91 | 1.47 | 0.84 |
| Limestone | 0.73 | 0.73 | 0.75 | 0.73 | 0.72 |
| Di-calcium phosphate | 1.28 | 1.28 | 1.28 | 1.32 | 1.36 |
| L-Lysine · HCL | 0.15 | 0.33 | 0.52 | 0.72 | 0.94 |
| DL-Methionine | 0.09 | 0.17 | 0.26 | 0.34 | 0.43 |
| L-Threonine | 0.03 | 0.12 | 0.20 | 0.30 | 0.41 |
| L-Tryptophan | 0.03 | 0.06 | 0.09 | 0.13 | 0.16 |
| L-Valine | – | – | 0.09 | 0.20 | 0.31 |
| L-Isoleucine | – | – | 0.03 | 0.13 | 0.22 |
| L-Phenylalanine | – | – | – | 0.06 | 0.16 |
| L-Histidine | – | – | – | 0.03 | 0.09 |
| L-Leucine | – | – | – | 0.01 | 0.19 |
| Mineral-vitamin premix[2] | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Salt | 0.38 | 0.38 | 0.38 | 0.38 | 0.38 |
| Choline chloride | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 |

[1] diets supplemented with standardized ileal digestible lysine at a dose of 0.98, 1.11, 1.23, 1.35 or 1.48%; [2] supplemented per kg of diet: IU: vit. A 8000, vit. D 32000, vit. E 20; mg: vit. $B_1$ 1.5, vit. $B_2$ 5.6, vit. $B_{12}$ 0.02, vit. $B_6$ 1.5, vit. K 32, calcium pantotenate 10, nicotinic acid 15, biotin 0.1, folic acid 0.6, Fe ($FeSO_4$) 100, Cu ($CuSO_4 \cdot 5H_2O$) 20, Zn ($ZnSO_4$) 100, Mn ($MnSO_4$) 60, I (KI) 0.3, Se ($Na_2SeO_3$) 0.3

**Table 2.** Calculated nutritional content of the experimental diets, as-fed basis[1]

| Indices | SID Lys[2], % | | | | |
|---|---|---|---|---|---|
| | 0.98 | 1.11 | 1.23 | 1.35 | 1.48 |
| CP, % | 18.50 (18.50) | 18.50 (18.24) | 18.50 (18.02) | 18.50 (18.34) | 18.50 (18.37) |
| NE, kcal/kg | 2510 | 2510 | 2510 | 2510 | 2510 |
| SID AAs, % | | | | | |
| lysine | 0.98 | 1.11 | 1.23 | 1.35 | 1.48 |
| methionine | 0.33 | 0.40 | 0.49 | 0.53 | 0.64 |
| methionine+cystine | 0.59 | 0.66 | 0.74 | 0.81 | 0.88 |
| threonine | 0.62 | 0.70 | 0.77 | 0.85 | 0.93 |
| tryptophan | 0.22 | 0.24 | 0.27 | 0.30 | 0.32 |
| isoleucine | 0.65 | 0.64 | 0.65 | 0.72 | 0.78 |
| valine | 0.78 | 0.76 | 0.84 | 0.92 | 1.00 |
| leucine | 1.42 | 1.40 | 1.37 | 1.37 | 1.48 |
| arginine | 0.98 | 0.96 | 0.93 | 0.98 | 0.83 |
| histidine | 0.43 | 0.42 | 0.41 | 0.43 | 0.47 |
| phenylalanine | 0.81 | 0.79 | 0.77 | 0.80 | 0.87 |
| Total AAs[3], % | | | | | |
| lysine | 1.11 (1.11) | 1.23 (1.19) | 1.35 (1.37) | 1.47 (1.46) | 1.59 (1.59) |
| methionine | 0.36 (0.38) | 0.43 (0.43) | 0.52 (0.53) | 0.59 (0.59) | 0.67 (0.70) |
| methionine+cystine | 0.68 (0.77) | 0.74 (0.82) | 0.82 (0.91) | 0.89 (0.96) | 0.96 (1.06) |
| threonine | 0.73 (0.75) | 0.81 (0.80) | 0.87 (0.87) | 0.95 (0.96) | 1.03 (1.02) |
| tryptophan | 0.25 (0.25) | 0.27 (0.27) | 0.30 (0.29) | 0.33 (0.32) | 0.35 (0.33) |
| isoleucine | 0.74 (0.72) | 0.73 (0.69) | 0.74 (0.69) | 0.80 (0.77) | 0.86 (0.83) |
| valine | 0.89 (0.91) | 0.88 (0.87) | 0.95 (0.93) | 1.03 (1.02) | 1.10 (1.10) |
| leucine | 1.61 (1.57) | 1.59 (1.51) | 1.56 (1.46) | 1.53 (1.48) | 1.65 (1.60) |
| arginine | 1.06 (1.18) | 1.04 (1.10) | 1.01 (1.06) | 0.96 (1.03) | 0.90 (0.97) |
| histidine | 0.48 (0.51) | 0.48 (0.47) | 0.47 (0.46) | 0.48 (0.46) | 0.52 (0.49) |
| phenylalanine | 0.91 (0.98) | 0.89 (0.96) | 0.87 (0.91) | 0.89 (0.94) | 0.96 (1.01) |
| Calcium,% | 0.70 | 0.70 | 0.70 | 0.70 | 0.70 |
| Available phosphorus, % | 0.36 | 0.36 | 0.36 | 0.37 | 0.37 |

CP – crude protein; NE – net energy; AAs – amino acids; [1] data in parentheses indicate the analysed composition; [2] see Table 1; [3] values for standardized ileal concentrations of AAs were estimated using standardized ileal digestible coefficients provided by NRC (2012) for AA and feed composition data for NE were also obtained from this source

## Sampling and measurements

On day 28, one pig with the average BW (20.45 ± 1.17 kg) in each pen was chosen and euthanized by a lethal dose of sodium pentobarbital (200 mg/kg BW) according to Chen et al. (2013). The abdomen was immediately opened to remove the jejunum, caecum and colon. Intestinal segment of the jejunum was collected and immediately frozen at –80 °C for quantitative Real-Time PCR analysis. Approximately 4 g of the digesta from the caecum and colon were kept in sterile tubes and immediately frozen at –80 °C until analysis of microbial DNA and short-chain fatty acids (SCFA) concentrations.

## Chemical analysis

Diet samples were ground through a 1-mm screen and analysed for crude protein (CP) according to standard methods (AOAC International, 1995). The AAs composition of ingredients and diets sample was measured by ion-exchange chromatography using a Hitachi L-8800 Amino Acids Analyzer (Tokyo, Japan).

## Detection of mRNA expression

Total RNA was isolated from jejunum (the entire alimentary canal without digesta) using Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's protocol. The concentration and purity of the RNA were detected spectrophotometrically (Beckman Coulter DU 800 Spectrophotometer, Beckman Coulter, Inc., Brea, CA, USA). The $OD_{260}$:$OD_{280}$ ratio (where OD – optical density) ranging from 1.8 to 2.0 in all samples was considered as suitable for further analysis. The integrity of RNA was measured by agarose gel electrophoresis and the 28S:18S ribosomal RNA band ratio was found to be ≥1.8. RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent kit (TaKaRa,

**Table 3.** Sequences of primers for the jejunum-related genes

| Gene Bank Acc. No. | Genes | Primer sequence (5'→3') (F – forward, R – reverse) | $A_T$[1], °C | Product size, bp |
|---|---|---|---|---|
| XM_021086047.1 | *ACTB* β-actin | F: TCTGGCACCACACCTTCT<br>R: TGATCTGGGTCATCTTCTCAC | 60 | 114 |
| NM_001012613.1 | *CAT1* cationic amino acids transporter 1; solute carrier family 7 member 1 (*SLC7A1*) | F: GAGCAAGACCAAACTCTCCTTC<br>R: AGCCTATCAGCATCCACACTG | 59 | 137 |
| NM_001164649.1 | *EAAC1* excitatory amino acids carrier 1, solute carrier family 1 member 1 (*SLC1A1*) | F: CAAACTGGGCCTTTACATGG<br>R: GTGTTGCTGAACTGGAGGAGAT | 60 | 170 |
| XM_003359855.4 | *B0AT1* system B0 neutral amino acids transporter; solute carrier family 6 member 19 (*SLC6A19*) | F: ACAACAACTGCGAGAAGGACTC<br>R: GCAGGTCAAACCCGTTGATAAG | 60 | 165 |
| NM_214347.1 | *PEPT1* peptide transporter T1; solute carrier family 15 member 1 (*SLC15A1*) | F: GATGCTGTGGTGTATCCTCTGA<br>R: CCTTTGGGGAAGACTGGAAGA | 60 | 155 |

[1] $A_T$ – annealing temperature

Dalian, China) following the manufacturer's guidelines. Expression levels of *B0AT1*, *CAT1*, *EAAC1* and *PEPT1* in jejunum were carried out by the Opticon DNA Engine (Bio-Rad, Hercules, CA, USA) and using SYBR Green PCR reagents (TaKaRa, Dalian, China). Primers for the selected genes (Table 3) were designed by Primer 6 Software (PREMIER Biosoft Int., Palo Alto, CA, USA) and synthesized commercially by Invitrogen (Shanghai, China). The Real-Time PCR procedure included the following cycle program: an initial pre-denaturation step at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 25 s, with a final extension at 72 °C for 5 min. A melting curve analysis was generated following each Real-Time quantitative PCR assay to check and verify the specificity and purity of all PCR products. β-actin (*ACTB*) was chosen as the reference gene transcript and used for normalization (Lai et al., 2005). The relative expression ratio of the target gene in comparison with the reference gene was calculated using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001). A standard curve was created from serial dilutions of one of the cDNA samples and drawn by plotting the natural log of the threshold cycle ($C_T$) against the natural log of the number of molecules. The standard curve of each gene was run in duplicate and triplicate to obtain reliable amplification efficiency. The relative expression of target genes in the 0.98% SID Lys group was set to be 1.0. Six samples were used for each treatment and each sample was measured in triplicate. The average of each triplicate value expressed as numbers of copies was used for subsequent statistical analysis.

## Microbial population determination

The digesta samples from caecum and colon were quickly collected, placed into sterile tubes, and stored at −80 °C until analysis. Microbial DNA from caecal and colonic digesta samples were isolated using an E.Z.N.A.™ Stool DNA Kit (Omega Bio-Tek, Doraville, CA, USA) according to the manufacturer's instructions. The fluorescent quantitative specific primers and probe of *Escherichia coli*, *Lactobacillus*, *Bifidobacterium* and *Bacillus* were designed followed their genetic sequence of the 16S rRNA. Primers and probe (Table 4) for total bacteria, *Bifidobacterium*, *E. coli*, *Lactobacillus* and *Bacillus* were used according to Diao et al. (2014). All the primers and probes were commercially synthesized by Invitrogen (Shanghai, China). The populations of total bacteria, *Bifidobacterium*, *E. coli*, *Lactobacillus* and *Bacillus* were measured through quantitative Real-Time PCR conducted with the use of CFX96 Real-Time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with optical-grade 96-well plates. To determine the total bacteria, the reaction mixture (25 µl) was composed of 12.5 µl SYBR Premix Ex Taq (Takara, Dalian, China), 1 µl of forward and 1 µl of reverse primers (100 nM), 9.5 µl nuclease-free water and 1 µl DNA. The PCR procedure was: an initial pre-denaturation step at 95 °C for 10 s; 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 25 s and extension at 72 °C for 60 s. The reaction volume for detecting *Lactobacillus*, *E. coli*, *Bifidobacterium* and *Bacillus* was 20 µl, composed of 1 µl probe enhancer solution, 0.3 µl probe (100 nM), 1 µl forward and 1 µl reverse primers (100 nM), 8 µl Real Master

**Table 4.** Sequences of primers and probes for hindgut bacteria

| Primer | Primers and probe sequences (5'→3')[1] | $A_T$[2], °C | Product size, bp |
|---|---|---|---|
| Total bacteria | F: ACTCCTACGGGAGGCAGCAG | 60.0 | 200 |
| | R: ATTACCGCGGCTGCTGG | | |
| *Escherichia coli* | F: CATGCCGCGTGTATGAAGAA | 59.5 | 96 |
| | R: CGGGTAACGTCAATGAGCAAA | | |
| | Probe: AGGTATTAACTTTACTCCCTTCCTC | | |
| *Lactobacillus* | F: GAGGCAGCAGTAGGGAATCTTC | 51.0 | 126 |
| | R: CAACAGTTACTCTGACACCCGTTCTTC | | |
| | Probe: AAGAAGGGTTTCGGCTCGTAAAACTCTGTT | | |
| *Bacillus* | F: GCAACGAGCGCAACCCTTGA | 59.5 | 92 |
| | R: TCATCCCCACCTTCCTCCGGT | | |
| | Probe: CGGTTTGTCACCGGCAGTCACCT | | |
| *Bifidobacterium* | F: CGCGTCCGGTGTGAAAG | 59.5 | 121 |
| | R: CTTCCCGATATCTACACATTCCA | | |
| | Probe: ATTCCACCGTTACACCGGGAA | | |

[1] F – forward primer, R – reverse primer; [2] $A_T$ – annealing temperature

Mix (Tiangen, Beijing, China), 7.7 µl nuclease-free water and 1 µl DNA. The reaction process consisted of predenaturation at 95 °C for 10 s and 50 cycles of denaturation at 95 °C for 5 s, annealing at 55–60 °C for 25 s and extension at 72 °C for 60 s. The copy numbers of total bacteria, *E. coli*, *Lactobacillus*, *Bifidobacterium* and *Bacillus* were quantified with $C_T$-values and standard curves. The respective standard curves were generated by constructing standard plasmids containing the 16s rRNA genes as described by Han et al. (2012). Deoxyribonucleic acid concentrations of standard plasmids were detected using a spectrophotometer (Beckman Coulter DU 800 Spectrophotometer, Beckman Coulter, Inc., Brea, CA, USA). A series of 10-fold dilution ($1 \times 10^9$ to $1 \times 10^1$ copies/µl) of plasmids DNA were used to construct their respective standard curves. Each standard curve was generated by a linear regression of the plotted points with the logarithm of template copy numbers as the abscissa and the $C_T$ values as the ordinate.

## Microbial metabolites measurement

The concentration of SCFA was determined by the gas chromatography and the method described by Diao et al. (2014). The digesta samples of caecum and colon were diluted 1:1 (wt/vol) with distilled water, centrifuged at 500 *g* for 10 min. The supernatant fraction (2 ml) was centrifuged at 12 000 *g* for 10 min at 4 °C, then 1 ml supernatant was transferred to a new centrifuge tube, then added 0.2 ml 25% meta-phosphoric acid, stood for 30 min, the supernatants were further centrifuged at 12 000 *g* for 10 min, 500 µl supernatant was transferred to another centrifuge tube, then 500 µl methanol was added, centrifuged at 12 000 *g* for 10 min again. The clean supernatant was analysed for SCFA (acetic acid, propionic acid and butyric acid) concentrations using a gas chromatographic system (CP-3800 GC, Varian, Inc., Walnut Creek, CA, USA).

## Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) for a randomized block design using the GLM procedure of SAS ver. 8.0 (SAS Institute, Inc., Cary, NC, USA). Orthogonal polynomial contrast coefficients were used to determine linear and quadratic effects of increasing SID Lys level on response criteria. For all data analysis, the individual piglet was used as the experimental unit. Differences among treatments were analysed by Duncan's multiple range test. Probability values less than 0.05 were considered significant, probability values at 0.05 to 0.1 were considered as tendency. All data are presented as means and standard error of the mean (SEM).

# Results

No mortality was noted in the current study. The analysed contents of CP and essential AAs in all diets were close to the calculated values indicating that diets were mixed properly (Table 2).

Average daily feed intake did not differ among animals (data not shown). However, the average

daily gain (ADG) and gain-to-feed ratio (G:F) increased linearly and quadratically from days 0 to 28 as SID Lys increased. The serum urea nitrogen (SUN) concentrations were the same on day 0, but they decreased linearly on day 14 and decreased quadratically on day 28 as SID Lys increased. The optimal SID Lys requirement for pigs weighting 8 to 20 kg fed 18.5% CP diet based on the average value of ADG, G:F and SUN was estimated at 1.31%.

## The mRNA levels of nutrient transporters in jejunum

There were no differences ($P > 0.05$) in *B0AT1*, *EAAC1* and *PEPT1* mRNA levels among the five groups (Table 5). However, the mRNA levels of *CAT1* in pigs fed the 1.35% SID Lys diet was higher ($P < 0.01$) in comparison to those fed the 0.98, 1.11 and 1.23% SID Lys diets, and it was similar to the 1.48% SID Lys group. Additionally, the *CAT1* (linear, $P < 0.01$), *EAAC1* (linear, $P < 0.05$) and *PEPT1* (linear, $P < 0.01$) mRNA levels increased as SID Lys levels increased in weaned pigs diet.

## Microbial populations in caecum and colon

There were significant differences ($P < 0.01$) among treatment groups for the *E. coli*, *Lactobacillus*, *Bacillus*, *Bifidobacterium* and total bacteria populations in caecal digesta, whereas these bacteria among the treatment groups in colonic digesta were not influenced ($P > 0.05$) (Table 6). The populations of *Lactobacillus*, *Bacillus*, *Bifidobacterium* as well as the total bacteria in caecal digesta increased (linear, $P < 0.01$) with increasing dietary SID Lys levels, at the same time, increasing SID Lys levels increased (linear, $P < 0.01$) the population of *E. coli* in caecal digesta. Furthermore, the populations of *Lactobacillus* (linear, $P < 0.05$) and *Bifidobacterium* (quadratic, $P < 0.05$) increased with increasing SID Lys levels in colonic digesta.

## Microbial metabolites in caecum

In the caecal digesta, the content of butyric acid was influenced ($P < 0.05$) by the different dietary SID Lys levels, whereas the acetic acid, propionic acid and total SCFA content was not influenced ($P > 0.05$) (Table 7). Moreover, the content of butyric acid was

**Table 5.** Effect of increasing dietary level of standardized ileal digestible lysine (SID Lys) on amino acids transporter genes expression in the jejunum of weaned pigs

| Gene | SID Lys[1], % | | | | | SEM[2] | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.98 | 1.11 | 1.23 | 1.35 | 1.48 | | ANOVA | linear[3] | quadratic[3] |
| *CAT1* | 1.00[b] | 1.16[b] | 1.24[b] | 2.02[a] | 2.04[a] | 0.10 | <0.01 | <0.01 | 0.48 |
| *B0AT1* | 1.00 | 0.91 | 0.90 | 1.12 | 0.83 | 0.15 | 0.73 | 0.76 | 0.84 |
| *EAAC1* | 1.00 | 1.48 | 1.32 | 1.45 | 1.36 | 0.28 | 0.23 | 0.05 | 0.08 |
| *PEPT1* | 1.00 | 0.89 | 1.09 | 1.15 | 1.37 | 0.19 | 0.18 | 0.01 | 0.90 |

[1] see Table 1; [2] SEM – standard error of mean; [3] linear and quadratic contrast for effect of SID Lys level; [ab] – means with different superscripts within a row are significantly different at $P < 0.05$

**Table 6.** Effect of increasing dietary level of standardized ileal digestible lysine (SID Lys) on hindgut bacteria in the caecal and colonic digesta of weaned pigs, lg(copies/g) [1]

| Indices | SID Lys[1], % | | | | | SEM[2] | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.98 | 1.11 | 1.23 | 1.35 | 1.48 | | ANOVA | linear[3] | quadratic[3] |
| Caecum | | | | | | | | | |
| *Escherichia coli* | 8.02[b] | 8.03[b] | 8.41[b] | 8.49[ab] | 9.28[a] | 0.27 | <0.01 | <0.01 | 0.32 |
| *Bacillus* | 9.59[b] | 9.73[b] | 10.13[a] | 10.09[a] | 10.06[a] | 0.05 | <0.01 | <0.01 | <0.01 |
| *Lactobacillus* | 8.08[c] | 8.40[bc] | 8.89[ab] | 8.84[ab] | 8.98[a] | 0.14 | <0.01 | <0.01 | 0.05 |
| *Bifidobacterium* | 9.34[b] | 9.71[ab] | 10.21[a] | 10.19[a] | 10.10[ab] | 0.18 | 0.01 | 0.01 | 0.07 |
| Total bacteria | 11.08[b] | 11.13[b] | 11.41[a] | 11.37[a] | 11.44[a] | 0.06 | <0.01 | <0.01 | 0.14 |
| Colon | | | | | | | | | |
| *Escherichia coli* | 7.86 | 7.94 | 7.78 | 8.05 | 8.48 | 0.29 | 0.34 | 0.15 | 0.48 |
| *Bacillus* | 9.97 | 9.97 | 9.96 | 9.83 | 9.82 | 0.06 | 0.15 | 0.06 | 0.64 |
| *Lactobacillus* | 8.91 | 8.87 | 9.12 | 9.09 | 9.19 | 0.12 | 0.34 | 0.04 | 0.88 |
| *Bifidobacterium* | 9.94 | 10.25 | 10.32 | 10.18 | 10.14 | 0.14 | 0.07 | 0.13 | 0.02 |
| Total bacteria | 11.44 | 11.58 | 11.50 | 11.36 | 11.47 | 0.06 | 0.19 | 0.41 | 0.65 |

[1] see Table 1; [2] SEM – standard error of mean; [3] linear and quadratic contrast for effect of SID Lys level; [abc] – means with different superscripts within a row are significantly different at $P < 0.05$

**Table 7.** Effect of increasing dietary level of standardized ileal digestible lysine (SID Lys) on the short-chain fatty acids (SCFA) concentrations in caecal digesta of weaned pigs

| Indices | SID Lys[1], % | | | | | SEM[2] | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.98 | 1.11 | 1.23 | 1.35 | 1.48 | | ANOVA | linear[3] | quadratic[3] |
| Acetic acid, mg/g | 4.20 | 3.78 | 3.81 | 3.49 | 3.51 | 0.30 | 0.52 | 0.09 | 0.58 |
| Propionic acid, mg/g | 2.25 | 2.40 | 2.19 | 2.26 | 1.84 | 0.15 | 0.18 | 0.09 | 0.23 |
| Butyric acid, mg/g | 0.70[b] | 0.90[ab] | 0.91[ab] | 1.18[a] | 0.80[ab] | 0.09 | 0.02 | 0.20 | 0.04 |
| Total SCFA, mg/g | 7.16 | 7.08 | 6.91 | 6.94 | 6.15 | 0.43 | 0.57 | 0.16 | 0.55 |

[1] see Table 1; [2] SEM – standard error of mean; [3] linear and quadratic contrast for effect of SID Lys level; [ab] – means with different superscripts within a row are significantly different at $P < 0.05$

increased (quadratic, $P < 0.05$) with increasing dietary SID Lys levels and the 1.35% SID Lys treatment showed a significantly higher content of butyric acid than the other treatments ($P < 0.05$).

## Discussion

Animal life is built of many gene expression processes. These processes are genetically preprogrammed, but dietary nutrients play a crucial role in activating these processes (Liao et al., 2015). It has been reported that regulation of nutrient transporters in the small intestine is connected with the nutrients present in the diet (Gilbert et al., 2008). Amino acids are transported by neutral, acidic or basic AAs transporters (Wu, 2013). B0AT1 is the major apical neutral amino acid transporter in the kidney and the small intestine (Bröer, 2008). CAT plays an important role in the transport and maintenance of homeostasis of basic AAs (arginine, histidine, lysine and ornithine) in the small intestine (Bröer et al., 2000; Hyde et al., 2003). Peptide transport which is $H^+$-dependent is one of the major routes of AA assimilation by the enterocyte (Chen et al., 2002). In the present study, the mRNA levels of *B0AT1*, *EAAC1* and *PEPT1* in jejunum were not influenced by dietary SID Lys levels. However, different dietary SID Lys levels affected the mRNA level of *CAT1* in jejunum, which is in line with the recent reports stating that diets with different Lys levels have different effects on the expression of cationic AAs transporters in the small intestine of weaned pigs (García-Villalobos et al., 2012; Wang et al., 2012). Moreover, the quantities of *CAT1* mRNA in the 1.35% SID Lys group were greater in jejunum, and the mRNA levels of *CAT1* linearly increased as dietary SID Lys increased significantly. Likewise, increasing mRNA levels of *EAAC1* and *PEPT1* were connected by increasing SID Lys. Thus, it may imply that diets with higher SID Lys content (i.e. 1.35% SID Lys) may improve the transport capacity of some AAs in the small intestine.

In the gastrointestinal tract the populations and species of microbes affect the nutrient digestibility and have an impact on gut health (Yang et al., 2010). It is widely known that *Lactobacilli*, *Bacillus* and *Bifidobacterium* are considered as beneficial gut bacteria, whereas *E. coli* are often harmful. Microbial population in the gastrointestinal tract is dependent on the diet composition (Yin et al., 2009; Blachier et al., 2010). It seems that the available amounts of substrates for bacterial proliferation in the gastrointestinal tract may be affected by reducing dietary protein levels and supplementating AAs to low protein diets (Dai et al., 2011). The results of the present study indicate that the populations of *E. coli*, *Lactobacilli*, *Bacillus* and *Bifidobacterium* total bacteria in the caecal digesta were influenced by dietary levels of SID Lys, and all of them increased linearly as dietary SID Lys increased. Furthermore, the results of *Lactobacilli*, *Bacillus* and *Bifidobacterium* populations may suggest that increasing dietary SID Lys levels decreased the amounts of substrates for bacterial proliferation which was beneficial for hindgut health. In the present study, the crystalline AA was increased as dietary SID Lys level increased and it is well known that free AA are completely absorbed to the end of the small intestine. It can be expected that protein content or other AA content in pig digesta reaching the large intestine was decreased with increasing SID Lys level. Indeed, in another study at our laboratory, it was found that the apparent total tract digestibilities of crude protein and all essential AAs in 1.35% SID Lys group were higher than in 1.23% SID Lys group (data unpublished). Moreover, it was noted that the higher levels of by L-Lys and maize with reduced soybean meal, the increased concentration of starch (and fibre) in the highest Lys diet. Protein fermentation can contribute to yield toxic metabolites (e.g., sulphur-containing compounds, ammonia and several phenolic and

indolic compounds) (Egert et al., 2006) whereas carbohydrate fermentation leads to the appearance of perceived health-promoting metabolites such as short-chain fatty acids and reduction of ammonia and amines in the pig hindgut (Scheppach, 1994; Pieper et al., 2012). These facts may be the main reason for the observed results. However, the results concerning *E. coli* were in the contrary. In the diets with high SID Lys level the imbalance of Lys can result in not successful utilisation of all protein (AA), leading to the degradation of protein/AA which can enter the hindgut and also produce the urea. We have also found increased plasma urea N in animals fed Lys or other AA imbalanced diets (data not shown) suggesting increased population of *E. coli* in the 1.48% SID Lys group.

Furthermore, in the current study it was found that populations of *Lactobacillus* and *Bifidobacterium* in the colonic digesta increased linearly and quadratically as SID Lys increased. Taking into account the results mentioned above, it is suggested that appropriately increased dietary SID Lys levels may have positive influence of the number of beneficial bacteria in the hindgut of weaned pigs. Moreover, further research is needed to reveal the effects of dietary SID Lys levels on the microbial population of weaned pigs.

Short-chain fatty acids (SCFA) are the major end products of the fermentation processes of intestinal microflora in the large intestine (Bergen and Wu, 2009), and are diet type- and microbial composition-dependent (van Beers-Schreurs et al., 1998). SCFA, particularly butyrate, are the major and preferred metabolic substrates for colonocytes providing at least 60–70% of their energy requirements necessary for their proliferation and differentiation (Suzuki et al., 2008). They can also maintain normal morphology of intestinal mucosal epithelial cells as well as the normal function of the intestinal mucosa barrier as well as inhibit intestinal inflammatory reaction (Ferreira et al., 2012). In the present study, the concentration of butyric acid increased quadratically with increasing SID Lys and maximize in 1.35% SID Lys group. Moreover, it was previously reported that a higher concentration of acetic and propionic acids can inhibit the activity of bacteria which produce butyric acid (Duncan et al., 2007). Interestingly, in current study the concentrations of acetic and propionic acids tended to decrease linearly. Thus, diet with 1.35% SID Lys may be beneficial for promoting the production of butyrate in the hindgut of weaned pigs.

## Conclusions

The cationic amino acids transporter 1 (*CAT1*) mRNA expression, the populations of bacteria and the content of butyric acid in weaned pigs were significantly influenced by different dietary standardized ileal digestible lysine (SID Lys) levels. When dietary SID Lys level was 1.35% it contributed, to some extent, to increasing absorption of amino acids and promoted the hindgut health of weaned pigs.

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