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Original Research Article

Growth performance, serum parameters, inflammatory responses, intestinal morphology and microbiota of weaned piglets fed 18% crude protein diets with different ratios of standardized ileal digestible isoleucine to lysine

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ABSTRACT

The present study was to explore the Ile requirement of piglets fed 18% crude protein (CP) diets. Two hundred and fifty 28-day-old Duroc × Landrace × Yorkshire piglets (8.37 ± 1.92 kg) were randomly divided into 5 dietary treatments (10 piglets per replicate, 5 barrows and 5 gilts per replicate) with 45%, 50%, 55%, 60%, 65% standardized ileal digestible (SID) Ile-to-Lys ratios, and the SID Lys was formulated to 1.19%. The experimental design consisted of two phases (d 1 to 14 and d 15 to 28). Results showed that average daily gain (ADG) had a tendency to quadratically increase as the SID Ile-to-Lys ratio increased ($P = 0.09$), and the optimum SID Ile-to-Lys ratios required to maximize ADG were 48.33% and 54.63% for broken-line linear model and quadratic polynomial model, respectively. Different SID Ile-to-Lys ratios had no significant effects on average daily feed intake and gain-to-feed ratio. Dry matter ($P < 0.01$), CP ($P = 0.01$), ether extract ($P = 0.04$), gross energy ($P < 0.01$) and organic matter ($P < 0.01$) digestibility increased quadratically. Serum total cholesterol levels decreased linearly ($P = 0.01$) and quadratically ($P < 0.01$); aspartate aminotransferase ($P < 0.01$), interleukin-1β ($P = 0.01$), and tumor necrosis factor-α ($P < 0.01$) levels decreased quadratically; immunoglobulin G ($P = 0.03$) and immunoglobulin M ($P = 0.01$) concentrations increased quadratically. Serum Ser levels decreased linearly ($P < 0.01$) and quadratically ($P = 0.01$); Glu ($P = 0.02$), Arg ($P = 0.05$), and Thr ($P = 0.03$) levels decreased quadratically; Gly ($P < 0.01$) and Leu ($P = 0.01$) levels decreased linearly; Ile ($P < 0.01$) concentration increased linearly. Duodenal villus height ($P < 0.01$) and villus height to crypt depth ratio ($P < 0.01$) increased quadratically. The deficiency or excess of Ile decreased short chain fatty acid-producing bacteria abundance and increased pathogenic bacteria abundance. Overall, taking ADG as the effect index, the optimum SID Ile-to-Lys ratios of piglets offered 18% CP diets were 48.33% and 54.63% based on two different statistical models, respectively, and the deficiency or excess of Ile negatively affected piglet growth rates and health status.

1. Introduction

Nitrogen pollution and dietary protein source shortage limit pig industrial development, so protein restriction is considered a promising strategy (Zhang and Piao, 2022). Additionally, weaning is a stressful event that negatively impacts digestion and absorption capacity, immune function and intestinal microbiota in piglets (Ma et al., 2021a). A reduction of dietary protein level can alleviate weaning stress of piglets; however, benefits of the reduction in protein levels are usually accompanied by sub-optimal growth of

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piglets, as organisms need to reduce protein synthesis rates to adapt to insufficient protein intake (Garlick et al., 1999). It has been reported that low protein diets containing functional amino acids (AA) can improve growth rate and nitrogen utilization of piglets (Figueroa et al., 2002; Lordelo et al., 2008; Torrazza et al., 2010). It has been well documented that except for Lys, Met, Thr and Trp, branched chain amino acids (BCAA) supplemented to low protein diets can improve piglet performance (Lordelo et al., 2008).

The BCAA including Leu, Ile and Val regulate key metabolic pathways that are necessary for maintenance, growth and immunity, and can only be offered through diets (Zhang et al., 2017; Zhou et al., 2018). The BCAA were reported to improve feed intake, AA utilization efficiency, immune function, intestinal development and microbial composition of piglets (Zhang et al., 2013a; Ren et al., 2015; Kwon et al., 2020; Habibi et al., 2021). Insufficient levels of BCAA in the diet may negatively affect growth rates of piglets (van Milgen et al., 2012). However, several studies demonstrated that excessive intake of AA reduced energy utilization efficiency (Just, 1982). When the level of AA in diets reaches the required level, the nitrogen utilization efficiency is the highest (van Milgen et al., 2012). As one of the BCAA, it is necessary to explore Ile requirements in pig diets. However, most studies focused on Ile requirements of pigs fed diets with blood products (van Milgen et al., 2012). Further research is necessary to explore requirements of Ile in piglets fed blood cell-free diets, and facilitate the preparation of diets with more reasonable AA levels. Additionally, physiological and nutritional functions of Ile still need to be clarified. We can further explore the recommended level of Ile by analyzing the impacts of Ile on serum biochemical indices and intestinal health of pigs. Therefore, this experiment aimed to estimate the optimal standardized ileal digestible (SID) level of Ile in 18% crude protein (CP) diets, and explore the effects of Ile on growth performance, serum biochemical indices and intestinal health of piglets.

2. Materials and methods

2.1. Animal ethics statement

The Institutional Animal Care and Use Committee of China Agricultural University approved the experimental procedures used in this study (Beijing, China). CJ Cheiljedang Corporation (Seoul, Korea) provided the Ile product in this experiment.

2.2. Experimental animals, design, diets, management and procedures

Two hundred and fifty 28-d-old Duroc × Landrace × Yorkshire piglets (8.37 ± 1.92 kg) were randomly divided into 5 groups according to a randomized complete block design based on body weight (BW) and gender. Each group contained 5 replicates (10 piglets per replicate), and 5 barrows and 5 gilts per replicate. The 250 piglets were fed 18% CP diets with SID Ile-to-Lys ratios of 45% (N45), 50% (N50), 55% (N55), 60% (N60) and 65% (N65). The SID AA levels were calculated by multiplying the SID coefficients provided by the NRC (2012) by AA levels of these ingredients. An experimental diet containing 1.19% SID Lys was prepared, which was lower than the value obtained from the NRC (2012). The Lys level aimed to ensure that Lys has a marginal limit on pigs and the SID Ile-to-Lys ratio requirement is not underestimated. Except Ile, the remaining indispensable AA-to-Lys ratios were set to exceed the NRC (2012) recommendation by 105% (Table 1). The experiment period consisted of phase 1 (d 1 to 14) and phase 2 (d 15 to 28). The piglets were raised in the pens with plastic floors and nipple drinkers, and had free access to water and feed throughout the period. The room temperature was controlled at 24 to 26 °C. During this experiment, the weaned piglets were vaccinated on d 7, 14 and 21, and the health status of the piglets was regularly monitored.

Table 1
Composition and nutrient levels of basal diets (%, as-fed basis).

Item	SID Ile-to-Lys ratio				
	45%	50%	55%	60%	65%
Corn	64.12	64.06	64.00	63.94	63.88
Soybean meal, 46% CP	9.80	9.80	9.80	9.80	9.80
Fish meal, 64.7% CP	2.50	2.50	2.50	2.50	2.50
Peanut meal	3.00	3.00	3.00	3.00	3.00
Corn gluten meal	5.00	5.00	5.00	5.00	5.00
Whey powder	10.00	10.00	10.00	10.00	10.00
Soy oil	1.00	1.00	1.00	1.00	1.00
Limestone	0.80	0.80	0.80	0.80	0.80
$CaHPO_3$	0.85	0.85	0.85	0.85	0.85
Salt	0.33	0.33	0.33	0.33	0.33
L-Lysine HCl	0.72	0.72	0.72	0.72	0.72
DL-Met	0.30	0.30	0.30	0.30	0.30
L-Thr	0.33	0.33	0.33	0.33	0.33
L-Tyr	0.12	0.12	0.12	0.12	0.12
Val	0.18	0.18	0.18	0.18	0.18
Ile	0.00	0.06	0.12	0.18	0.24
ZnO	0.20	0.20	0.20	0.20	0.20
Cr_2O_3	0.25	0.25	0.25	0.25	0.25
Vitamin-mineral premix[1]	0.50	0.50	0.50	0.50	0.50
Chemical composition					
CP[2]	17.78	17.74	17.80	17.82	17.85
DM[2]	88.66	89.35	88.73	88.60	88.66
EE[2]	3.10	3.10	3.05	3.07	3.00
GE[2], MJ/kg	16.26	16.45	16.24	16.18	16.25
OM[2]	83.55	84.41	83.47	83.52	83.83
SID Lys[3]	1.19	1.19	1.19	1.19	1.19
SID Met[3]	0.59	0.59	0.59	0.59	0.59
SID Thr[3]	0.82	0.82	0.82	0.82	0.82
SID Trp[3]	0.24	0.24	0.24	0.24	0.24
SID Ile[3]	0.54	0.60	0.66	0.72	0.77
SID Leu[3]	1.49	1.49	1.49	1.49	1.49
SID Val[3]	0.84	0.84	0.84	0.84	0.84
SID Ile-to-Lys ratio[3]	0.45	0.50	0.55	0.60	0.65
SID Lys-to-Leu ratio[3]	1.25	1.25	1.25	1.25	1.25

SID = standardized ileal digestible; CP = crude protein; DM = dry matter; EE = ether extract; GE = gross energy; OM = organic matter.
[1] Premix provided the following per kilogram of diets for weaned piglets: vitamin A, 12,000 IU; vitamin D_3, 2,500 IU; vitamin E, 30 IU; vitamin K_3, 3.0 mg; vitamin B_{12}, 0.012 mg; riboflavin, 4.0 mg; pantothenic acid, 15.0 mg; niacin, 30.0 mg; choline chloride, 400.0 mg; folacin, 0.7 mg; vitamin B_1, 1.5 mg; vitamin B_6, 3 mg; Mn, 40.0 mg; Fe, 75.0 mg; Zn, 75.0 mg; Cu, 100.0 mg; I, 0.3 mg; Se, 0.3 mg.
[2] Analyzed values.
[3] Values were calculated according to NRC (2012).

2.3. Sample collection

We collected about 1000 g feces of each pen from d 26 to 28, then the samples were mixed completely and kept in an oven drying at 65 °C for 72 h. The collected feed and fecal samples were ground to pass through a 1-mm sieve and kept at 4 °C for further analysis. On d 28, approximately 5 mL of blood was sampled from each barrow with BW closest to the mean pen BW per pen using vacuum tubes, then centrifuged at 3000 × *g* for 15 min for collecting the supernatant. Subsequently, one barrow per pen (with BW closest to the mean BW in each pen) was slaughtered, and the samples of pancreas, ileum, chyme of ileum, cecum and colon were collected and rapidly placed in liquid nitrogen. The samples of the middle parts of the duodenum, jejunum and ileum were also collected and fixed with 4% paraformaldehyde for hematoxylin and eosin staining.

2.4. Growth performance, diarrhea rate and nutrient digestibility

The BW of piglets and feed consumption of each pen were recorded to calculate the average daily gain (ADG), average daily

feed intake (ADFI) and gain-to-feed ratio. During the experiment, the fecal consistency of piglets was assessed and evaluated every day to calculate the diarrhea rates as previously reported (Shang et al., 2021a).

The proximate composition ash, CP, ether extract (EE) and dry matter (DM) of the feed and fecal samples were determined according the Association of Official Agricultural Chemists (AOAC, 2012). The gross energy (GE) was determined by using the adiabatic oxygen bomb calorimeter (Parr 6300 Calorimeter, Moline, IL, USA). The chromium (Cr) concentration was analyzed using anatomic absorption spectrometry (Z-5000; Hitachi, Tokyo, Japan) as Williams et al. (1962) described. The apparent total tract digestibility (ATTD) of nutrients was calculated as follows:

$$\text{ATTD of nutrients (\%)} = [1 - (Cr_{diets} \times Nutrient_{feces})/(Cr_{feces} \times Nutrients_{diets})] \times 100\%$$

For analyzing AA, excluding Met, Trp and Cys, feed samples were first treated with 6 mol/L HCl for 24 h at 110 °C, and AA composition was determined using an automatic amino acid analyzer (Hitachi High-Technologies Co., Japan, L-8900). Cys and Met were measured as cysteic acid and methionine sulfone after hot acid hydrolysis and quantitative oxidation with performic acid. The Trp level was examined by high performance liquid chromatography (Agilent 1200 Series; Aligent, Santa Clara, CA, USA) after hydrolysis in 4 mol/L LiOH for 22 h at 110 °C.

2.5. Serum biochemical indices and amino acids

The Sykam S433 amino acid analyzer was applied to determine serum AA concentrations as previously described (Sedgwick et al., 1991). Serum biochemical indices including urea nitrogen (BUN), glucose (GLU), triglyceride (TG), total cholesterol (TC), creatine kinase (CK), high density lipoprotein (HDL), low density lipoprotein (LDL), lactate dehydrogenase (LDH), total protein (TP), albumin (ALB), globulin (GLB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were determined by commercially available kits (Laibo Tairui Technology Development Co., Ltd, Beijing, China).

2.6. Digestive enzyme activity

For determination of pancreatic enzyme activity, approximately 1 mg of pancreatic tissue samples was homogenized on ice with 9 mL of phosphate buffer saline. The homogenized tissues were centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatants were carefully collected, and the double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used to measure activities of α-amylase, lipase, chymotrypsin and trypsin, using ELISA kits (Laibo Tairui Technology Development Co., Ltd, Beijing, China). The total concentrations of protein in samples were measured using a bicinchoninic acid protein assay kit (Jiancheng Bioengineering Institute, Nanjing, China).

2.7. Intestinal morphology

For analyzing intestinal morphology, samples were embedded in paraffin after dehydration with ethanol solution, then sliced into sections of 4 μm by a microtome (Leica, Wetzlar, Germany) and stained by hematoxylin and eosin to determine villus height (VH), crypt depth (CD), and VH to CD ratio. The examination of sections was performed at least in 10 well-oriented intact villi and their associated crypts by light microscopy.

2.8. Immunoglobulin and cytokine levels

Serum immunoglobulin A (IgA), immunoglobulin M (IgM), immunoglobulin G (IgG), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) levels were measured by ELISA kits (Laibo Tairui Technology Development Co., Ltd, Beijing, China).

2.9. Tight junctional protein level

The real-time PCR analysis was performed to analyze occludin, claudin-1 and zonula occludens-1 (*ZO-1*) relative mRNA expression levels of ileal samples. Trizol reagent (Invitrogen, USA) was used for the extraction of total RNA, and quality and quantity of extracted RNA were assessed by a NanoDrop spectrophotometer. The PrimeScript RT Reagent kit (TaKaRa, Dalian, China) was applied to perform reverse transcription. The PCR procedure consisted of predenaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 10 s, and annealing at 60 °C for 30 s. The PCR reaction mixture (10 μL) contained cDNA (1 μL), SYBR Green Master Mix (1 μL), ROX Reference Dye (0.2 μL), and forward and reverse primers (0.2 μL × 2) which are shown in Table 2. The relative expression levels of each gene were calculated by using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001).

2.10. 16S RNA sequencing

Total DNA was extracted from all ileal, cecal and colonic digesta samples using the Stool DNA Kit (Omega Bio-tek, Norcross, GA, USA). The method for DNA sequencing was carried out as previously reported (Shang et al., 2019). QIIME (version 1.17) was applied for demultiplexing and quality-filtering of raw sequences. The sequences were clustered into operational taxonomic units (OTUs) at a similarity level of 97%.

2.11. Short chain fatty acid (SCFA) concentration

The ileal, cecal and colonic SCFA contents were analyzed according to the method previously reported (Shang et al., 2019). Intestinal digesta was added to 8 mL of distilled water, and the mixture was then centrifuged (12,000 × *g*, 10 min). The collected supernatant was filtered through a 0.22-μm membrane, and then detected by an Ion Chromatography system (DIONEX ICS-3000, Thermo Fisher, Waltham, MA, USA).

2.12. Statistical analysis

The results in this experiment were analyzed using SAS 9.4 software (SAS Inst. Inc., Cary, NC, USA). For growth performance and diarrhea rates, the individual pen was considered as an

Table 2
Primer sequences of housekeeping and target genes concerned with intestinal barrier function.

Item	Primer sequences (5′ to 3′)
β-Actin	F : TACGCCAACACGGTGCTGTC
	R : GTACTCCTGCTTGCTGATCCACAT
Occludin	F : ATGCTTTCTCAGCCAGCGTA
	R : AAGGTTCCATAGCCTCGGTC
Claudins	F : CAAAACCTTCGCCTTCCAG
	R : TCCCCACATTCGAGATGATTAC
ZO-1	F : GAGGATGGTCACACCGTGGT
	R : GGAGGATGCTGTTGTCTCGG

ZO-1 = zonula occludens-1; F = forward primer; R = reverse primer.

experimental unit, and when analyzing other data, the individual pig was considered as an experimental unit. The homogeneity of variance and normal distribution were analyzed by the process of HOVTEST and PROC UNIVARIATE NORMAL, and we performed the χ^2 contingency test to analyze diarrhea rates. The linear and quadratic regression analyses were applied to evaluate the dose-response effect of SID Ile-to-Lys ratio. The differences were considered as significant at $P < 0.05$, the highly significant difference was regarded as $P < 0.01$, and $0.05 \leq P < 0.10$ indicted a significance of trend. The estimates of the Ile requirement for optimum performance were determined by subjecting the ADG data to the broken-line linear model, $y = L + U \times (R-x)$, where $(R-x)$ is zero when $x > R$, and quadratic polynomial model, $y = U(x-R)^2 + L$, according to the formula reported by Clark et al. (2017). In these equations, y is the response of the piglet, x is the SID Ile-to-Lys ratio of the dietary treatment, U is the slope of the curve, L is the inflection point, R is the requirement of Ile. For the analysis of 16S rRNA gene sequencing data, the Chao and Shannon diversity indices were analyzed using R software (version 3.6.3), and differences were analyzed using the one-way ANOVA method. The linear discriminant analysis effect size (LEfSe) was applied to analyze differences in microbiota composition, and the biomarker was identified with linear discriminant analysis (LDA), with scores higher than 2.

3. Results

3.1. Growth performance and diarrhea rate

As illustrated in Table 3, from d 1 to 14, ADG showed a quadratic increase as the SID Ile-to-Lys ratio increased ($P = 0.02$). From d 1 to 28, ADG tended to increase quadratically as the SID Ile-to-Lys ratio increased ($P = 0.09$), and when piglets were fed the diet with 55% SID Ile-to-Lys ratio, the ADG was the highest. However, during all stages, ADFI, gain-to-feed ratio and diarrhea rates (Fig. 1) were not significantly affected by the dietary treatments. Because different ratios of SID Ile to Lys only significantly affected the ADG, broken-line linear and quadratic polynomial model analyses were chosen to determine the optimal SID Ile-to-Lys ratio. As shown in Fig. 2, the ADG reached the highest, when the SID Ile-to-Lys ratios were 48.33% and 54.63% based on a broken-line linear model and a quadratic polynomial model, respectively.

3.2. Nutrient digestibility

The ATTD of DM ($P < 0.01$), CP ($P = 0.01$), EE ($P = 0.04$), GE ($P < 0.01$) and OM ($P < 0.01$) quadratically enhanced as the SID Ile-to-Lys ratio increased (Table 4).

Table 3
Effects of SID Ile-to-Lys ratios on weaned piglet growth performance[1].

Item	SID Ile-to-Lys ratio					SEM	*P*-value	
	45%	50%	55%	60%	65%		Linear	Quadratic
Day 1 to 14								
ADG[2], g	384	418	424	411	392	8.7	0.83	0.02
ADFI[3], g	538	563	593	565	542	11.6	0.91	0.13
Gain-to-feed ratio[4]	0.71	0.72	0.72	0.73	0.73	0.009	0.49	0.92
Day 14 to 28								
ADG, g	574	612	621	602	591	11.4	0.77	0.19
ADFI, g	908	962	975	945	903	26.2	0.87	0.22
Gain-to-feed ratio	0.63	0.66	0.64	0.64	0.66	0.018	0.64	0.94
Day 1 to 28								
ADG, g	479	516	523	507	493	9.6	0.76	0.09
ADFI, g	724	761	784	755	723	13.9	0.94	0.14
Gain-to-feed ratio	0.66	0.68	0.67	0.67	0.68	0.009	0.31	0.84

SID = standardized ileal digestible; SEM = standard error of the mean.
[1] $n = 5$.
[2] Average daily gain (ADG) = (body weight gain of the pen/piglets' number)/days.
[3] Average daily feed intake (ADFI) = (feed intake of the pen/piglets' number)/days.
[4] Gain-to-feed ratio = body weight gain of the pen/feed intake of the pen.

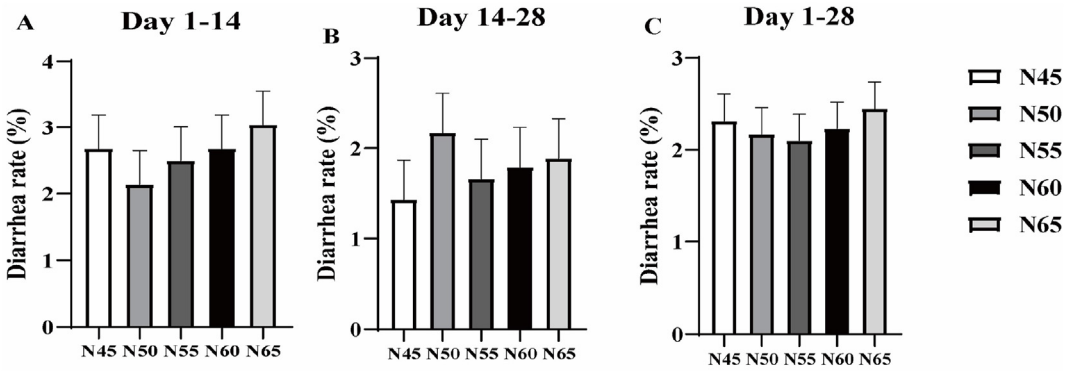

Fig. 1. Effects of standardized ileal digestible (SID) Ile-to-Lys ratios on the diarrhea rates of weaned piglets. The results are presented as the mean and SEM. (A) Day 1 to 14, (B) day 14 to 28, and (C) day 1 to 28 of the experiment. The CP content of five treatments was consistent at 17.8%, and SID Ile-to-Lys ratios were 45% (N45), 50% (N50), 55% (N55), 60% (N60) and 65% (N65). CP = crude protein; SEM = standard error of the mean.

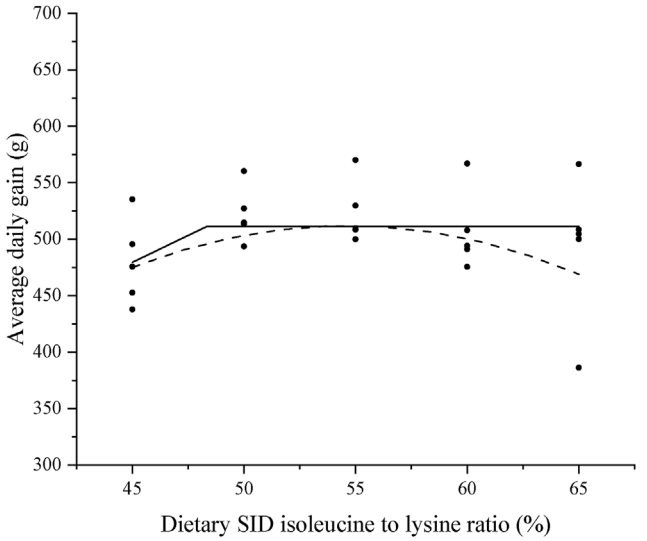

Fig. 2. The optimum standardized ileal digestible (SID) Ile-to-Lys ratio determined by subjecting the average daily gain (ADG) data to the broken-line and quadratic polynomial models. The quadratic model is as follows: if $x \leq$ requirement, $y = -0.40\ (x - 54.63)^2 + 511.98$, where estimated SID Ile-to-Lys ratio is 54.63% to maximize ADG. The broken-line model is as follows: if $x \leq$ requirement, $y = 511.2 - 9.54 \times (48.33 - x)$, where estimated break-point is SID Ile-to-Lys ratio 48.33%.

Table 4
Effects of SID Ile-to-Lys ratios on nutrient digestibility of weaned piglets (%)[1].

Item	SID Ile-to-Lys ratio					SEM	*P*-value	
	45%	50%	55%	60%	65%		Linear	Quadratic
DM	76.44	81.97	82.91	80.72	78.88	0.883	0.17	<0.01
CP	53.39	63.89	65.67	63.47	58.16	3.052	0.33	0.01
EE	47.37	57.86	55.13	58.23	50.39	2.905	0.58	0.04
GE	74.03	78.27	80.37	81.25	76.49	1.564	0.11	<0.01
OM	79.53	84.23	85.08	83.41	81.30	0.848	0.28	<0.01

SID = standardized ileal digestible; SEM = standard error of the mean; DM = dry matter; CP = crude protein; EE = ether extract; GE = gross energy; OM = organic matter.
[1] $n = 5$.

3.3. Serum AA and BUN levels

In Table 5, as the ratio of SID Ile-to-Lys increased, serum BUN ($P < 0.01$; $P < 0.01$), Ser ($P < 0.01$; $P = 0.01$) levels decreased linearly and quadratically. Additionally, serum Gly ($P < 0.01$) and Leu ($P = 0.01$) contents decreased linearly, Gln ($P = 0.02$), Arg ($P = 0.05$) and Thr ($P = 0.03$) concentrations reduced quadratically as the SID Ile-to-Lys ratio increased. Serum Ile ($P < 0.01$) concentration enhanced linearly with increasing SID Ile-to-Lys ratio. Additionally, different SID Ile-to-Lys ratios did not significantly affect other serum AA levels.

3.4. Serum biochemical indices

As the SID Ile-to-Lys ratio increased, serum TC ($P = 0.01$; $P < 0.01$) level decreased linearly and quadratically, and serum AST ($P < 0.01$) level decreased quadratically (Table 6).

3.5. Digestive enzyme activity

The trypsin ($P = 0.01$) and amylase ($P = 0.04$) activities increased quadratically with the increase of SID Ile-to-Lys ratio (Table 7). Additionally, different ratios of SID Ile to Lys did not significantly affect lipase and chymotrypsin activities.

3.6. Intestinal morphology

In Table 8 and Fig. 3, intestinal morphology of weaned piglets are presented. As SID Ile-to-Lys ratio increased, duodenal VH ($P < 0.01$) and ratio of VH to CD ($P < 0.01$) increased in a quadratic way.

3.7. Immunoglobulin and cytokine levels

As shown in Table 9, IL-1β ($P = 0.01$) and TNF-α ($P < 0.01$) contents decreased in a quadratic manner with an increase of SID Ile-to-Lys ratio. Additionally, IgG ($P = 0.03$) and IgM ($P = 0.01$) concentrations increased quadratically.

3.8. Tight junctional protein level

As for tight junctional protein levels (Fig. 4), among the five treatments, piglets from the N55 group had the highest mRNA expression level of occludin ($P < 0.05$).

3.9. Intestinal microbiota

The cecal microbiota composition of piglets are shown in Fig. 5. The Venn (Fig. 5A) result showed that 401 OTUs were shared, and 23, 27, 11, 16 and 38 OTUs were unique in the N45, N50, N55, N60 and N65 groups. As for alpha diversity, no differences in the Chao and Shannon indices were observed among the five treatments (Fig. 5B and C). Phyla Firmicutes and Bacteroidetes were the most abundant among the five treatments (Fig. 5D). Lactobacillaceae, Lachnospiraceae, Prevotellaceae, Ruminococcaceae, Oscillospiraceae and Clostridiaceae were dominant families (Fig. 5E). At the genus level, the top 50 microbial communities of cecal digesta are shown in Fig. 5F. To further investigate effects of various Ile-to-Lys ratios on the cecal microbiota composition of piglets, the biomarkers among different groups were identified by the LefSe analysis (Fig. 5G). Among the five treatments, the N45 group had a higher abundance of phyla Deinococcota, order Burkholderiales, family Nitrosomonadaceae and genera *Nitrosomonas*; whereas the N50 group had higher order Clostridia_vadinBB60_group abundance; the N55 group had more abundant genera *Catenisphaera, norank_f__Prevotellaceae, norank_f__Ruminococcaceae* and *norank_f__Bacteroidales*; and pigs from the N65 group had a higher abundance of genera *Romboutsia*, *Terrisporobacter* and *Family_XIII_UCG_001* ($P < 0.05$). Between N45 and N55 (Fig. 5H), a higher abundance of phyla Proteobacteria, Chloroflexi, order Flavobacteriales, Kapabacteriales, Ardenticatenales, Thermomicrobiales, family Eubacterium_coprostanoligenes_group, Burkholderiaceae, Nitrosomonadaceae, Sphingomonadaceae, Saprospiraceae and genera *Desulfovibrio* was observed in the N45 group, and N55 had a higher abundance of order Oscillospirales, class Negativicutes, family Coriobacteriaceae and genera *norank_f__Ruminococcaceae*, *Fournierella* ($P < 0.05$).

The colonic microbiota of piglets from various groups is shown in Fig. 6. As shown in, 455 OTUs were shared, and 31, 19, 20, 12 and 18 OTUs were unique in the N45, N50, N55, N60 and N65 groups, respectively. The Chao and Shannon indexes were also not significantly different among all the treatments (Fig. 6B and C). The microbiota composition was mostly composed of phyla Firmicutes and Bacteroidetes (Fig. 6D). At the family level, Lactobacillaceae, Prevotellaceae, Lachnospiraceae, Ruminococcaceae, Oscillospiraceae, Muribaculaceae and Clostridiaceae were the dominant families (Fig. 6E). The microbial-community heatmap of the top 50 genera is shown in Fig. 6F. Among all the groups (Fig. 6G), the LefSe analysis showed that phyla Patescibacteria and family Saccharimonadaceae were more abundant in pigs fed the N50 diet, and

Table 5
Effects of SID Ile-to-Lys ratios on serum urea nitrogen and amino acid concentrations of weaned piglets (μmol/L) [1].

Item	SID Ile-to-Lys ratio					SEM	*P*-value	
	45%	50%	55%	60%	65%		Linear	Quadratic
BUN, mmol/L	4.23	2.14	2.00	2.28	2.84	0.247	<0.01	<0.01
Asn	73.63	69.10	47.15	72.43	73.70	10.233	0.92	0.15
Gln	699.50	547.80	459.30	630.00	807.00	90.038	0.20	0.02
His	33.73	20.23	27.43	26.25	26.45	5.183	0.61	0.34
Ser	292.00	192.75	164.00	160.00	177.25	22.750	<0.01	0.01
Asp	101.55	106.98	98.23	88.73	142.20	17.677	0.55	0.44
Glu	273.00	303.75	286.50	270.25	417.50	41.986	0.05	0.13
Gly	1982.5	1417.5	1265.5	1057.0	1125.0	66.54	<0.01	0.06
Arg	340.75	234.25	238.00	298.75	331.75	40.654	0.73	0.05
Cys	12.98	8.94	5.74	4.90	11.48	6.409	0.70	0.27
Pro	292.00	276.50	228.75	273.75	291.00	25.591	0.96	0.13
Tyr	72.15	70.40	60.08	57.10	58.48	6.604	0.20	0.71
Lys	533.00	505.50	366.80	485.30	528.50	91.439	0.93	0.29
Met	159.25	130.93	101.48	91.78	146.18	25.908	0.47	0.09
Val	363.50	320.75	263.00	287.25	316.50	69.613	0.57	0.40
Phe	89.55	72.60	83.45	83.68	82.55	7.546	0.92	0.56
Trp	52.48	35.18	39.40	42.48	42.00	8.093	0.66	0.38
Thr	361.00	323.50	283.25	240.00	344.00	32.162	0.25	0.03
Ala	677.50	593.25	492.25	603.25	621.75	63.890	0.64	0.11
Ile	33.78	62.90	76.85	111.58	134.88	18.960	<0.01	0.90
Leu	237.00	198.25	182.25	170.50	169.00	16.067	0.01	0.24

SID = standardized ileal digestible; SEM = standard error of the mean; BUN = urea nitrogen.
[1] $n = 5$.

Table 6
Effects of SID Ile-to-Lys ratios on serum biochemical parameters of weaned piglets[1].

Item	SID Ile-to-Lys ratio					SEM	*P*-value	
	45%	50%	55%	60%	65%		Linear	Quadratic
GLU, mmol/L	5.67	5.79	6.19	5.79	6.39	0.214	0.94	0.11
TG, mmol/L	0.52	0.48	0.39	0.48	0.38	0.042	0.05	0.74
TC, mmol/L	2.22	1.72	1.54	1.48	1.78	0.116	0.01	<0.01
CK, U/L	990.20	1050.00	712.20	627.00	1335.00	41.366	0.79	0.20
HDL, mmol/L	0.48	0.70	0.49	0.60	0.68	0.059	0.14	0.84
LDL, mmol/L	1.19	1.54	1.46	1.25	1.31	0.110	0.91	0.10
LDH, U/L	636.43	465.29	457.44	563.61	390.25	69.017	0.06	0.65
TP, g/L	51.28	52.13	63.08	51.98	50.81	4.314	0.94	0.13
ALB, g/L	25.42	21.74	22.87	23.98	23.17	1.702	0.65	0.34
GLB, g/L	40.91	26.33	26.30	25.63	26.80	5.339	0.10	0.13
AST, U/L	75.47	33.39	50.04	48.21	71.57	6.016	0.79	<0.01
ALT, U/L	56.04	44.38	41.86	47.55	40.04	5.071	0.06	0.35
ALP, U/L	362.79	333.57	276.24	269.55	316.42	28.679	0.11	0.08

SID = standardized ileal digestible; SEM = standard error of the mean; GLU = glucose; TG = triglyceride; TC = total cholesterol; CK = creatine kinase; HDL = high density lipoprotein; LDL = low density lipoprotein; LDH = lactate dehydrogenase; TP = total protein; ALB = albumin; GLB = globulin; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase.
[1] $n = 5$.

Table 7
Effects of SID Ile-to-Lys ratios on pancreatic digestive enzyme activities of weaned piglets (U/g) [1].

Item	SID Ile-to-Lys ratio					SEM	*P*-value	
	45%	50%	55%	60%	65%		Linear	Quadratic
Trypsin	3.82	5.45	8.27	5.86	3.96	0.881	0.88	0.01
Lipase	24.44	34.08	35.23	43.08	24.38	6.852	0.71	0.10
Amylase	15.49	23.74	23.83	22.75	15.82	3.212	0.98	0.04
Chymotrypsin	9.59	13.59	13.21	18.14	9.69	2.070	0.55	0.06

SID = standardized ileal digestible; SEM = standard error of the mean.
[1] $n = 5$.

pigs in N60 showed a higher abundance of genera *Allisonella*, and piglets in N65 presented an increased abundance of genera *Candidatus_Soleaferrea* ($P < 0.05$). Between N45 and N55 (Fig. 6H), the abundance of phyla Spirochaetota, order Clostridia_vadinBB60_group and Monoglobales, family Anaerovoracaceae, genera *Butyrivibrio, Eisenbergiella, Lachnospiraceae_XPB1014_group, Eubacterium_nodatum_group, Lachnospiraceae_UCG-010, Negativibacillus, Candidatus_Soleaferrea, norank_f__Erysipelotrichaceae, Prevotellaceae_UCG-004, Clostridium_sensu_stricto_1* increased in N45, while the abundance of phyla Bacteroidota, genera *Subdoligranulum*, *Fournierella*, and *Prevotella* was enhanced in the N55 group ($P < 0.05$).

Table 8
Effects of SID Ile-to-Lys ratios on intestinal morphology of weaned piglets[1].

Item	SID Ile-to-Lys ratio					SEM	*P*-value	
	45%	50%	55%	60%	65%		Linear	Quadratic
Duodenum								
VH, μm	220.00	346.20	447.61	324.30	270.49	30.821	0.40	<0.01
CD, μm	363.16	366.10	341.07	338.06	379.26	27.845	0.96	0.34
VH-to-CD ratio	0.61	0.96	1.36	0.97	0.72	0.140	0.59	<0.01
Jejunum								
VH, μm	354.15	366.82	305.47	317.38	327.01	35.537	0.34	0.59
CD, μm	300.37	307.01	366.00	310.83	298.15	25.466	1.00	0.19
VH-to-CD ratio	1.19	1.26	0.84	1.02	1.12	0.163	0.43	0.27
Ileum								
VH, μm	212.98	185.44	189.71	219.32	206.16	26.541	0.82	0.61
CD, μm	191.27	240.83	224.38	194.30	207.73	30.034	0.88	0.43
VH-to-CD ratio	1.12	0.77	0.87	1.12	1.09	0.143	0.57	0.22

SID = standardized ileal digestible; SEM = standard error of the mean; VH = villus height; CD = crypt depth.
[1] $n = 5$.

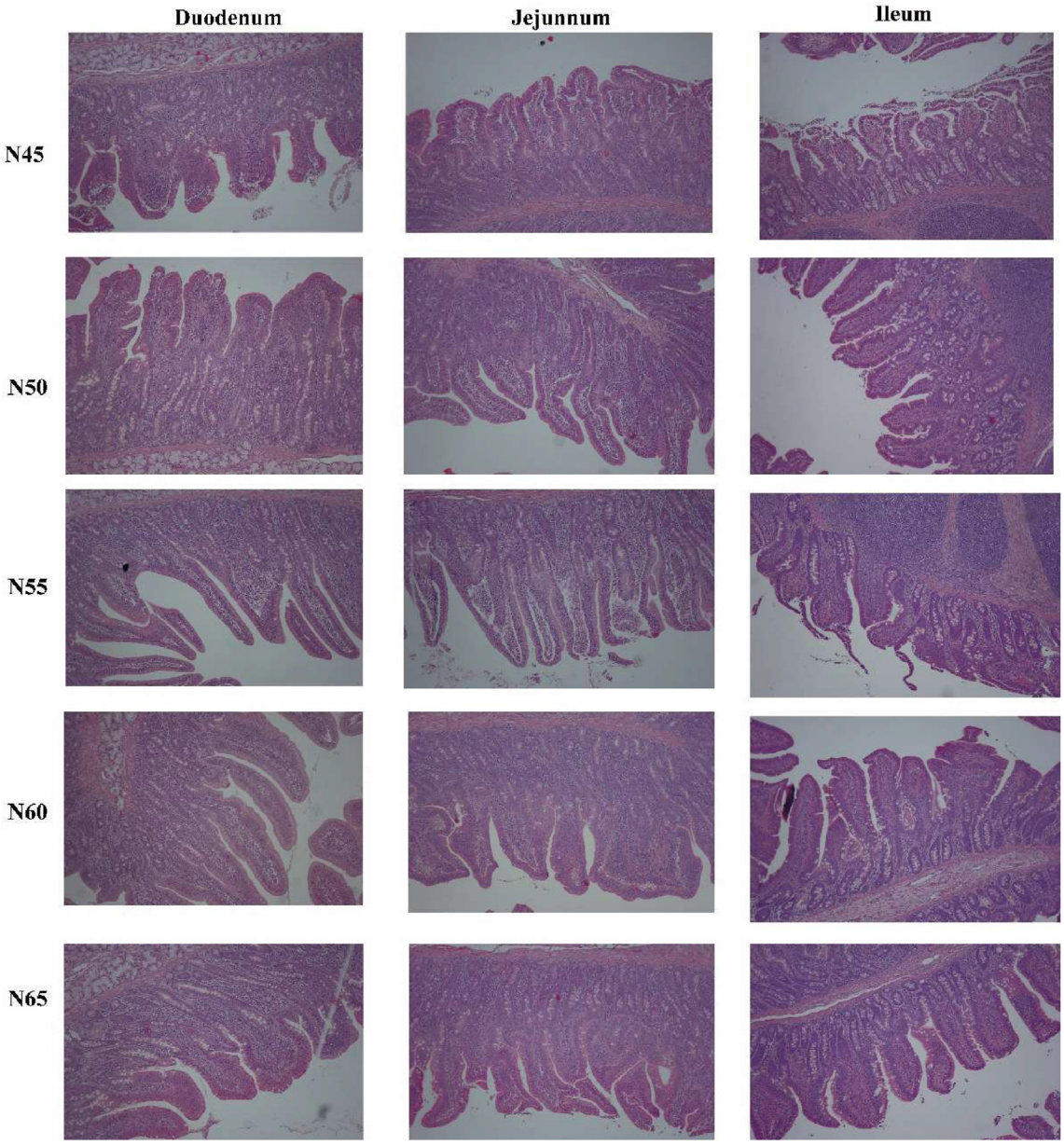

Fig. 3. Representative histological micrographs in piglets fed diets with different standardized ileal digestible (SID) Ile-to-Lys ratios. The CP content of five treatments was consistent at 17.8%, and SID Ile-to-Lys ratios were 45% (N45), 50% (N50), 55% (N55), 60% (N60) and 65% (N65). CP = crude protein.

Table 9
Effects of SID Ile-to-Lys ratios on inflammatory factors of weaned piglets[1].

Item	SID Ile-to-Lys ratio					SEM	*P*-value	
	45%	50%	55%	60%	65%		Linear	Quadratic
IgA, μg/mL	14.11	14.02	15.06	13.51	13.64	0.411	0.24	0.14
IgG, mg/mL	6.51	6.93	7.25	6.64	6.66	0.194	0.99	0.03
IgM, μg/mL	6.08	6.38	6.33	6.09	5.84	0.144	0.08	0.01
IL-1β, ng/L	108.45	96.23	99.69	106.21	107.44	3.105	0.39	0.01
IL-6, ng/L	33.20	34.29	34.04	33.77	34.90	0.509	0.06	0.98
IL-10, ng/L	19.02	18.60	17.68	19.44	19.69	0.737	0.39	0.18
TNF-α, ng/L	54.93	52.11	49.44	53.15	54.68	1.008	0.90	<0.01

SID = standardized ileal digestible; SEM = standard error of the mean; Ig = immunoglobulin; IL = interleukin; TNF = tumor necrosis factor.
[1] $n = 5$.

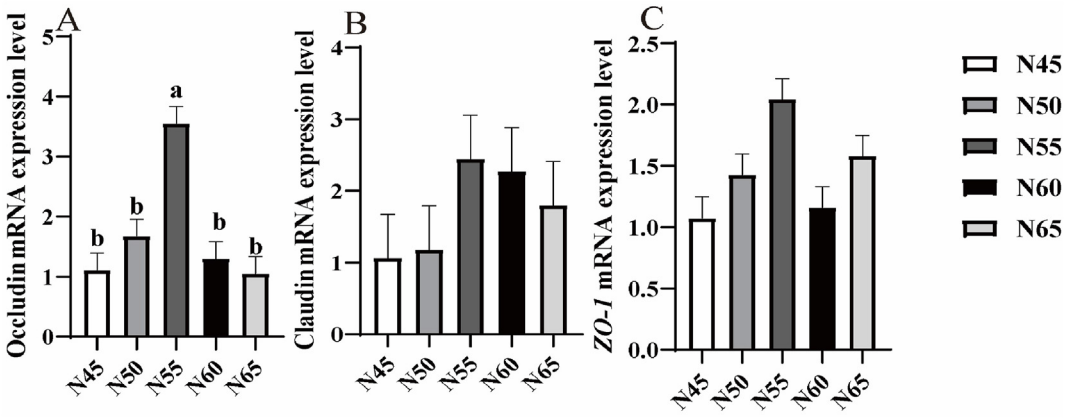

Fig. 4. Effects of standardized ileal digestible (SID) Ile-to-Lys ratios on mRNA expression level of tight junctional proteins in the ileum of weaned piglets. The results are presented as the mean and SEM. [a, b] Mean values with different letters differ at $P < 0.05$. (A) Occludin; (B) claudin; (C) zonula occludens-1 (*ZO-1*). The CP content of five treatments was consistent at 17.8%, and SID Ile-to-Lys ratios were 45% (N45), 50% (N50), 55% (N55), 60% (N60) and 65% (N65). SEM = standard error of the mean. CP = crude protein.

3.10. SCFA concentration

Cecal and colonic SCFA contents of piglets from the five treatments are shown in Table 10. As the SID Ile-to-Lys ratio increased, cecal acetate ($P = 0.01$; $P = 0.02$) concentration increased linearly and quadratically, and colonic acetate ($P < 0.01$) and butyrate ($P = 0.01$) levels increased in a quadratic way.

4. Discussion

Ile is traditionally regarded as an essential AA for pigs, because it cannot be synthesized in the body (van Milgen et al., 2012). The growth performance and health status of pigs have been demonstrated to be positively affected by appropriate intake of Ile (van Milgen et al., 2012; Wu et al., 2013). In accordance with the ideal protein concept, the requirement of Ile is defined as the ratio of Ile to Lys, Lys is generally considered the first limiting AA, while Lys should be the second limiting AA when evaluating Ile requirements (Soumeh et al., 2014; van Milgen et al., 2012). The diet containing 1.19% SID Lys was prepared in this study to ensure that Lys was marginally limiting, and the Ile requirement would not be underestimated. It has been demonstrated that the diets that included 11.4 g SID Lys/kg were formulated to evaluate the Ile requirement in 8 to 15 kg pigs, the SID Lys level of the experimental diet was 93% of values obtained from the Lys requirement (Soumeh et al., 2014).

Most studies focused on the Ile requirement of pigs fed diets with blood products due to low level of Ile in blood products, studies concerning Ile requirement of pigs fed diets without blood products are still limited (van Milgen et al., 2012). Recently, some researchers have performed experiments to explore the Ile requirement of piglets fed blood cell-free diets. In pigs fed 15.34% CP diet, optimal SID Ile-to-Lys ratios were 0.52, 0.52, 0.52 using a quadratic regression model based on ADFI, ADG and gain-to-feed ratio, respectively, and using the quadratic broken-line model, when the SID Ile-to-Lys ratios were 0.50, 0.53, 0.54, the ADFI, ADG and gain-to-feed ratio reached the greatest, respectively (Norgaard et al., 2013). The meta-analysis showed that the SID Ile-to-Lys ratio was at least 50% when pigs were fed blood cell-free diets (van Milgen et al., 2012). However, Soumeh et al. (2014) found that to maximize ADG and ADFI, the SID Ile-to-Lys ratios should be 0.52 and 0.48, respectively, in order to minimize feed conversion ratio for piglets fed diets containing 16.28% CP. Barea et al. (2009) demonstrated the SID Ile-to-Lys ratio for piglets fed diets without blood products was not greater than 50%. In this experiment, the estimated SID Ile-to-Lys ratio to obtain the highest ADG was 54.63% with a quadratic polynomial model, and when using a broken-line linear model, the SID Ile-to-Lys ratio was 48.33% for the greatest ADG. Different optimum SID Ile-to-Lys ratios based on different statistical models were observed, because a reduction was observed after maximum ADG, the quadratic polynomial model might be better than the broken-line linear model for analyzing these data. Our results are similar to the studies concerning the SID Ile-to-Lys ratio requirement (between 0.48 and 0.54) for piglets fed diets without blood products. The ratio of SID Ile to Lys did not linearly or quadratically affect ADFI, but numerically the 45% and 65% SID Ile-to-Lys ratio led to the lowest ADFI, suggesting that insufficient or excessive supply of Ile could also result in negative impacts on piglet ADFI. The gain-to-feed ratio of piglets among all treatment groups were not significantly different. Soumeh et al. (2014) also reported that the gain-to-feed ratio of piglets was not

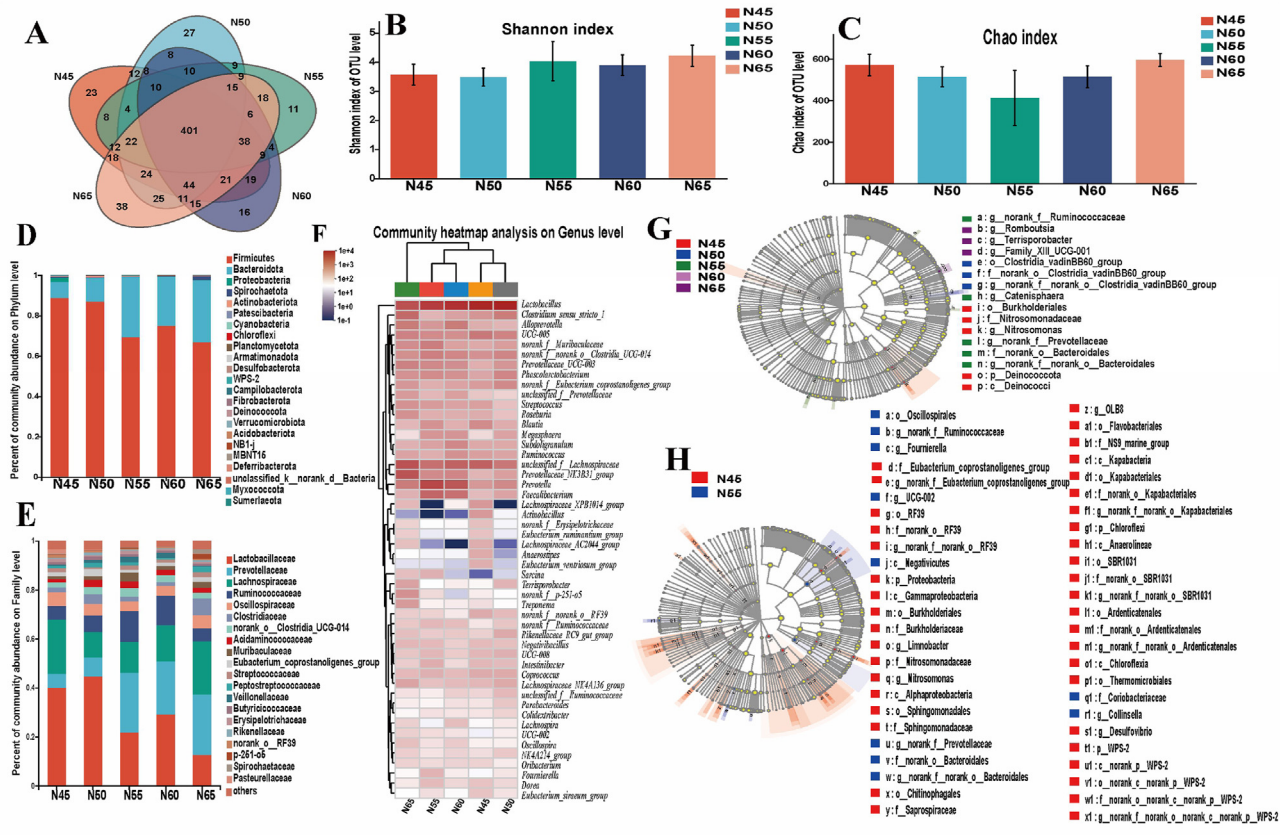

Fig. 5. Effects of standardized ileal digestible (SID) Ile-to-Lys ratios on cecal microbiota of weaned piglets. (A) Venn diagram. (B) Shannon index. (C) Chao index. (D, E) Barplot analysis of microbial composition at the phylum and family levels. (F) Heatmap analysis of microbial composition at the genus level. (G) LEfSe analysis from phylum to genus level among the five treatments. (H) LEfSe analysis from phylum to genus level between the N45 and N55 groups. The CP content of five treatments was consistent at 17.8%, and SID Ile-to-Lys ratios were 45% (N45), 50% (N50), 55% (N55), 60% (N60) and 65% (N65). CP = crude protein.

significantly affected by the ratio of Ile to Lys. The insufficient supply of BCAA in diets has been reported to negatively impact pig growth rate and health status (van Milgen et al., 2012). In this study, when the SID Ile-to-Lys ratio was 45%, which was lower than the optimal SID Ile-to-Lys ratio of 55%, 9.19% reduction in ADG was observed. In previous reports, when pig diets had a 10% decrease in the Ile content, a 21% decrease in ADG and 15% decrease in ADFI were observed (van Milgen et al., 2012). Moreover, excessive BCAA intake might lead to enhanced metabolism of BCAA, and thus negatively affect pig performance (Soumeh et al., 2014). The pigs fed a diet containing excessive Ile were reported to showed significantly reduced ADFI and ADG compared with pigs fed a diet with non-excessive levels of Ile (Norgaard et al., 2013). It was speculated that negative effects of excessive Ile might be related to AA interactions. Wiltafsky et al. (2009) also found that a too high ratio of SID Ile to Lys could lead to a reduction in performance, and speculated that the production of α-keto-β-methylvalerate by an excessive amount of Ile might activate branched-chain keto acid dehydrogenase complex (BCKDH), where BCKDH could catalyze all irreversible steps of BCAA catabolism, and thus limit the metabolism of Leu.

In this study, Ile deficiency resulted in a decrease of nutrient digestibility, and the reduction might partly explain reduced growth performance in the N45 group. We observed that Ile deficiency negatively affected intestinal morphology and decreased pancreatic digestive enzyme activity in piglets. The improvement in digestive enzyme activity and intestinal morphology may be an indicator for increasing nutrient digestibility; whereas decreased digestive enzyme activity and impaired intestinal morphology are not conducive to nutrient absorption, leading to a reduction in nutrient digestibility (Yuan et al., 2021; Zhu et al., 2021). In addition, CP digestibility was lower than most reported CP digestibility values in pigs. The reduced CP digestibility was speculated to be related to the occurrence of the weaning stress, which could result in changes in the development and function of the small intestine, disrupting digestion and absorption capacity, and finally leading to decreased nutrient digestibility (Tang et al., 2022). The low CP digestibility might also be associated with the dietary CP level, as Jie et al. (2020) reported that weaned piglets fed 17% CP diets showed lower CP digestibility compared to piglets fed 19% CP diets. Additionally, peanut meal was used in this experiment, whereas it has been reported that the application of peanut meal is limited due to anti-nutritional factors and imbalance in amino acid profile (Li et al., 2014, 2023). Until now, knowledge on effect of Ile on nutrient digestibility of piglets is limited; however, BCAA have been demonstrated to have a crucial role in nutrients digesting and absorbing in the intestine (Zhang et al., 2013b).

Dietary AA intake might affect serum AA concentration, and serum AA levels are regarded as an indicator for the measurement of AA status in the body (Sedgwick et al., 1991). In this study, alterations in serum Ser, Gly, Leu, Glu, Arg, Thr and Ile levels were observed. Soumeh et al. (2014) found that as the SID Ile-to-Lys ratio increased, Ile content increased linearly, and Leu, Gly and Ser concentrations decreased linearly. Wiltafsky et al. (2009) reported that serum Ser and Gly concentrations decreased as the ratio of SID Ile to Lys increased. Therefore, dietary Ile intake is crucial to

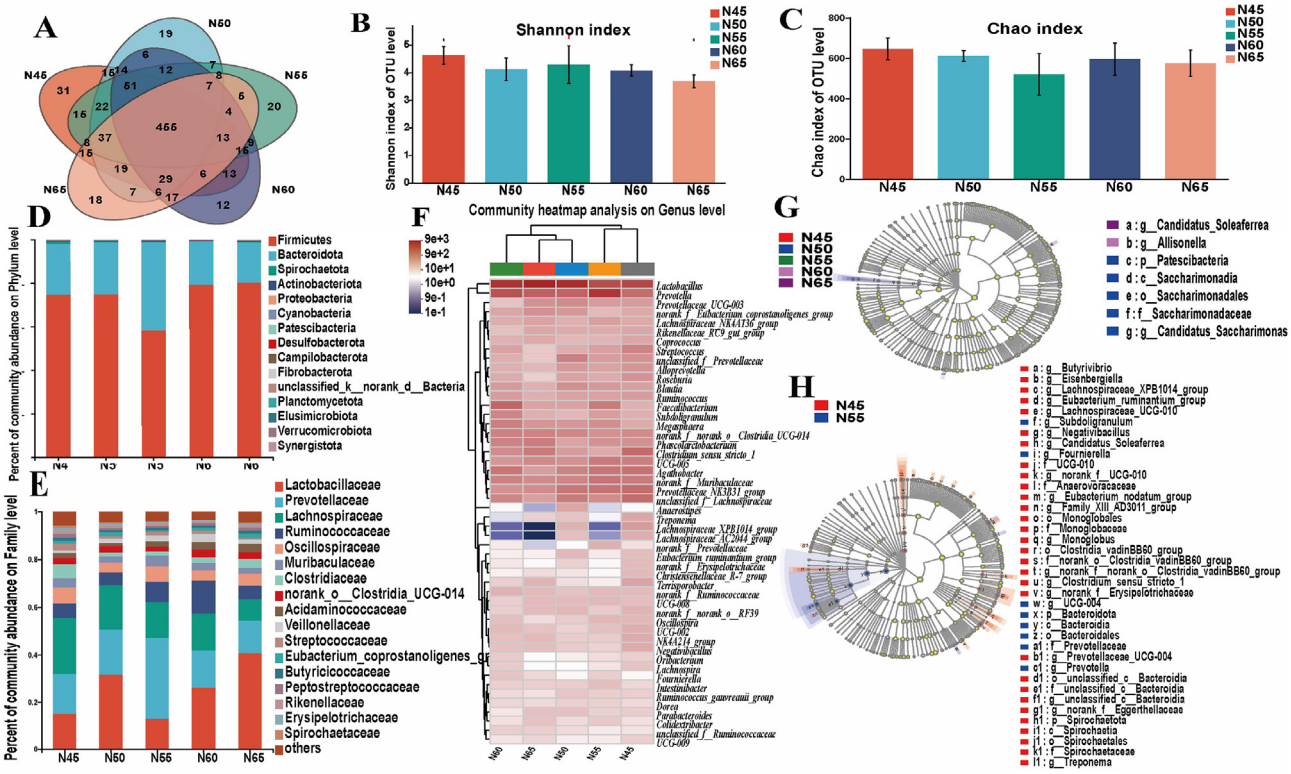

Fig. 6. Effects of standardized ileal digestible (SID) Ile-to-Lys ratios on colonic microbiota of weaned piglets. (A) Venn diagram. (B) Shannon index. (C) Chao index. (D, E) Barplot analysis of microbial composition at the phylum and family levels. (F) Heatmap analysis of microbial composition at the genus level. (G) LEfSe analysis from phylum to genus level among the five treatments. (H) LEfSe analysis from phylum to genus level between the N45 and N55 groups. The CP content of five treatments was consistent at 17.8%, and SID Ile-to-Lys ratios were 45% (N45), 50% (N50), 55% (N55), 60% (N60) and 65% (N65). CP = crude protein.

Table 10
Effects of SID Ile-to-Lys ratios on short chain fatty acid concentrations of weaned piglets (mg/kg) [1].

Item	SID Ile-to-Lys ratio					SEM	*P*-value	
	45%	50%	55%	60%	65%		Linear	Quadratic
Cecum								
Acetate	4742.2	6185.3	5679.6	5776.6	5877.5	110.44	0.01	0.02
Propionate	1987.8	2165.0	2504.6	2267.8	2735.9	68.56	0.02	1.00
Butyrate	1114.1	1241.9	1515.3	1324.1	1532.6	52.77	0.09	0.61
Isovalerate	234.24	260.59	236.81	242.11	381.82	37.331	0.06	0.13
Colon								
Acetate	4527.3	5507.5	6433.4	5462.3	5133.8	95.42	0.22	<0.01
Propionate	1825.7	2114.6	2051.9	2394.3	2750.0	24.65	0.01	0.51
Butyrate	1149.5	1461.5	2120.7	1459.4	1337.0	72.84	0.52	0.01
Isovalerate	311.56	328.18	430.04	444.75	403.62	45.568	0.10	0.33

SID = standardized ileal digestible; SEM = standard error of the mean.
[1] $n = 5$.

physiological functions of pigs, and the importance of balancing BCAA in maintaining growth and health status of pigs should be emphasized (Duan et al., 2016). BUN is a serum byproduct of protein catabolism and a crucial indicator of AA utilization efficiency (Lv et al., 2018; Wang et al., 2011). In the current study, increasing dietary SID Ile-to-Lys ratio resulted in a linear and quadratic decrease in BUN level. The increase in BUN concentration is associated with a decrease in protein synthesis and enhancement of protein catabolism, and its level is negatively related to AA and protein utilization efficiency (Heo et al., 2008). A lack of enough Ile leads to decreased protein synthesis, which may promote deamination of other AA, followed by an increase in BUN level (Kwon et al., 2019). Previous research showed that BUN level decreased as Ile content increased, and high efficiency of AA utilization was observed when the intake of Ile reached the requirement (Parr et al., 2003). However, compared with N55, the BUN concentration increased in the piglets from the N65 group, indicating that excessive intake of Ile also negatively affected protein utilization efficiency.

Serum biochemical parameters can reflect the metabolic function in the body (Prvulovic et al., 2007). GLU is the basic energy and carbon source of most eukaryotic cells, as it can be oxidized to provide energy, and has a broad impact on cell function (Gaster et al., 2000). The AA could participate in the regulation of GLU level (Doi et al., 2003). Among all AA, BCAA (especially Ile) have been proven to improve GLU consumption and utilization (Doi et al., 2003). In normal rats, Ile could inhibit the increase of blood GLU concentration (Doi et al., 2003). Studies also have shown that

Ile intake alone could increase the intake of GLU in pigs (Zhang et al., 2016). However, consistent with the research of Zhang et al. (2016), Ile did not significantly affect blood GLU level in this study. The blood sampling time may have affected these results; continuously measuring blood GLU level of pigs with vascular catheter might be a more appropriate sampling method. Additionally, insufficient or excessive BCAA might negatively affect lipid metabolism (Zhang et al., 2017). Serum TG and TC contents are related to lipid absorption, and HDLC and LDLC levels are associated with lipid decomposition and transport (Wang et al., 2011). The enhancement in TC levels in the N45 and N65 groups, indicating that insufficient intake or excessive intake of Ile may affect lipid metabolism of pigs. Oxidative metabolism by Ile could reduce lipid accumulation in cells (Solon-Biet et al., 2019). Additionally, excessive BCAA intake may lead to an increase in the catabolism of all BCAA, and studies have shown that excessive Leu or Ile might have an antagonistic effect, and negatively affect the function of adipose tissue and lipid metabolism (Morales et al., 2016; Zhang et al., 2021).

Higher intestinal VH indicates a larger epithelial surface area, and is crucial to nutrient absorption by the intestine, while villous atrophy can increase CD. Therefore, the ratio of VH to CD can reflect health of the intestine (Wang et al., 2020). In the current study, duodenal VH and the ratio of VH to CD of piglets increased quadratically as the ratio of Ile to Lys increased. Previous studies found that BCAA could improve intestinal morphology (Chang et al., 2015). During the process of ammonia transfer, BCAA could provide AA for the synthesis of other AA, particularly glutamic acid and aspartic acid, which are regarded as main fuels for protein turnover and nutrient transport in the small intestinal mucosal cells (Chang et al., 2015; Zhou et al., 2018). In addition, Ile has been demonstrated to upregulate expression levels of the monosaccharide transporters and promote monosaccharide absorption in the intestine of pigs, thereby potentially promoting intestinal maturation (Zhang et al., 2016).

The integrity of intestinal barrier can be evaluated by analyzing expression levels of tight junction proteins including claudin-1, occludin and *ZO-1* (Mao et al., 2011; Mao et al., 2011). The intact intestinal mucosal barrier can prevent harmful bacteria and toxins from entering the body (Chen et al., 2018; Li et al., 2019). In this study, occludin mRNA expression levels were remarkably affected by the appropriate dietary SID Ile-to-Lys ratio. It also has been reported that BCAA can increase tight junction protein transcript abundance, and thus inhibit the colonization of pathogenic bacteria (Ma and Ma, 2019). The improved performance of piglets might be related to the improvement of barrier function, because intestinal mucosal barriers are the first line of defense against pathogens. Additionally, serum AST activities are considered indicator of tissue damage (Nyblom et al., 2004; Obaleye et al., 2007). The higher level of serum AST in piglets from the N45 and N65 groups indicated that the intestine of pigs fed diets containing insufficient or excessive Ile showed high membrane permeability (Ramsay et al., 2018). In this study, IL-1β and TNF-α contents were affected by different ratios of SID Ile to Lys, these inflammatory factors have been previously reported to be related to intestinal mucosal barrier damage, as the occurrence of inflammatory responses may damage intestinal barrier function. It has been reported that insufficient BCAA could negatively affect immune responses and increase sensitivity to harmful agents (Zhang et al., 2017). Ren et al. (2019) found that Ile may inhibit the increase of serum endotoxin and IL-6 levels to alleviate infection induced by *E. coli*. Immunoglobulins IgG, IgA and IgM are crucial to immune status, and key parts of humoral immunity (Ma et al., 2021a). Serum IgG and IgM contents increased as the ratio of Ile to Lys increased, and the increase in immunoglobulin secretion in turn can strengthen mucosal barrier function. It was reported that adding BCAA to diets could increase IgA and sIgA contents in piglets, thereby improving immune system function (Ren et al., 2015).

Intestinal microbiota is crucial to growth performance and health status of piglets (Xiong et al., 2019). The alpha diversity, such as Chao and Shannon indices, could be an indicator for evaluating intestinal microbiota (Zhang et al., 2020). No differences in alpha diversity among the 5 treatments were presented. In fact, dietary free AA were considered to be absorbed in the upper intestinal tract, and most studies showed that BCAA affected the microbial composition in the small intestine (Dai et al., 2010; Luise et al., 2023). However, Liao (2021) reported that supplementing AA may affect the overall absorption rate of AA by the host and the availability of AA by intestinal microbiota, and previous reports showed that BCAA could affect the microbiota in the hindgut of pigs (Hu et al., 2019; Luise et al., 2023). We also observed that different ratios of Ile to Lys could affect the abundance of specific bacteria. Deficient or excess intake of Ile inhibits the colonization of beneficial bacteria such as Oscillospirales, Bacteroidota and *Prevotella.* BCAA supplementation has been reported to promote the colonization of *Prevotellaceae_UCG-004* of pigs, and BCAA significantly increased the abundance of *Prevotella* in the colon of mice (Yang et al., 2016; Luise et al., 2023). Previous studies reported a positive correlation between *Prevotella* abundance and blood BCAA circulation, and *Prevotella ruminicola,* a species of *Prevotella,* plays an important role in protein digestion and AA absorption (Broderick, 1996; Yue et al., 2019). Thus, the alterations in the abundance of *Prevotella* might be associated with the balance of AA intake. Although alterations in the abundance of BCAA-utilizing bacteria were observed, the source of BCAA for bacterial utilization is still not clear, and changes in the microbiota in the large intestine of piglets by Ile supplementation might be an indirect effect. *Prevotellaceae* shows high fiber utilizing capacity, and it is considered a SCFA-producing bacterium (Bernad-Roche et al., 2021). Oscillospirales and Bacteroidota were also demonstrated to be able to produce SCFA (Ahrens et al., 2021; Yu et al., 2017). Additionally, we also observed that as the SID Ile-to-Lys ratio increased, cecal acetate concentration increased linearly and quadratically, and colonic acetate and butyrate levels increased in a quadratic way. These changes in SCFA concentrations might be related to alterations in SCFA-producing bacteria abundance. Thus, Ile supplementation could modulate SCFA concentration in the large intestine of piglets. SCFA are regarded as an important energy source of intestine cells, contributing to the maturation of the immune system and the balance of anti/pro-inflammatory cells. They play an important role in intestinal barrier function, and inhibit the colonization of harmful bacteria (Ahrens et al., 2021; Bernad-Roche et al., 2021; Ma et al., 2021b; Morrison and Preston, 2016). In the current study, Ile deficiency or excess also increased the abundance of harmful bacteria such as Proteobacteria, Burkholderiales, *Desulfovibrio, Monoglobaceae*, *Negativibacillus*, *Candidatus_Soleaferrea, Clostridium_sensu_stricto_1*, *Terrisporobacter* and *norank_f_Erysipelotrichaceae*. Burkholderiales are an order of Proteobacteria, and like all Proteobacteria, they include some pathogenic bacteria (Wang et al., 2018). Increased *Negativibacillus* abundance is related to the occurrence of gut dysbiosis (Wang et al., 2021). *Candidatus_Soleaferrea* is associated with inflammatory diseases (Cao et al., 2021). Pathogenic bacteria *Clostridium_sensu_stricto_1* was demonstrated to be related to inflammation in the intestine (Shang et al., 2021b). *Terrisporobacter,* as an emerging anaerobic pathogen, could be harmful to the gut (Chen et al., 2021). *norank_f_Erysipelotrichaceae* was reported to be significantly positively correlated with TNF-α and IL-1β levels (Li et al., 2022). Thus, higher abundance of beneficial bacteria and lower abundance of harmful bacteria in the N55 group might explain the improvement in immune function and growth performance of piglets. At present, the knowledge about

effects of BCAA on intestinal microbiota is limited, and the effects of Ile on the microbiota in the large intestine of piglets still need to be further explored.

5. Conclusion

The optimum SID Ile-to-Lys ratio required to maximize ADG was 48.33% based on the broken-line linear model and 54.63% based on the quadratic polynomial model. Ile supplementation could improve serum biochemical parameters, immune response, intestinal morphology and microbiota, and in turn increase growth performance and nutrient digestibility. Deficiency or excess Ile in diets might result in poor performance and health status of piglets.

Author contributions

Jian Wang: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft. **Sujie Liu:** Investigation, Data curation. **Jiayu Ma:** Investigation, Visualization. **Xiaoli Dong:** Investigation. **Shenfei Long:** Formal analysis, Investigation. **Xiangshu Piao:** Supervision, Funding acquisition.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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