Effects of *Bacillus subtilis* DSM32315 supplementation and dietary crude protein level on performance, gut barrier function and microbiota profile in weaned piglets¹

Wenjie Tang,[†] Ye Qian,[†] Bing Yu,[†] Tao Zhang,[‡] Jun Gao,[‡] Jun He,[†] Zhiqing Huang,[†] Ping Zheng,[†] Xiangbing Mao,[†] Junqiu Luo,[†] Jie Yu,^{†,2} and Daiwen Chen^{†,2}

[†]Animal Nutrition Institute, Sichuan Agricultural University, and Key Laboratory of Animal Disease-Resistance Nutrition, Ministry of Education of China, Chengdu 611130, Sichuan, P.R. China; and [‡]Evonik Degussa (China) Co., Ltd., Beijing 10028, P.R. China

ABSTRACT: Seventy-two piglets aged at 25 d were chosen to investigate the effects of *Bacillus subtilis* DSM32315 supplementation in diets with different protein levels on growth performance, intestinal barrier function, and gut microbiota profile in a 42-d trial. The animals were allotted to four treatment groups in a randomized complete block design involving a 2 (protein levels) × 2 (probiotic levels) factorial arrangement of treatments. Two protein levels included the high CP (HP) diets (0 to 14 d, 20.5%; 15 to 42 d, 19.5%) and the low CP (LP) diets (0 to 14 d, 18%; 15 to 42 d, 17%), and added probiotic (PRO) levels included at 0 and 500 mg/kg diet. Two interactions between CP and PRO for ADG ($P < 0.01$) and F/G ($P < 0.05$) were observed in phase 1. Within the piglets given the LP diet, probiotic supplementation increased ADG and decreased F/G ratio. Likewise, there were interactions between CP and PRO on the digestibility of CP ($P < 0.01$) and EE ($P < 0.05$), and probiotic supplementation increased the digestibility of CP and ether extract (EE) of piglets fed with LP diet, but that was not the case for piglets fed with HP diet. Furthermore, there were interactions between CP and PRO on villus

height ($P < 0.01$) and villus height:crypt depth ratio ($P < 0.05$) in ileum. Piglets fed with LP diet containing probiotic had the greatest villus height and villus height:crypt depth ratio in ileum among treatments. There were also main effects of PRO on the propionic acid ($P < 0.05$) and butyric acid ($P < 0.05$), and the concentrations of propionic acid and butyric acid in colonic digesta were increased with the inclusion of probiotic in diet. Piglets fed with LP diet containing probiotic had the greatest population of *Bacillus* and *Bifidobacterium* ($P < 0.05$) in colon. In addition, there were interactions between CP and PRO on the mRNA expressions of occludin-1 ($P < 0.05$), epidermal growth factor (EGF) ($P < 0.05$), and insulin-like growth factor 1 receptor (IGF-1R) ($P < 0.05$). The LP fed piglets plus probiotic exhibited the greatest mRNA expressions of occludin-1, EGF, and IGF-1R in ileum compared with other treatments. In conclusion, moderate dietary protein restriction combining with the addition of *B. subtilis* DSM32315 synergistically increased growth performance, altered hindgut bacterial composition and metabolites, maintained intestinal barrier function in ileum of piglets.

Key Words: *Bacillus subtilis* DSM32315, gut health, gut microbiota profile, low protein diet, pigs

© The Author(s) 2019. Published by Oxford University Press on behalf of the American Society of Animal Science. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com.

J. Anim. Sci. 2019.97:2125–2138

doi: 10.1093/jas/skz090

¹This study was supported by the Key Project in Sichuan Science and Technology Pillar Program (2016NZ0006), China Agricultural Technology System (CARS-35), and funded in part by Evonik Degussa (China) Co., Ltd. (Beijing, China)

²Corresponding authors: yujie@sicau.edu.cn

Received December 14, 2018.

Accepted March 15, 2019.

INTRODUCTION

High dietary CP level and imbalanced AA in diets generate enormous waste of protein and severe environmental nitrogen pollution in swine industry (Leek et al., 2007). Feeding pigs low CP diet is considered as one of the potential

strategies to maintain the gastrointestinal tract health of pigs and reduce nitrogen emission (Kong et al., 2007; Opapeju et al., 2009), but the effects of low dietary CP on performance and gut health have not been well documented. Additionally, recent evidences (Hermes et al., 2009; Pieper et al., 2012) showed that the improvement of intestinal barrier function through moderate dietary protein might be associated with the composition of gut microbiota. Probiotic is a live microbial feed additive with beneficial effects on the gut health and serves as a growth promoter in livestock production (Kechagia et al., 2013). In the past decades, many studies have shown that *Bacillus* supplementation can improve nutrient absorption, pathogen resistance, and immune-modulatory activity (Hong et al., 2009; Altmeyer et al., 2014; Khochamit et al., 2015; Elshaghabee et al., 2017; Makarenko et al., 2018). At weaning, piglets suffer from nutritional, environmental, immunological, and social stresses (Kim et al., 2012). Feeding weaned pigs with high CP diet would likely result in intestinal dysfunction (Salazarlindo et al., 2004). At this stage, the probiotic supplementation might potentially alleviate the risk of high protein diets and increase the benefit of low protein diets. Studies focusing on the impact of low protein diet with probiotic supplementation on growth performance and gut health of pigs are needed. The objective of this study was to evaluate the effects of probiotic supplementation on performance, barrier function and gut microbiota profile of weaned pigs weaned pigs in two dietary protein levels.

MATERIALS AND METHODS

Animal, Housing, and Experimental Design

The experiment protocol was approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University. Seventy-two healthy [Duroc× (Landrace×Yorkshire)] weaned piglets (25-d of age) with BW of 7.61 ± 0.55 kg were used in a 42-d trial. Piglets were assigned into four treatment groups in a randomized complete block design involving a 2 (protein levels) × 2 (probiotic levels) factorial arrangement of treatments. Two protein levels included high CP (HP) (0 to 14 d, 20.5%; 15 to 42 d, 19.5%) and low CP (LP) (0 to 14 d, 18%; 15 to 42 d, 17%), and added probiotic (*Bacillus subtilis* DSM32315) levels included 0 and 500 mg/kg diet. Each treatment had six replicates with three pigs per replicate. Piglets within same replicate were housed together in one pen (1.5 ×

1.5 m²). The room temperature was maintained at 26 to 28 °C and relative humidity was controlled at 65% to 75% (Liu et al., 2019). All piglets had free access to water and feed. The experiment was conducted at the Teaching and Research Base of Animal Nutrition Institute, Sichuan Agricultural University.

Experimental Diets

A corn–soybean meal-based diet was formulated according to the recommended nutrient requirements for pigs weighing 7 to 11 kg and 11 to 25 kg (NRC, 2012). Experimental diets included HP diet and LP diet. The HP supplemented 500 g/t GutCare PY1 and the LP diet supplemented 500 g/t GutCare PY1. The GutCare PY1 was composed of *B. subtilis* DSM32315 and calcium carbonate. The *B. subtilis* spore count in the GutCare PY1 was 2×10^9 CFU/g. The nursery period was divided into two phases, days 1 to 14 and 15 to 42. Compositions and analyzed nutrient levels of the diets were presented in Table 1. All diets were free of antibiotics.

Sample Collections and Preparation

Experimental diets were sampled and stored at −20 °C for chemical analysis of CP, DM, ether extract (EE), crude ash, calcium, phosphorus, and gross energy (GE). From days 39 to 42, fecal samples were collected with disposable gloves during defecation to avoid falling to the ground and put them into the sample bag immediately, and then 10 mL of 5% H₂SO₄ solution was added to 100 g of each fresh fecal sample for fixing excreta nitrogen (Liu et al., 2018). Fecal samples treated as described above were dried in a forced air oven (60 °C) for 72 h, and subsequently grounded by high rotated speed disintegrator FW-100 (Taisite Instrument Co., Tianjin, China) to pass through a 1-mm screen for analyses of the apparent total tract digestibility (ATTD) of nutrients (Yan et al., 2018). At days 0, 14, and 42 of the experiment, BW and feed consumption were recorded. At the end of the experiment, one pig with the average BW in each pen was slaughtered after euthanasia. After the abdomen was opened, the pH value of the ileal and colonic digesta was measured immediately. Ileal and colonic digesta were collected into sterile containers and stored at −80 °C pending for measurement of microbial quantity (quantitative PCR), volatile basic nitrogen (VBN), and VFA. The histological samples from jejunum and ileum were

Table 1. Ingredients and nutrient composition of the basal diet(as-fed basis)

Item	0–14 d		15–42 d	
	CP 20.5%	CP 18%	CP 19.5%	CP 17%
Ingredients, %				
Corn (CP 7.8%)	29.11	31.60	31.37	33.74
Extruded corn	29.12	31.61	31.37	33.75
Soybean meal (CP 46%)	13.00	10.00	15.00	15.00
Extruded soybean	6.00	5.00	7.00	5.00
Fish meal (CP 62.5%)	5.00	3.50	—	—
Plasma protein powder	2.19	1.90	—	—
Whey powder	5.00	5.00	3.00	3.00
Soybean protein concentrate	3.00	2.50	6.08	2.23
Soybean oil	1.80	2.25	0.80	1.30
Sucrose	3.00	3.00	2.00	2.00
Limestone	0.87	0.88	0.78	0.80
Dicalcium phosphate	0.15	0.39	0.62	0.64
Salt	0.20	0.20	0.25	0.25
L-Lys·HCl	0.45	0.70	0.54	0.77
DL-Met	0.26	0.34	0.28	0.35
L-Thr	0.18	0.29	0.21	0.32
L-Trp	0.10	0.14	0.10	0.13
L-Val	0.12	0.25	0.15	0.27
Chloride choline	0.10	0.10	0.10	0.10
Vitamin premix ¹	0.05	0.05	0.05	0.05
Mineral premix ²	0.30	0.30	0.30	0.30
Total	100.00	100.00	100.00	100.00
<i>Nutrient levels³</i>				
DE, Mcal/kg	3.54	3.54	3.49	3.49
CP, %	20.62	18.05	19.44	17.03
Calcium, %	0.78	0.79	0.71	0.70
Total phosphorus, %	0.53	0.54	0.49	0.49
SID lysine	1.44	1.43	1.32	1.31
SID methionine + cysteine	0.84	0.83	0.80	0.79
SID threonine	0.88	0.90	0.79	0.80
SID tryptophan	0.30	0.32	0.27	0.27
SID valine	0.97	0.97	0.89	0.89
SID isoleucine	0.74	0.69	0.68	0.56
SID leucine	1.56	1.53	1.39	1.24
SID histidine	0.47	0.44	0.42	0.39

¹Vitamin premix provides the following per kilogram of diets: VA, 6000 IU; VD₃, 400 IU; VE, 10 IU; VK₃, 2 mg; VB₁, 0.8 mg; VB₂, 6.4 mg; VB₆, 2.4 mg; VB₁₂, 12 µg; folic acid, 0.2 mg; nicotinic acid, 14 mg; D-pantothenic acid, 10 mg.

²Mineral premix provided the following per kilogram of diets: Fe (ferrous sulfate) 100 mg, Cu (copper sulfate) 6 mg, Mn 4 mg, Zn (zinc sulfate) 100 mg, I (potassium iodide) 0.14 mg.

³All nutrient levels except digestible energy were analyzed by Health and Nutrition of Evonik Industries AG, Germany.

SID, standard ileal digestible.

rapidly fixed into 10% buffered formalin for villus height and crypt depth determination. The mucosa of ileum was sequentially obtained through carefully scraping of the mucosa layer (flushed with ice-cold saline) with a glass microscope slide, and then snap-frozen in liquid nitrogen for analyzing relative mRNA expressions of tight junction-related genes and intestinal development-related genes in the ileum of piglets.

ATTD and Digesta pH Value

The ATTD was evaluated by using AIA as the digestibility indicator. The AIA in diets and fecal samples were assayed by the method adapted from McCarthy et al., (1974) with modifications. Briefly, 10 g of crushed feed or feces was boiled in 150 mL HCl for 30 min, then filtered through ash-less filter paper, and washed with boiling water until free of

acid and finally ashed at 550 °C in the muffle furnace for 6 h. All diets and fecal samples were analyzed for DM (method 930.15, AOAC, 1995), ash (method 942.05, AOAC, 1995), EE (method 945.16, AOAC, 1995), Ca (method 927.02, AOAC, 1995), P (method 995.11, AOAC, 1995), CP (method 990.03, AOAC, 1995) (International, 1995), and GE. The GE concentration of feed and feces was measured by using bomb calorimetry (Parr Instrument 1563, Moline, IL). The digestibility of chemical constituents was calculated using the following formula:

$$\text{ATTD (\%)} = (100 - A_1 \times A_2^{-1} \times F_2 \times F_1^{-1} \times 100) \times 100\%,$$

where A_1 is the AIA content of the diet, A_2 is the AIA content of the feces, F_1 is the nutrient content of the diet, and F_2 is the nutrient content of the feces (Van et al., 1996).

The digesta pH value was measured with a pH meter (PHS-3C PH, Shanghai, China).

Detection of VFA in Ileal and Colonic Contents by Gas Chromatography

The concentration of VFA in the digesta samples was determined by gas chromatography. Briefly, about 1.0 g of thawed ileal and colonic digesta was suspended in 1.5 mL of ultrapure water in a centrifuge tube for 30 min. The entire sample was centrifuged at $12,000 \times g$ for 10 min. One milliliter supernatant was transferred to a sterile tube, and then mixed with 0.2 mL 25% metaphosphoric acid and 23.3 μL 210 mmol/L crotonic acid simultaneously, the sterile tubes were centrifuged again for 10 min after placed the sterile tubes in ice-bath for 30 min. The mixture of 500 μL supernatant and 500 μL methanol was homogenized for 10 min in

another sterile tube. After that, the mixture was centrifuged at $12,000 \times g$ for 10 min at 4 °C. The supernatant was injected into a gas chromatographic system (VARIAN CP-3800, America) to separate and quantify the VFA.

Determination of VBN in Ileal and Colonic Digesta

Volatile basic nitrogen content of digesta was measured following the Chinese National Standard GB/T 5,009.44 (2003) with modifications. Briefly, 10.0 g of intestinal contents was suspended in 100 mL distilled water in the Erlenmeyer flask for 30 min. After filtering the suspension, the blend of 5 mL of filtrate and 5 mL of 10 g/L magnesia was injected into reaction chamber of Kjeldahl distillate unit. Steam distillation was performed using Kjeldahl distillate unit for 5 min. The distillate was absorbed by 10 mL of 20 g/L boric acid, and then titrated with 0.01 mol/L HCl.

Gut Microbial Population Determination

Microbial genomic DNA from ileal and colonic digesta samples were extracted using E.Z.N.A Stool DNA Kit (Omega Bio-Tek, Doraville, GA) according to the offered manual. The fluorescent quantitative-specific primers and probe for total bacteria, *Escherichia coli*, *Lactobacillus*, *Bifidobacterium*, and *Bacillus* (Table 2) were obtained from Qi et al., (2013) and Fierer et al., (2005) with modifications of annealing temperature. All the primers and probe were purchased from Invitrogen (Shanghai China). The total bacteria, *E. coli*, *Lactobacillus*, *Bifidobacterium*, and *Bacillus* were quantified by quantitative PCR

Table 2. Primer and probe sequences used for real-time quantitative PCR analysis of selected microbial populations in ileal and colonic digesta samples

Items	Primer/probe name and sequence, 5' to 3'	Size, bp	Annealing temperature, °C
<i>Bifidobacterium</i>	SQ-F, CGCGTCCGGTGTGAAAG	121	55.0
	SQ-R, CTTCCCGATATCTACACATTCCA		
	SQ-P, (FMA) ATTCCACCGTTACACCGGGAA (BHQ-1)		
<i>Lactobacillus</i>	RS-F, GAGGCAGCAGTAGGGAATCTTC	126	53.0
	RS-R, CAACAGTTACTCTGACACCGTTCTTC		
	RS-P, (FMA) AAGAAGGGTTTCGGCTCGTAAACTCTGTT (BHQ-1)		
<i>Bacillus</i>	YB-F, GCAACGAGCGCAACCCCTTGA	92	53.0
	YB-R, TCATCCCCACCTTCCTCCGGT		
	YB-P, (FMA) CGGTTTGTCACCGGCAGTCACCT (BHQ-1)		
<i>Escherichia coli</i>	DC-F, CATGCCGCGTGTATGAAGAA	96	55.0
	DC-R, CGGGTAACGTCAATGAGCAAA		
	DC-P, (FMA) AGGTATTAACTTTACTCCCTTCCTC (BHQ-1)		
Total bacteria	Eub338F, ACTCCTACGGGAGGCAGCAG	200	61.5
	Eub518R, ATTACCGCGGCTGCTGG		

with the method adapted from [Diao et al. \(2014\)](#). Copies per sample were calculated with Ct-values and standard curve as presented by [Chen et al. \(2013\)](#). Bacterial copies were transformed ($\log_{10}$) before statistical analysis.

Total RNA Extraction and Reverse Transcription Reaction

Total RNA was isolated using the TRIzol reagent (Invitrogen) by means of manufacture's protocol. The purity and concentration of total RNA were determined spectrophotometrically using a Beckman Coulter DU 800, and the optical density $OD_{260}:OD_{280}$ ratio ranged from 1.8 to 2.0 in all the samples. The RNA integrity was determined by 1% agarose gel electrophoresis. The reverse transcription reaction was made by RT Reagents Kit with gDNA Eraser (TaKaRa) by the manufacturer's instructions.

Real-Time Quantitative PCR

The relative mRNA expressions of tight junction-related genes and intestine tract development-related genes in ileum of weaned piglets were measured by real-time quantitative PCR with the CFX96 Real-Time PCR Detection System (Bio-Rad) as previously described ([Zhao et al., 2014](#)). The gene-specific primers used in the present study are shown in [Table 3](#), which were purchased from TaKaRa Biotechnology (Dalian, China). The β -actin was chosen as the reference gene, which was used to normalize the variance in amounts of RNA

input in the reaction. The relative gene expressions compared with the reference gene were calculated following previous procedures ([Livak and Schmittgen, 2001](#)).

Intestinal Morphology Measurement

Samples of jejunal and ileal tissues from each piglet were fixed in polyformaldehyde for morphology measurement. The tissues were excised, dehydrated, and embedded following standard procedures ([Pluske et al., 2010](#)). The tissues in paraffin block were cut into four transverse sections (5 μ m), and then installed on glass slides and stained with eosin and haematoxylin. The specimens were examined by the Olympus CK 40 Microscope at 40 $\times$ magnification. The villus height and crypt depth were measured and analyzed using Image-pro plus 6.0 (Media Cybernetics, Inc, Rockville, MD).

Statistical Analysis

The effect of diet on different variables was analyzed as a 2×2 factorial with the general linear model procedures of the SAS (Version 9.0; SAS Institute, Gary, NC). The factors of the models included the main effects of dietary CP (HP or LP) and probiotic treatment (supplemented or unsupplemented with *B. subtilis* DSM 32315) as well as their interaction following this model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}.$$

where Y_{ijk} is the dependent variable, μ is the overall mean, α_i is the effect of dietary CP, β_j is the effect of

Table 3. Primer sequences for quantitative real-time polymerase chain reaction of genes

Genes	Primer sequence, 5' to 3'	Size, bp	Accession number
<i>ZO-1</i>	Forward: CTGAGGGAATTGGGCAGGAA	105	NM-009386.2
	Reverse: TCACCAAAGGACTCAGCAGG		
<i>Occludin-1</i>	Forward: CAGGTGCACCCTCCAGATTG	92	NM-008756.2
	Reverse: GGACTTTCAAGAGGCCTGGAT		
<i>Claudin-1</i>	Forward: ATTTTCAGGTCTGGCTATCTTAGTTGC	214	NM-001244539.1
	Reverse: AGGGCCTTGGTGTGGGTAA		
<i>EGF</i>	Forward: ATCTCAGGAATGGGAGTCAACC	94	NM-031197.2
	Reverse: TCACTGGAGGATGGAATACAGC		
<i>GLP-2</i>	Forward: ACTCACAGGGCACGTTTACCA	110	NM-019810.4
	Reverse: AGGTCCCTTCAGCATGTCTCT		
<i>IGF-1</i>	Forward: CTGAGGAGGCTGGAGATGTACT	128	NM-010113.4
	Reverse: CCTGAACTCCCTCTACTTGTGTTTC		
<i>IGF-1R</i>	Forward: TTCGCCAGATCCTAGGGGAG	123	NM-001314010.1
	Reverse: TCCCAGCTTTGATGGTCAGG		
β -Actin	Forward: TCTGGCACCACACCTTCT	86	NM-007393.3
	Reverse: TGATCTGGGTCATCTTCTCAC		

ZO-1, zonula occludens 1 protein; EGF, epidermal growth factor; GLP-2, glucagon-like peptide-2; IGF-1, insulin-like growth factor 1; GF-1R, insulin-like growth factor 1 receptor.

probiotic, $(\alpha\beta)_{ij}$ is the interaction between protein levels and probiotic. ε_{ijk} represents the unexplained random error. When P -value for interaction was $P < 0.05$, multiple comparisons of the means were performed using SAS adjusted by Tukey Kramer. $P < 0.05$ was considered to indicate statistical significance, and $P < 0.10$ was considered to indicate statistical tendency.

RESULTS

Growth Performance

In phase 1, the ADFI and F/G were not affected by dietary CP (Table 4), but two interactions between CP and PRO for ADG ($P < 0.01$) and F/G ($P < 0.05$) were observed. The ADG of pigs fed the LP diet plus *B. subtilis* DSM32315 tended to be increased ($P < 0.010$) compared with other treatments. The F/G was decreased ($P < 0.01$) when the diets were supplemented with *B. subtilis* DSM32315, and it was also lower in pigs fed the LP diet as compared with the HP diet. The ADFI ($P < 0.05$) in phase 1 was increased in pigs fed diets containing *B. subtilis* DSM32315. During phase 2, no interactions between CP and PRO for ADFI, ADG, and F/G were observed. ADFI was not affected by dietary CP or *B. subtilis* DSM32315, but the ADG of pigs fed diets containing *B. subtilis*

DSM32315 was increased ($P < 0.05$). The F/G of pigs fed diets containing *B. subtilis* DSM32315 was decreased ($P < 0.05$). Overall, pigs fed diets containing *B. subtilis* DSM32315 had greater ($P < 0.05$) ADG and lower ($P < 0.01$) F/G than pigs fed diets without *B. subtilis* DSM32315.

Apparent Total Tract Digestibility

The apparent total tract digestibility of crude ash, Ca, and P was not affected by dietary protein level or *B. subtilis* DSM32315 (Table 5). But, there were interactions between CP and PRO on CP ($P < 0.010$) and EE ($P < 0.05$). The ATTD of CP ($P < 0.01$) and EE ($P < 0.05$) was increased in pigs fed the LP diet containing *B. subtilis* DSM32315 compared with other treatments. There was a main effect of PRO on the DM ($P < 0.05$), with pigs fed *B. subtilis* DSM32315 having greater digestibility of DM than the others.

Digesta pH Value

The pH of jejunal digesta was not affected by dietary protein level or *B. subtilis* DSM32315 (Table 6). There was an interaction between CP and PRO on pH of colonic digesta ($P < 0.05$), and pigs fed the HP diet plus *B. subtilis* DSM32315

Table 4. Effect of probiotic supplementation in diets with different protein level on growth performance in weaned piglets¹

Items	Diets ²				SEM	P-value ³		
	HP		LP			CP	PRO	CP×PRO
	NA	PRO	NA	PRO				
Initial BW, kg	7.61	7.60	7.61	7.61	0.10	0.991	0.973	0.991
Phase 1, days 1 to 14								
ADFI, g/d	378.96	354.26	375.92	371.80	3.36	0.226	0.022	0.092
ADG ⁴ , g/d	255.68 ^a	248.64 ^a	250.48 ^a	270.93 ^b	2.74	0.065	0.141	<0.01
F/G ⁵ , g/d	1.48 ^c	1.43 ^b	1.50 ^c	1.37 ^a	0.01	0.246	<0.01	0.024
Phase 2, days 15 to 42								
ADFI, g/d	727.28	736.95	721.97	736.15	8.45	0.866	0.513	0.901
ADG, g/d	453.21	473.11	441.25	485.35	7.54	0.992	0.036	0.406
F/G, g/d	1.61	1.56	1.64	1.52	0.02	0.861	0.010	0.189
Overall, days 1 to 42								
ADFI, g/d	611.17	609.39	606.63	614.70	5.49	0.968	0.791	0.678
ADG, g/d	387.37	398.29	377.66	413.30	5.22	0.758	0.021	0.195
F/G, g/d	1.56	1.53	1.61	1.49	0.01	0.696	<0.01	0.104

Means with different superscripts (a, b, c) in the same row differ at $P < 0.05$.

¹Values are the means of six replicates per treatment.

²HP, high CP diet; LP, low CP diet; NA, no additive; PRO, probiotic of *Bacillus subtilis* DSM32315.

³CP, main effect of the level of dietary CP; PRO, main effect of the inclusion of *Bacillus subtilis* DSM32315 in the diets.

⁴ADG, average daily BW gain.

⁵F/G = the ratio of average daily feed intake: ADG.

Table 5. Effect of probiotic supplementation in diets with different protein level on apparent total tract digestibility of nutrients in weaned piglets¹

Items	Diets ²				SEM	<i>P</i> -value ³		
	HP		LP			CP	PRO	CP × PRO
	NA	PRO	NA	PRO				
CP	76.76 ^{ab}	77.57 ^b	74.94 ^a	80.02 ^c	0.51	0.665	<0.01	<0.01
DM	82.08	83.76	82.53	85.16	0.45	0.267	0.015	0.566
Crude ash	46.24	43.92	43.44	49.07	1.35	0.642	0.571	0.147
Ether extract	68.27 ^{ab}	70.37 ^b	67.48 ^a	73.98 ^c	0.67	0.128	<0.01	0.022
Ca	48.65	50.91	45.34	52.12	1.53	0.736	0.157	0.469
P	31.42	28.53	27.37	33.27	1.63	0.918	0.658	0.204
Gross energy	82.32	82.87	81.23	83.55	0.65	0.147	0.051	0.227

Means with different superscripts (a, b, c) in the same row differ at $P < 0.05$.

¹Values are the means of six replicates per treatment.

²HP = high CP diet; LP = low CP diet; NA = no additive; PRO = probiotic of *Bacillus subtilis* DSM32315.

³CP, main effect of the level of dietary CP; PRO, main effect of the inclusion of *Bacillus subtilis* DSM32315 in the diets.

Table 6. Effect of probiotics supplementation in diets with different protein level on intestinal pH value in weaned piglets¹

Items	Diets ²				SEM	<i>P</i> -value ³		
	HP		LP			CP	PRO	CP×PRO
	NA	PRO	NA	PRO				
Jejunum	6.37	5.86	6.12	5.80	0.11	0.482	0.066	0.663
Ileum	6.70	6.33	6.79	6.55	0.09	0.721	0.039	0.900
Colon	6.05 ^b	6.57 ^a	6.09 ^b	5.93 ^b	0.03	0.039	0.203	0.025

Means with different superscripts (a, b) in the same row differ at $P < 0.05$.

¹Values are the means of six replicates per treatment.

²HP = high CP diet; LP = low CP diet; NA = no additive; PRO = probiotic of *Bacillus subtilis* DSM32315.

³CP = main effect of the level of dietary CP; PRO = main effect of the inclusion of *Bacillus subtilis* DSM32315 in the diets.

had greater pH of colon than the others. The ileal digesta pH was greater ($P < 0.05$) in pigs fed diets without *B. subtilis* DSM32315.

Small Intestine Morphology

There was an interaction between CP and PRO on villus height in jejunum ($P = 0.01$), and pigs fed the LP diet containing *B. subtilis* DSM32315 had greater villus height than the other treatments (Table 7). There were also main effects of PRO on the crypt depth ($P < 0.05$) and villus height: crypt depth ($P < 0.01$) in jejunum. Pigs fed with *B. subtilis* DSM32315 had greater villus height: crypt depth ratio and lower crypt depth compared with pigs fed without *B. subtilis* DSM32315. In ileum, interactions between CP and PRO for villus height ($P < 0.01$) and villus height: crypt depth ratio ($P < 0.05$), and pigs fed the LP diet plus *B. subtilis* DSM32315 had greater villus height and villus height: crypt depth ratio than the other treatments. The crypt depth in ileum was not affected by dietary protein level or *B. subtilis* DSM32315.

Concentration of Intestinal VFAs and VBN

The VBN, propionate, and butyrate of ileal digesta were not affected by dietary protein or *B. subtilis* DSM32315 (Table 8), but in ileal digesta, an interaction between CP and PRO for acetic acid of ileal digesta ($P < 0.01$), and the acetic acid of ileal digesta was greater for pigs fed the LP diet containing *B. subtilis* DSM32315 compared with other treatments. There was a main effect of PRO ($P < 0.05$) on content of VBN of colonic digesta, and the VBN of colonic digesta was decreased in pigs fed diets containing *B. subtilis* DSM32315. The propionic acid and butyric acid of colonic digesta were increased ($P < 0.05$) in pigs fed diets containing *B. subtilis* DSM32315.

Intestinal Microflora

Ileal and colonic microflora data were shown in Table 9. The populations of *Bacillus* ($P < 0.01$) and *Bifidobacterium* ($P < 0.05$) in ileum of probiotic groups were increased. In colonic content,

Table 7. Effect of probiotics supplementation in diets with different protein level on morphology of small intestine of weaned piglets¹

Items	Diets ²				SEM	P-value ³		
	HP		LP			CP	PRO	CP×PRO
	NA	PRO	NA	PRO				
Jejunum								
Villus height, μm	404.73 ^{ab}	439.23 ^b	369.41 ^a	505.97 ^c	13.460	0.394	<0.01	0.010
Crypt depth, μm	189.85	157.87	169.34	163.01	4.526	0.351	0.027	0.126
Villus height: crypt depth	2.16	2.81	2.21	3.10	0.103	0.198	<0.01	0.363
Ileum								
Villus height, μm	413.45 ^a	422.15 ^a	382.91 ^a	483.89 ^b	10.478	0.322	<0.01	<0.01
Crypt depth, μm	170.88	163.39	165.04	161.61	2.560	0.478	0.312	0.704
Villus height: crypt depth	2.45 ^a	2.60 ^{ab}	2.32 ^a	3.00 ^b	0.083	0.272	<0.01	0.042

Means with different superscripts (a, b, c) in the same row differ at $P < 0.05$.

¹Values are the means of six replicates per treatment.

²HP = high CP diet; LP = low CP diet; NA = no additive; PRO = probiotic of *Bacillus subtilis* DSM32315.

³CP = main effect of the level of dietary CP; PRO = main effect of the inclusion of *Bacillus subtilis* DSM32315 in the diets.

Table 8. Effect of probiotic supplementation in diets with different protein level on microorganism metabolic products in intestine content of weaned piglets¹

Items	Diets ²				SEM	<i>P</i> -value ³		
	HP		LP			CP	PRO	CP×PRO
	NA	PRO	NA	PRO				
Ileal contents								
VBN, mg/kg	91.373	87.368	81.070	88.918	3.922	0.601	0.818	0.480
Acetic acid, mg/kg	7.145 ^a	7.024 ^a	6.933 ^a	9.001 ^b	0.247	0.026	0.015	<0.01
Propionic acid, mg/kg	1.037	1.065	0.794	1.039	0.057	0.245	0.237	0.342
Butyric acid, mg/kg	0.219	0.221	0.205	0.226	0.015	0.877	0.709	0.739
Colonic contents								
VBN, mg/kg	416.133	384.162	403.445	379.467	5.993	0.440	0.020	0.721
Acetic acid, mg/kg	48.687	50.995	46.808	53.929	2.181	0.908	0.310	0.610
Propionic acid, mg/kg	23.976	25.459	22.186	30.441	1.186	0.465	0.034	0.130
Butyric acid, mg/kg	13.703	14.401	11.604	15.851	0.606	0.772	0.037	0.124

Means with different superscripts (a, b) in the same row differ at $P < 0.05$.

¹Values are the means of six replicates per treatment.

²HP = high CP diet; LP = low CP diet; NA = no additive; PRO = probiotic of *Bacillus subtilis* DSM32315.

³CP = main effect of the level of dietary CP; PRO = main effect of the inclusion of *Bacillus subtilis* DSM32315 in the diets.

VBN, volatile basic nitrogen.

there were interactions between CP and PRO for the populations of *Bacillus* ($P < 0.05$) and *Bifidobacterium* ($P < 0.05$), and pigs fed with the LP diet plus probiotic had greater population of *Bacillus* in colonic content compared with treatment of LP diet without probiotic and treatment of HP diet without probiotic, but it was similar to those fed HP diet with probiotic. In colonic content, pigs fed with the LP diet plus probiotic had greater population of *Bifidobacterium* than treatment of HP diet without probiotic, but it was similar to those fed HP diet with or without probiotic.

Real-Time Quantitative PCR

The mRNA expressions of ileal mucous ZO-1, occluding-1, epidermal growth factor (EGF), glucagon-like peptide-2 (GLP-2), and insulin-like growth factor 1 receptor (IGF-1R) in probiotic groups were greater ($P < 0.05$) than that of the other groups (Table 10). However, decreasing dietary protein concentration had no significant influence on the expression of ileum tight junction mRNA and ileum lumen development-related genes of piglets. The mRNA expression of occludin-1, EGF, and IGF-1R was not increased when dietary protein

Table 9. Effect of probiotic supplementation in diets with different protein level on the ileal and colonic bacterial community of weaned piglets¹ (log (copies·g⁻¹))

Items	Diets ²				SEM	<i>P</i> -value ³		
	HP		LP			CP	PRO	CP × PRO
	NA	PRO	NA	PRO				
Ileal								
Total bacteria	10.972	10.918	10.827	11.057	0.054	0.976	0.430	0.211
<i>Bacillus</i>	7.248	7.358	7.155	7.483	0.046	0.846	0.013	0.191
<i>Lactobacillus</i>	6.073	6.260	6.137	6.390	0.064	0.454	0.098	0.795
<i>Bifidobacterium</i>	6.302	6.380	6.238	6.478	0.040	0.820	0.049	0.300
<i>Escherichia coli</i>	6.798	6.773	6.602	6.753	0.095	0.594	0.755	0.664
Colon								
Total bacteria	11.340	11.425	11.258	11.373	0.055	0.570	0.396	0.898
<i>Bacillus</i>	8.823 ^b	9.090 ^{bc}	8.530 ^a	9.182 ^c	0.065	0.228	<0.01	0.028
<i>Lactobacillus</i>	7.700	8.023	7.760	8.120	0.087	0.649	0.057	0.915
<i>Bifidobacterium</i>	6.790 ^{ab}	6.807 ^{ab}	6.447 ^a	7.102 ^b	0.076	0.850	0.015	0.020
<i>Escherichia coli</i>	6.492	6.557	6.870	6.317	0.106	0.743	0.255	0.153

Means with different superscripts (a, b, c) in the same row differ at $P < 0.05$.

¹Values are the means of six replicates per treatment.

²HP = high CP diet; LP = low CP diet; NA = no additive; PRO = probiotic of *Bacillus subtilis* DSM32315.

³CP = main effect of the level of dietary CP; PRO = main effect of the inclusion of *Bacillus subtilis* DSM32315 in the diets.

Table 10. Effect of probiotic supplementation in diets with different protein level on the relative mRNA expression of tight junction-related genes and intestine tract development-related genes in ileum of weaned piglets¹

Items	Diets ²				SEM	P-value ³		
	HP		LP			CP	PRO	CP×PRO
	NA	PRO	NA	PRO				
ZO-1	1.000	1.237	0.938	1.532	0.104	0.560	0.048	0.375
Occludin-1	1.000 ^a	1.163 ^{ab}	0.755 ^a	1.592 ^b	0.098	0.575	<0.01	0.049
Claudin-1	1.000	1.252	1.057	1.612	0.106	0.314	0.059	0.461
EGF	1.000 ^{ab}	1.268 ^{ab}	0.693 ^a	1.734 ^c	0.110	0.627	<0.01	0.029
GLP-2	1.000	1.125	0.762	1.655	0.113	0.457	0.015	0.060
IGF-1	1.000	1.207	1.172	1.632	0.098	0.122	0.086	0.497
IGF-1R	1.000 ^a	1.430 ^b	0.832 ^a	1.822 ^c	0.100	0.400	<0.01	0.042

Means with different superscripts (a, b, c) in the same row differ at $P < 0.05$.

¹Values are the means of six replicates per treatment.

²HP = high CP diet; LP = low CP diet; NA = no additive; PRO = probiotic of *Bacillus subtilis* DSM32315.

³CP = main effect of the level of dietary CP; PRO = main effect of the inclusion of *Bacillus subtilis* DSM32315 in the diets.

ZO-1 = zonula occludens 1 protein; EGF = epidermal growth factor; GLP-2 = glucagon-like peptide-2; IGF-1 = insulin-like growth factor 1; IGF-1R = insulin-like growth factor 1 receptor.

was reduced alone in the diet, but did increase in the probiotic combination ($P < 0.05$). In addition, there were interactions between CP and PRO for the mRNA expressions of ileal occludin-1, EGF, and IGF-1R, and piglets fed with LP diet plus *B. subtilis* DSM32315 had greater EGF and IGF-1R mRNA expressions than other treatments. Piglets fed with LP diet plus *B. subtilis* DSM32315 had greater occludin-1 mRNA expressions than groups without probiotic, but it was similar to those fed the HP diet with probiotic.

DISCUSSION

Manipulating dietary CP content has been recognized as one of the nutritional means to improve gastrointestinal health in weaned pigs (Nyachoti et al., 2006; Heo et al., 2008; Wellock et al., 2008). The inclusion of *Bacillus* probiotic strains in pig feed has also been proven as one of the nonpharmaceutical methods to improve performance and health (Fuller, 1989; Wang et al., 2009; Lee et al., 2014). The mechanisms of spore forming *Bacillus* could enhance health of host include synthesis

of proteases (Makarenko et al., 2018). Therefore, whether the *B. subtilis* could improve the utilization of diet CP to satisfy the need of growth of piglets, while exploiting the benefits of the LP diets at the same time. In this study, piglets fed with the HP diet did not show superior performance compared with those fed the LP diet with or without probiotic supplementation. These data indicated that moderate dietary protein restriction may not be detrimental to piglets. Nevertheless, piglets that received probiotic showed a significantly lower F/G ratio. Wang et al. (2011) reported that the consumption of *B. subtilis* strains decreased F/G ratio in growing pigs. Furthermore, the LP diet with the *B. subtilis* DSM32315 inclusion further improved the performance of piglets. This observation shows the synergetic effects of the LP diet and probiotic on the performance in piglets. Similar results were obtained by Bhandari et al. (2010) that pigs fed with LP diet plus probiotic got greater growth performance compared with other treatments. The enhanced performance observed with the LP diet containing *B. subtilis* DSM32315 might be due to further improvement of dietary digestibility and better adaptation to weaning. Simultaneously, the better performance might be related to amelioration of the intestinal integrity and the microbial profile of the piglets.

The digestibilities of CP and EE were increased for piglets offered the LP diets with *B. subtilis* DSM32315 in the present study, which was supported by Min et al. (2004). Previous study in broilers had similar results that the retention of GE and CP had linear response to increasing levels of dietary *B. subtilis* LS. In contrast, there was a report that *Bacillus*-based multi-microbes had little effect on the digestibility of CP in grower-finisher pigs (Wang et al., 2009). The variation in response probably reflected the difference in animal models and experimental conditions. Interestingly, besides the effects of probiotic, groups fed with LP diet and probiotic had greater digestibility of CP and EE than HP diets plus probiotic. If intestinal digestive function of weaned piglets is not well development, then moderate dietary protein restriction could alleviate the pressure of protein indigestive. The positive influence of *Bacillus* strains on the growth could be mediated by the production of extracellular enzymes (Makarenko et al., 2018). In particular, proteases could promote digestibility of CP which could be synergy with LP to enhanced gut health. In addition, the greater nutrient digestibility would relate to the improved intestinal digestive and absorptive functions. But more studies

are needed to investigate the synergistic effect of protein reduction and probiotic on gut health.

Furthermore, reduction of the pH in intestine, enhanced proteolytic digestion and restrained the growth of pathogenic bacteria (Kirchgessner et al., 1993). The HP diet plus probiotic had the greatest pH value, which might suggest that greater protein level hinder the effect of probiotic on the intestinal digestive function.

The jejunum and ileum play a pivotal role in the nutrient digestion, absorption, and transportation, and the function of absorption mainly depends on the villus in the intestinal lumen (Low et al., 1978). Therefore, the mucosal morphology would be related to nutrient digestion, absorptive capacity and growth performance of the animal. In the present study, pigs fed probiotic showed a greater villous height, which was in accordance with Lee et al. (2014), who provided converging evidence for a significant linear effect of feeding *B. subtilis* LS 1 to 2 on the villus height and villus height: crypt depth ratio of jejunum and ileum of pigs, and thus improved nutrient absorption and growth performance of piglets. Furthermore, supplementation of *B. subtilis* DSM32315 in the LP diet increased villus height: crypt depth ratio in ileum, indicating more favorable mucosa structure and larger absorptive area of luminal villous, which might result in adequate mucosal digestive enzyme development and better digestibility (Pluske et al., 1996). This observation showed the synergetic effect of LP diet and *B. subtilis* DSM32315 on maintaining and strengthening the structure of small intestinal villi, which suggested the superior gut integrity and luminal compartment environment compared with HP treatments. The increase in growth performance and ATTD of the nutrients inversely supported the greater ratio of villus height to crypt depth that impact on absorptive capacity for nutrients in LP diet plus *B. subtilis* DSM32315 group.

Gastrointestinal microbial populations play multiple roles in nutrition and gut health. In the present study, qPCR analysis showed that the bacterial species were not significantly affected by reduced of dietary protein, while the *B. subtilis* DSM32315 administration modified the intestinal bacterial components, as increasing *Lactobacillus* and *Bifidobacterium*, which was in line with the results of the study in the piglets fed with different probiotic products (Choi et al., 2011). At present study, these findings further indicated moderate reduction of dietary protein would slightly influence gut microflora, but *B. subtilis* DSM32315 improved the intestinal balance by inhibiting harmful microorganisms

and favoring beneficial microorganisms. Meanwhile, the increase of population in the *Bacillus* in the intestinal lumen highlighted the ability of *B. subtilis* DSM32315 to colonize piglet intestinal tract. The previous studies had reported that *Bifidobacterium* was the predominant populations in the large intestine (Jing et al., 2010), which could be the reason for the difference in *Bifidobacterium* species observed only in the colon as the results showed. The intestinal microbiota had closely impact on animal health via maintaining the intestinal barrier function. Thus, dietary *B. subtilis* DSM32315 supplementation modified the intestinal microbiota structures, which could also be a reason that it enhanced the growth performance of weaned pigs.

The VFAs, mainly acetate, propionate, and butyrate, were produced by fermentation of undigested dietary proteins and fibers (Dai et al., 2011; Neis et al., 2015). The data in our study demonstrated that concentrations of VFAs in both ileum (acetate) and colon (propionic acid and butyric acid) were increased by consuming *Bacillus subtilis* DSM32315, suggesting a stronger bacterial fermentation caused by probiotic supplementation. However, there was no significant differences of VFAs concentration with a reduction in dietary protein, which was consistent with result from Hermes et al., (2009), who reported that feeding piglets with LP diet had no significant difference in VFAs compared with the HP diet. VFAs have profound effects on metabolism and gut health. Acetate and propionate are energy substrates for peripheral tissues (Tremaroli and Bäckhed, 2016). Therefore, diets with *B. subtilis* DSM32315 might increase the energy produced by bacteria fermentation for influencing host metabolism. Butyrate has been considered as allowing colonic cells to proliferate and contribute to the maintenance of healthy gastrointestinal barrier function. Currently, Tremaroli and Bäckhed (2016) discovered that butyrate may also strongly influence the microbial environment and ecology by communicating with host cell, like instructing colonic cell to “breathe” oxygen through activating β -oxidation to protect the host against the expansion of potentially pathogenic bacteria to the intestinal lumen (Cani, 2017). The synergistic effect between the LP diet and the probiotic used in the current study on favoring beneficial microorganisms was probably a result of different mechanisms by which they produced the butyrate in colon. In the present study, the addition of *B. subtilis* DSM32315 to the diets significantly modified the concentration of butyrate in the colon, which might further indicate its pleiotropic actions on host cell

metabolism and intestine development. In addition, our study also suggested that the effect of probiotic on microbial activity by changing intestinal content of VBN in colon. VBN is low molecular organic polycations produced by decomposing undigested proteins by intestinal spoilage microbiota (Dong et al., 1996). The finding suggested that supplementation of probiotic in diet might inhibit the spoilage bacteria, that produced VBN, in the intestinal of piglets. The fact that the addition of *B. subtilis* DSM32315 in LP diets not only changed the gut microflora but also enhanced the concentration of propionic acid in the colon opens new options to elaborate the synergistic effects between LP and *B. subtilis* DSM32315 on gut health.

Intestinal permeability has been considered as an indicator of intestinal epithelial barrier function. This barrier was primarily regulated by a well-organized system of an epithelial junctional complex referred to as the tight junction. Occludin-1, ZO-1, and claudin-1 were the most critical components in the structural and functional organization of tight junctions (Fanning et al., 1998). Ileal mRNA expression of the intestinal tight junction proteins such as ZO-1 was up regulated when *B. subtilis* DSM32315 was added to the diets. Occludin-1 was an integral membrane protein of the epithelia tight junction, having functional importance in maintaining the integrity and barrier function of the tight junction (Holmes et al., 2006), which was the greatest expression in the group fed with LP plus probiotic diet compared with other treatments. Our study indicated that *B. subtilis* DSM32315 inclusion in LP diet had more tendency to ameliorate intestinal permeability than in the HP diet. The increasing mRNA expression of occluding-1 would be regulated by variable bacterial community, but further research is needed to verify this promotion.

EGF was a potent stimulant of cell migration (restitution) and cell proliferation, both of which were important in reestablishing epithelial continuity, and EGF also reduced injury and stimulated repair in animal models of gastric and small intestinal injury (Playford, 1995). In our study, it was that LP diet with *B. subtilis* DSM32315 facilitated the EGF mRNA expression in ileum, which was consistent with the effect on the morphology of the ileum. GLP-1 was associated with intestinal growth and adaptation in a variety of parental nutrition-induced intestinal atrophy and inflammatory bowel disease, which was also demonstrated to enhance intestinal adaptation (Dubé and Brubaker, 2007). Our study showed that the LP diet supplemented with *B. subtilis* DSM32315 increased the GLP-2

and IGF-1R gene expression in the ileal mucosa, which verified that the combination of LP and *B. subtilis* DSM32315 might enhance intestinal epithelial growth factors, proliferation, and differentiation of the ileum via upregulating the GLP-2 and IGF-1R gene expression in ileum of piglets, and further improved the morphology of the ileum.

In conclusion, our study indicated that the LP diet worked synergically with the *B. subtilis* DSM32315 to improve growth performance, protein digestibility, gut integrity and luminal morphology, and hindgut microbial composition and fermentation. These findings may provide a potential nutritional strategy to improve performance and gut health of weaning piglets.

Conflict of interest statement. None declared.

LITERATURE CITED

- Altmeyer, S., S. Kröger, W. Vahjen, J. Zentek, and L. Scharek-Tedin. 2014. Impact of a probiotic bacillus cereus strain on the jejunal epithelial barrier and on the NKG2D expressing immune cells during the weaning phase of piglets. *Vet. Immunol. Immunopathol.* 161:57–65. doi:10.1016/j.vetimm.2014.07.001
- AOAC. 1995. Official methods of analysis. 16th ed. Washington, DC: AOAC International.
- Bhandari, S. K., F. O. Opapeju, D. O. Krause, C. M. Nyachoti, D. Torrallardona, J. Brufau, E. Esteve-Garcia, R. Lizardo, J. Gasa, and J. F. Aguilera. 2010. Dietary protein level and probiotic supplementation effects on piglet response to *Escherichia coli* K88 challenge: performance and gut microbial population. *Livest. Sci.* 133:185–188. doi:10.1016/j.livsci.2010.06.060
- Cani, P. D. 2017. Gut cell metabolism shapes the microbiome. *Science.* 357:548–549. doi:10.1126/science.aao2202
- Chen, H., X. Mao, J. He, B. Yu, Z. Huang, J. Yu, P. Zheng, and D. Chen. 2013. Dietary fibre affects intestinal mucosal barrier function and regulates intestinal bacteria in weaning piglets. *Brit. J. Nutr.* 110:1–12. doi:10.1017/S0007114513001293
- Choi, J. Y., P. L. Shinde, S. L. Ingale, J. S. Kim, Y. W. Kim, K. H. Kim, I. K. Kwon, and B. J. Chae. 2011. Evaluation of multi-microbe probiotics prepared by submerged liquid or solid substrate fermentation and antibiotics in weaning pigs. *Livest. Sci.* 138:144–151. doi:10.1016/j.livsci.2010.12.015
- Dai, Z. L., G. Wu, and W. Y. Zhu. 2011. Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. *Front. Biosci. (Landmark Ed.)* 16:1768–1786. doi:10.2741/3820
- Diao, H., P. Zheng, B. Yu, J. He, X. B. Mao, J. Yu, and D. W. Chen. 2014. Effects of dietary supplementation with benzoic acid on intestinal morphological structure and microflora in weaned piglets. *Livest. Sci.* 167:249–256. doi:10.1016/j.livsci.2014.05.029
- Dong, G., A. Zhou, and F. Yang. 1996. Effect of dietary protein levels on the large intestinal protein putrefaction and diarrhoea in early-weaned pigs. *Acta Vet. Zootech. Sin.* 27:293–302.
- Dubé, P. E., and P. L. Brubaker. 2007. Frontiers in glucagon-like peptide-2: multiple actions, multiple mediators. *Am. J. Physiol. Endoc.* 293:460–465. doi:10.1152/ajpendo.00149.2007
- Elshaghabee, F. M. F., N. Rokana, R. D. Gulhane, C. Sharma, and H. Panwar. 2017. *Bacillus* as potential probiotics: status, concerns, and future perspectives. *Front. Microbiol.* 8:1490. doi:10.3389/fmicb.2017.01490
- Fanning, A. S., B. J. Jameson, L. A. Jesaitis, and J. M. Anderson. 1998. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J. Biol. Chem.* 273:29745–29753. doi:10.1074/jbc.273.45.29745
- Fierer, N., J. A. Jackson, R. Vilgalys, and R. B. Jackson. 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* 71:4117–4120. doi:10.1128/AEM.71.7.4117-4120.2005
- Fuller, R. 1989. Probiotics in man and animals. *J. Appl. Bacteriol.* 66:365–378. doi:10.1111/j.1365-2672.1989.tb05105.x
- Heo, J. M., J. C. Kim, C. F. Hansen, B. P. Mullan, D. J. Hampson, and J. R. Pluske. 2008. Effects of feeding low protein diets to piglets on plasma urea nitrogen, faecal ammonia nitrogen, the incidence of diarrhoea and performance after weaning. *Arch. Anim. Nutr.* 62:343–358. doi:10.1080/17450390802327811
- Hermes, R. G., F. Molist, M. Ywazaki, M. Nofrarias, A. Gomez de Segura, J. Gasa, and J. F. Pérez. 2009. Effect of dietary level of protein and fiber on the productive performance and health status of piglets. *J. Anim. Sci.* 87:3569–3577. doi:10.2527/jas.2008-1241
- Holmes, J. L., C. M. Van Itallie, J. E. Rasmussen, and J. M. Anderson. 2006. Claudin profiling in the mouse during postnatal intestinal development and along the gastrointestinal tract reveals complex expression patterns. *Gene Expr. Patterns* 6:581–588. doi:10.1016/j.modgep.2005.12.001
- Hong, H. A., R. Khaneja, N. M. Tam, A. Cazzato, S. Tan, M. Urdaci, A. Brisson, A. Gasbarrini, I. Barnes, and S. M. Cutting. 2009. *Bacillus subtilis* isolated from the human gastrointestinal tract. *Res. Microbiol.* 160:134–143. doi:10.1016/j.resmic.2008.11.002
- Jing, W., B. G. Sun, Y. P. Cao, and C. T. Wang. 2010. In vitro fermentation of xylooligosaccharides from wheat bran insoluble dietary fiber by Bifidobacteria. *Carbohydr. Polym.* 82:419–423. doi:10.1016/j.carbpol.2010.04.082
- Kechagia, M., D. Basoulis, S. Konstantopoulou, D. Dimitriadi, K. Gyftopoulou, N. Skarmoutsou, and E. M. Fakiri. 2013. Health benefits of probiotics: a review. *ISRN Nutr.* 2013:481651. doi:10.5402/2013/481651
- Khochamit, N., S. Siripornadulsil, P. Sukon, and W. Siripornadulsil. 2015. Antibacterial activity and genotypic-phenotypic characteristics of bacteriocin-producing *Bacillus subtilis* KKU213: potential as a probiotic strain. *Microbiol. Res.* 170:36–50. doi:10.1016/j.micres.2014.09.004
- Kim, J. C., C. F. Hansen, B. P. Mullan, and J. R. Pluske. 2012. Nutrition and pathology of weaner pigs: nutritional strategies to support barrier function in the gastrointestinal tract. *Anim. Feed Sci. Technol.* 173:3–16. doi:10.1016/j.anifeedsci.2011.12.022

- Kirchgessner, M., F. X. Roth, and U. Eidelsburger. 1993. Nutritive efficiency of tartaric acid and malic acid in the rearing of piglets. *J. Anim. Physiol. Anim. Nutr.* 16:93–108.
- Kong, X. F., G. Y. Wu, Y. P. Liao, Z. P. Hou, H. J. Liu, F. G. Yin, T. J. Li, R. L. Huang, Y. M. Zhang, and D. Deng. 2007. Effects of Chinese herbal ultra-fine powder as a dietary additive on growth performance, serum metabolites and intestinal health in early-weaned piglets. *Livest. Sci.* 108:272–275. doi:10.1016/j.livsci.2007.01.079
- Lee, S. H., S. L. Ingale J. S., Kim, K. H. Kim, A. Lokhande, E. K. Kim, I. K. Kwon, Y. H. Kim, and B. J. Chae. 2014. Effects of dietary supplementation with *Bacillus subtilis* LS 1–2 fermentation biomass on growth performance, nutrient digestibility, cecal microbiota and intestinal morphology of weanling pig. *Anim. Feed Sci. Technol.* 188:102–110. doi:10.1016/j.anifeedsci.2013.12.001
- Leek, A. B., E. T. Hayes, T. P. Curran, J. J. Callan, V. E. Beattie, V. A. Dodd, and J. V. O'Doherty. 2007. The influence of manure composition on emissions of odour and ammonia from finishing pigs fed different concentrations of dietary crude protein. *Bioresour. Technol.* 98:3431–3439. doi:10.1016/j.biortech.2006.11.003
- van Leeuwen, P., A. Veldman, S. Boisen, K. Deuring, G. J. Van Kempen, G. B. Derksen, M. W. Verstegen, and G. Schaafsma. 1996. Apparent ileal dry matter and crude protein digestibility of rations fed to pigs and determined with the use of chromic oxide (cr2o3) and acid-insoluble ash as digestive markers. *Br. J. Nutr.* 76:551–562.
- Liu, J. B., P. C. Xue, S. C. Cao, J. Liu, L. Chen, and H. F. Zhang. 2018. Effects of dietary phosphorus concentration and body weight on postileal phosphorus digestion in pigs. *Anim. Feed Sci. Technol.* 242:86–94. doi:10.1016/j.anifeedsci.2018.06.003
- Liu, J. B., H. L. Yan, S. C. Cao, J. Liu, Z. X. Li, and H. F. Zhang. 2019. The response of performance in grower and finisher pigs to diets formulated to different tryptophan to lysine ratios. *Livest. Sci.* 19:123–134. doi:10.1016/j.livsci.2019.01.016
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods.* 25:402–408. doi:10.1006/meth.2001.1262
- Low, A. G., I. G. Partridge, and I. E. Sambrook. 1978. Studies on digestion and absorption in the intestines of growing pigs. 2. Measurements of the flow of dry matter, ash and water. *Br. J. Nutr.* 39:515–526. doi:10.1079/BJN19780068
- Makarenko, M. S., V. A. Chistyakov, A. V. Usatov, M. S. Mazanko, E. V. Prazdnova, A. B. Bren, I. F. Gorlov, Z. B. Komarova, and M. L. Chikindas. 2018. The impact of *Bacillus subtilis* KATMIRA1933 supplementation on telomere length and mitochondrial DNA damage of laying hens. *Probiotics Antimicrob. Protein.* 7:1–6. doi:10.1007/s12602-018-9440-9
- Mccarthy, J. F., F. X. Aherne, and D. B. Okai. 1974. Use of HCl insoluble ash as an index material for determining apparent digestibility with pigs. *Can. J. Anim. Sci.* 54:107–109. doi:10.4141/cjas74-016
- Min, B. J., O. S. Kwon, K. S. Son, J. H. Cho, W. B. Lee, J. H. Kim, B. C. Park, and I. H. Kim. 2004. The effect of bacillus and active yeast complex supplementation on the performance, fecal bacillus counts and ammonia nitrogen concentrations in weaned pigs. *J. Anim. Sci.* 82:26–28.
- Neis, E. P., C. H. Dejong, and S. S. Rensen. 2015. The role of microbial amino acid metabolism in host metabolism. *Nutrients.* 7:2930–2946. doi:10.3390/nu7042930
- Nyachoti, C. M., F. O. Omogbenigun, M. Rademacher, and G. Blank. 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. *J. Anim. Sci.* 84:125–134. doi:10.2527/2006.841125x
- Opapeju, F. O., D. O. Krause, R. L. Payne, M. Rademacher, and C. M. Nyachoti. 2009. Effect of dietary protein level on growth performance, indicators of enteric health, and gastrointestinal microbial ecology of weaned pigs induced with postweaning colibacillosis. *J. Anim. Sci.* 87:2635–2643. doi:10.2527/jas.2008-1310
- Pieper, R., S. Kröger, J. F. Richter, J. Wang, L. Martin, J. Bindelle, J. K. Htoo, D. von Smolinski, W. Vahjen, J. Zentek, et al. 2012. Fermentable fiber ameliorates fermentable protein-induced changes in microbial ecology, but not the mucosal response, in the colon of piglets. *J. Nutr.* 142:661–667. doi:10.3945/jn.111.156190.
- Playford, R. J. 1995. Peptides and gastrointestinal mucosal integrity. *Gut* 37:595–597. doi:10.1136/gut.37.5.595
- Pluske, J. R., I. H. Williams, and F. X. Aherne. 1996. Maintenance of villous height and crypt depth in piglets by providing continuous nutrition after weaning. *J. Anim. Sci.* 62:131–144. doi:10.1017/S1357729800014417
- Qi, H., Xiang, Z., G. Han, B. Yu, Z. Huang, and D. Chen. 2013. Effects of different dietary protein sources on cecal microflora in rats. *Afri. J. Biotechnol.* 10:3704–3708. doi:10.5897/AJB102677
- Salazar-Lindo, E., S. Allen, D. R. Brewster, E. J. Elliott, A. Fasano, A. D. Phillips, I. R. Sanderson, P. I. Tarr and Latin American Society for Pediatric Gastroenterology, Hepatology and Nutrition. 2004. Intestinal infections and environmental enteropathy: working group report of the second world congress of pediatric gastroenterology, hepatology, and nutrition. *J. Pediatr. Gastroenterol. Nutr.* 39(Suppl. 2):S662–S669. doi:10.1097/00005176-200406002-00013
- Standards Press of China. 2003. Method for analysis of hygienic standard of meat and meat products. GB/T. 5009.44, Beijing (China).
- Tremaroli, V., and F. Bäckhed. 2016. Functional interactions between the gut microbiota and host metabolism. *Nature.* 71:242–249. doi:10.1038/nature11552
- Van, L. P., A. Veldman, S. Boisen, K. Deuring, G. J. M. Van Kempen, G. B. Derksen, M. W. A. Verstegen, and G. Schaafsma. 1996. *Brit. J. Nutr.* 76:551–562. doi:10.1079/bjn19960062
- Wang, Y., J. H. Cho, Y. J. Chen, J. S. Yoo, Y. Huang, H. J. Kim, and I. H. Kim. 2009. The effect of probiotic BioPlus 2B® on growth performance, dry matter and nitrogen digestibility and slurry noxious gas emission in growing pigs. *Livest. Sci.* 120:35–42.
- Wang, S. P., L. Yang, X. S. Tang, L. C. Cai, G. Liu, X. F. Kong, F. Blachier, and Y. L. Yin. 2011. Dietary supplementation with high-dose *Bacillus subtilis* or *Lactobacillus reuteri* modulates cellular and humoral immunities and improves performance in weaned piglets. *J. Food Agric. Environ.* 9:181–187. doi:10.1016/j.livsci.2008.04.018
- Wellock, I. J., P. D. Fortomaris, J. G. Houdijk, and I. Kyriazakis. 2008. Effects of dietary protein supply, weaning age and experimental enterotoxigenic *Escherichia coli* infection

- on newly weaned pigs: performance. *Animal*. 2:825–833. doi:10.1017/S1751731108001559
- Yan, H. L., S. C. Cao, J. Liu, H. F. Zhang, and J. B. Liu. 2018. Effect of feed intake level on the determination of apparent and standardized total tract digestibility of phosphorus for growing pigs. *Anim. Feed Sci. Technol.* 246:137–143. doi:10.1016/j.anifeedsci.2018.10.012
- Zhao, Y., B. Yu, X. Mao, J. He, Z. Huang, P. Zheng, J. Yu, G. Han, X. Liang, and D. Chen. 2014. Dietary vitamin D supplementation attenuates immune responses of pigs challenged with rotavirus potentially through the retinoic acid-inducible gene I signalling pathway. *Br. J. Nutr.* 112:381–389. doi:10.1017/S000711451400097X