Effect of Dietary Amylose/amylopectin Ratio on Diarrhea and Gut Microorganism of Weaned Pigs Challenged With Escherichia Coli Lipopolysaccharide

can yang
Hunan Normal University  https://orcid.org/0000-0001-8973-6994

min wang
Hunan Normal University

xiaowu tang
Hunan vocational technical college of environment and biology

huansheng yang (yhs@hunnu.edu.cn)
Hunan Normal University  https://orcid.org/0000-0003-1164-5771

fengna li
Institute of Subtropical Agriculture Chinese Academy of Sciences

yancan wang
Hunan Normal University

yulong yin
Hunan Normal University

Research

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Abstract

**Background:** Dietary amylose/amylopectin ratio (DAR) plays important role in piglets' immune system. It is controversial whether diarrhea could be relieved by changing DAR in weaning piglets.

**Methods:** A total of 60 (Landrace × Yorkshire) castrated male pigs (initial body weight 6.51 ± 0.64 kg) were allotted to 5 groups (1 pig/cage and 12 replicates/group) in a randomized complete block design according to their BW. Piglets were fed ad libitum with diets different in DAR (0.00, 0.20, 0.40, 0.60, 0.80) for 28 days. Feed transition occurs at day 15. 100 μg/kg body weight lipopolysaccharides or same amount of saline were injected via abdomen on day 29, 12 h before slaughter.

**Results:** Diarrhea rate and diarrhea degree were higher when DAR was 0.40 than in groups 0.20 and 0.80 during third week (P<0.05). Iso-valeric acid of ileum decreased after LPS stress (P<0.05). Cecal iso-butyrate acid and iso-valeric acid concentrations increased after LPS stress (P<0.05). Iso- and valeric acid concentration of ileal chyme was higher in group 0.20 compared with other groups (P<0.05). Cecal acetic acid and propionic acid concentration were higher in group 0.40 than in group 0.20 (P<0.05). Alpha diversity of cecal microbial representative by goods coverage was higher in group 0.40 when compared with group 0.20 (P<0.05). At the genus level, the abundance of *Ruminococcaceae_NK4A214_group* and *Anaerotruncus* in cecal chyme of Group 0.20 was significantly higher than that in Group 0.40 (P<0.05), with no significant difference compared with other groups (P>0.05). The abundance of *Cetobacterium* was significant lower in cecal chyme from Group 0.20 compared with pigs from Group 0.40(P<0.01), with no significant difference compared with other groups (P>0.05). Diarrhea rate during third week negative correlated with abundance of *Rikenellaceae_RC9_gut_group* and *X.Eubacterium_coprostanoligenes_group* (P<0.05).

**Conclusions:** Diarrhea happened when weaned piglets received diet with DAR 0.40 during feed transition, but they will regulate cecal microorganism and metabolites such as acetic acid and propionic acid to restore their health.

Background

The microbiota plays a pivotal role in regulating many aspects of immunity, protecting the host against pathogenic microbes and producing vitamins and other essential nutrients [1]. There is mounting evidence pointing to marked alteration of the structure and function of the gut microbiome and its role in the pathogenesis of diarrhoea-associated systemic inflammation. The gut microbiota exerts a primordial role promoting fermentation of fermentable fiber for production of short-chain fatty acids (SCFA). SCFA is vital nutrients for the colonic epithelial cells and the regulatory T lymphocytes (T-reg) that are essential for the maintenance of the immunological self-tolerance and limitation of the inflammatory response [2]. Diet rich in fermentable fiber promotes increased production of SCFAs which can contribute to the integrity of the intestinal epithelial barrier and attenuate local and systemic inflammation in chronic kidney disease rats [3]. High dietary total fiber intake (59% wt/wt HAMRS2) was associated with lower risk of inflammation and mortality in kidney disease and the associations were stronger in magnitude in those with kidney disease compared with individual without chronic kidney disease [4].

The ratio of SCFA production is dependent on fermentation substrate, the microbial composition, and colonic transit time. Weaning is a stage during which piglets have to face pathogenic challenges because pathways refer to innate system response were changed during this stage [5]. During weaning, piglets experience a radical change in diet and environment, milk from the sow is replaced by solid grain, this is likely very critical for the immature digestive and immune system of weanling pigs. The syndrome of postweaning diarrhea is a consequence of this situation. The most significant network altered by weaning is associated with antimicrobial and inflammatory response [5]. As the ratio of amylose to amylopectin raises, the enzymatic digestibility of the starch decreases because of the contact of amylose with amylopectin, thus diminishing the accessibility of enzymes to hydrolyze starch molecules [6]. Resistant starch which cannot be digested in small intestine can be fermented to SCFA, and then affect inflammation response during weaning stress. It was hypothesized that given an immune challenge, piglets receiving diet high in dietary amylose/amylopectin ratio (DAR) will have well-balanced immune responses that suppress potentially harmful inflammation compared to challenged control pigs. Therefore, in the current study, the aim was to determine the response of piglets receiving diet different in DAR to an immune system challenge with *E.coli* lipopolysaccharide (LPS) on gut microbial composition and their metabolites during post-weaning period.

Methods

Experimental procedure in this study was reviewed and approved by the Animal Care and Use Committee of the Hunan international joint laboratory of animal intestinal ecology and health, Hunan Normal University.

**Animals and diets**

Sixty castrated male pigs (Landrace × Yorkshire) with an initial average body weight (BW) of 6.51 ± 0.64 kg were selected, blocked by BW and allotted to five dietary treatments with 12 cages per treatment and 1 pig per metabolic cage. The experimental diet was formulated on the basis of nutrient requirements established by the NRC [7] for 7.11 kg pigs. The A, B, C, D, E diets differed only in DAR, 0.00, 0.20, 0.40,0.60 or 0.80 respectively (Table 1). Nursing diets were provided in a 2-phase feeding program. PhaseⅠlasted from d 1 to d 14. PhaseⅡlasted from d 15 to 28. Pigs were fed ad libitum. Water was provided ad libitum. On day 29, 12 h before slaughter, 6 pigs every treatment were challenged with lipopolysaccharides (LPS, from Escherichia coli O55:B5, Sigma Chemical Inc., St Louis, MO, USA, L2880) whereas an equivalent amount of sterile saline was administered to other 6 pigs. LPS was dissolved in sterile saline and administered intraperitoneal injection at 100 μg/kg body weight. One pig from A, B, D, E died 11 h after LPS stress.

**Slaughter surveys and sampling**
Diarrhea of piglets was recorded every day during the experimental period. On day 29, 12 h post-challenge, pigs were euthanized. Immediately after removal of the gastrointestinal tract from the carcass, digesta samples from stomach, proximal duodenum, distal jejunum, end of ileum, cecal and colon were taken. Mucosa from jejunum and ileum were sampled using glass slide scraping intestinal wall. A 5 cm section of cecal and colon was fixed by immersion in 10% buffer neutral formalin. In addition, the remaining digesta samples underwent determination of pH value (Testo 206, pH meter, Testo AG, Lenzkirch, Germany). Mucosa and chyme samples were stored at -80°C until required for analysis.

Analysis

Diarrhea incidence

Fecal consistency was scored as follows: 0 = normal, 5 = liquid. Diarrhea degree was calculated as the sum of the fecal scores for every piglet each week. Piglets diarrhea rates were recorded daily and calculated as follows: Diarrhea rate = total number of pigs with diarrhea/(total number of pigs × experimental days) × 100, where total number of pigs with diarrhea was defined as the number of pigs with diarrhea observed on each day.

Intestinal morphology

Fixed intestine was dehydrated, embedded, sectioned and stained with hematoxylin and eosin. The mean crypt depth was quantified using a 40 × combined magnification, and an image processing and analysis system (Version 1, Leica Imaging Systems Ltd., Cambridge, UK). A minimum of 20 crypts were randomly chosen and measured per subject. Crypt depth was measured using a light microscope fitted with an image analysis system (AxioScope A1, Carl Zeiss, Germany).

Volatile fatty acid analysis

Volatile fatty acid (VFA) determination was conducted on metaphosphoric acid derived samples using gas chromatographic methods described by Mathew [8]. Gas chromatography (Agilent Technologies 7890B GC System; AGILENT) and DB-FFAP column (30 m×250 μm×0.25 μm) were used for determination of propionate, acetate, butyrate, valerate, isobutyrate, and isovalerate concentrations.

Gene expression analysis by RT-qPCR

Total RNA was isolated from mucosa using RNAiso Plus (TaKaPa) and then reverse transcription reactions were performed using RT reagent kit (TaKaPa). All the procedures were carried out as described by the manufacturer's protocol. The quantity and quality of RNA were determined using NanoDrop ND-2000 spectrophotometer system (Thermo Fisher Scientific). Real-time RT-PCR primers were designed to assay genes related to tight connection and inflammation (Table 2). 18S were used as reference gene. Real-time RT-PCR for target genes were performed on MyIQ instrument (Bio-Rad, Hercules, California) using SYBR Green quantitative PCR mix (TaKaRa).

16S ribosomal RNA sequencing

Total genome DNA from cecal digesta of saline stressed piglets was extracted using CTAB/SDS method. The hypervariable regions V3-V4 of the 16S rRNA genes were chosen for pyrosequencing to investigated the taxonomic compositions of the microbial community. The PCR amplification was conducted in triplicate utilizing the barcoded universal bacterial primers. Triplicates were pooled, and the PCR amplicons were sequenced using IlluminaHiSeq2500 platform and 250 bp paired-end reads were generated according to previous study [9, 10]. The raw sequence data were processed and analyzed with a QIIME software package. Then, sequences with a threshold of 97% similarity were assigned to the same operational taxonomic units (OTUs) on the basis of representative sequences using Uparse v7.0.1001 and GreenGene Database. R software (version 2.15.3) with the 'vegan' package was used to perform bacterial analysis of Bray-Curtis dissimilarities based on the levels of changed gut microbiota.

Statistical analysis

Gene expression data from replicate measurement within the same RNA extraction were averaged and analyzed using Livak’s (2001) [11] method to measure the difference between the five DAR diets. Data of diarrhea occurrence, microorganism were examined by single factor design using GLM procedure. Other data were analyzed by double factor with the DAR and LPS stress as main effects, using general linear model (GLM) procedure of SAS 8.0. Duncan differences were determined to compare differences among the groups. Results were presented as means ± sem. Differences with \( P<0.05 \) were considered to be significant.

Results

Diarrhea occurrence

Diarrhea rate and diarrhea degree were higher in group 0.40 than in group 0.20 and 0.80 during third week \( (P<0.05) \). No statistical difference of diarrhea rate and degree could be observed between different experimental treatments during first, second, fourth and total four weeks \( (P>0.05) \) (Table 3).

pH value and VFA of digesta

No significant difference of pH could be observed between five groups in digesta of stomach, jejunum and ileum \( (P>0.05) \) (Table 4). Cecal pH was affected by DAR and LPS stress, pH value was higher in group 0.20 than in groups 0.80 and 0.00 \( (P>0.05) \), but with no significant difference when compared with groups 0.40 and 0.60 \( (P>0.05) \). Cecal and colonic pH value increased after LPS stress \( (P<0.05) \). No effect of interaction between DAR and LPS stress was found on pH value.
The DAR had no significant effect on SCFA such as acetic acid, propionic acid, iso-butyrate acid, iso-Valeric acid and total amount of SCFA concentration in jejunum (P>0.05) (Table 5). Jejunal butyrate acid was affected by DAR, it was higher in group 0.80 compared with groups 0.00, 0.40, 0.60 (P>0.05). Jejunal butyrate acid increased in group 0.00 and 0.20 but decreased in groups 0.60 and 0.80 after LPS stress (P>0.05). Valeric acid concentration in jejunum increased after LPS stress in groups 0.00, 0.20, 0.80 but decreased in groups 0.40 and 0.60 (P<0.05). Ileum acetic acid, propionic acid, butyrate acid, iso-butyrate acid, valeric acid and total amount of SCFA were not affected by DAR or LPS stress except that iso-valeric acid of ileum decreased after LPS stress (P<0.05). Cecal SCFA except butyrate acid was affected by DAR (P<0.05). Acetic acid, propionic acid and total SCFA concentrations were higher in group 0.40 compared with groups 0.00, 0.60, 0.80 (P<0.05). Iso-butyrate acid and iso-valeric acid concentrations increased after LPS stress (P<0.05), they were both higher in group 0.20 than in other groups (P<0.05). Valeric acid concentration was higher in group 0.20 compared with groups 0.00, 0.60, 0.80 (P<0.05). Total amount of SCFA was higher in group 0.40 when compared with groups 0.00, 0.60, 0.80 (P<0.05), but with no significant difference compared with group 0.20 (P>0.05). Colonic SCFA except valeric acid was affected by LPS stress but not DAR, all of SCFA such as acetic acid, propionic acid, iso-butyrate acid, butyrate acid, iso-valeric acid decreased after LPS stress (P<0.05).

Crypt depth of large intestine
Crypt depth of cecal was lower in group 0.80, lower than other groups except group 0.40 (P<0.05). Crypt depth of colon was not affected by DAR and LPS stress (P>0.05) (Table 6).

Expression of genes related to gut health
Expression of genes such as claudin, zo-1 in mucosa were showed in Table 7. DAR did not alter the mRNA expression of ZO-1, JL-1, TNFα in mucosa of jejunal, the occludin, ZO-1 in mucosa of ileum (P>0.05). LPS stress caused lower mRNA expression of claudin in jejunal (P<0.05). Claudin mRNA expression was higher in group 0.60 when compared with other groups (P<0.045) in jejunl, and it was higher in group 0.20 than in other groups in mucosa of ileum (P<0.01). Ingestion of diet with DAR 0.00 resulted in lowest TNFα mRNA levels in ileum mucosa, lower than groups 0.40, 0.60 and 0.80 (P<0.05).

The bacterial community composition in the cecum
The reads for each sample is in the range of 70049 to 96176. After quality trimming and chimera checking, each sample has 77296±7459 tags with a minimum length of 410 nucleotides, a maximum length of 426. 791 OUTs were shared by the five groups, and 169, 211, 194, 368, 247 OTUs were only found in ceca of group 0.00, 0.20, 0.40, 0.60, 0.80 respectively (Fig. 1). No significant differences were found on Shannon, Simpson, ACE, PD, whole tree indices and beta diversity of bacteria between different DAR groups (Fig. 2 A and Fig. 2 B). Alpha diversity representative by chao1 of group 0.40 tended to be lower than group 0.60 (P=0.053), with no significant difference compared with other groups (P<0.05). Alpha diversity representative by goods coverage of groups 0.20 and 0.40 was lower than group 0.60 (P<0.05), with no significant difference compared with other groups (P<0.05).

At the phylum level, Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes were predominately found in the cecal samples from different DAR groups. No significant difference was found in phylum between different DAR groups (P>0.05) (Fig. 3A). At phyla level, no significant difference was found between different DAR groups (P>0.05). At the genus level, the abundance of Ruminococcaceae_NK4A214_group and Anaerotruncus in cecal chyme of Group 0.20 was significantly higher than that in Group 0.60 (P<0.05), with no significant difference compared with other groups (P>0.05) (Fig. 3B). The abundance of Cetobacterium was significant lower in cecal chyme from Group 0.20 compared with pigs from Group 0.60 (P<0.01), with no significant difference compared with other groups (P>0.05).

Relationship between bacterial abundance and apparent indicators
According Fig 4, diarrhea rate during third week was negative correlated with abundance of Rikenellaceae_RC9_gut_group and X.Eubacterium_coprostanolgenes_group (P<0.05). Abundance of Ruminococcaceae_UCG_002, Ruminococcaceae_NK4A214_group was positive correlated with cecal total SCFA, acetic acid, propionic acid, iso-butyrate acid, iso-valeric acid and valeric acid concentrations (P<0.05). Abundance of Anaerotruncus was positive correlated with cecal iso-valeric acid concentration (P<0.05). Abundance of Ruminococcaceae_UCG_005, Prevotellaceae_NK3B31_group.Leeia was positive correlated with serum cholesterol concentrations (P<0.05).

Discussion
An inappropriate balance between pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α), and the anti-inflammatory cytokines such as IL-10 would lead to inflammation in local tissues such as bowel [12]. Elevation in expression of the pro-inflammatory cytokines such as TNFa and IL-6 are the hallmark of acute bowel inflammation [12]. Therefore, our cytokine profiling results suggest that acute bowel inflammation had occurred in Group 0.40 but not in Group 0.00 and 0.20 under 100 μg/kg of lipopolysaccharide (LPS) stress in weaning pigs. This is inconsistent with other reports. High level of amylase is associated with high resistant starch (RS) level. Chronic kidney disease rats consuming diets supplemented with amylopectin exhibited inflammation, activation of NFκB, upregulation of pro-inflammatory, pro-oxidant molecules, impaired nuclear transcription factor (Nrf2) activity, down-regulation of antioxidant enzymes, and disruption of colonic epithelial tight junction, but the high resistant starch diet significantly attenuated these abnormalities [14]. RS supplementation has proven to be effective in reducing markers of inflammation in the state of the disease [15]. Studies found a reduction of TNF-α concentrations in prediabetes patients supplemented with 45 g/d high-amylose maize for 12 weeks [16, 17]. Supplementation of HAM-RS2 20 g/d in the first month and 25 g/d during the second month led to a decrease in serum urea, IL-6, TNFα in end-stage renal disease patients [17]. Moreover, consumption of retrograded high-amylose corn resistant starch at 15% may protect the colon from developing inflammation by enhancing IL-10 abundance in pigs, but not affected TNFα and IL-6 abundance in colon [18]. Though gut injury was observed in piglets that received 60 [19] or 100 [20] μg/kg of E.coli LPS and injection of LPS stimulated the production of cytokines such as IL-1, TNF-α and IFN-γ [21]. But 11 h post-challenge showed...
no effect of LPS on TNFa mRNA expression in jejunal and ileal mucosa of weaning pigs in our result. The acute bowel inflammation in Group 0.40 was due to severe diarrhea happened during the last week before LPS stress. Diarrhea resulted adverse effect on average daily gain (ADG) and feed efficiency (F:G) during the next week (data not shown).

Prebiotic approaches also typically fail to consider the potential to cause detrimental changes in individuals in whom the gut microbiota is already substantially disrupted, such as the selective promotion of potentially pathogenic taxa. Phylum Proteobacteria abundance was higher in Group 0.40. Previous studies indicated that the abnormal increase of Prevotellaceae abundance exacerbated the occurrence of inflammation [22]. Genus Sutterella belongs to Prevotellaceae phylum, and it has been found to elevated in feces from dogs with acute hemorrhagic diarrhea [23]. The genus Rikenellaceae RC9 gut group was significantly increased in the high-fat diet with high-dose genistein mice group [24] and in isoproterenol-induced acute myocardial ischemia group [25]. In the present study, we observed the significant negative correlation between abundance of Rikenellaceae RC9 gut group and diarrhea rate during third week. Thus, the increase of Rikenellaceae RC9 gut group might associate with the gut inflammation. Although piglets from Group 0.40 suffered severe diarrhea, they got the same ADG and feed intake as other groups during the whole four experimental weeks. This result, in part, be due to SCFAs’ modulating inflammation response. In this study, 11 h post-LPS- challenge pigs showed a significant reduction in acetate, propionic acid, butyric acid and total short-chain fatty acid (SCFA) in colonic digesta. Supplementation of diet with DAR 0.40 in weaning piglets resulted in a significant increase in these SCFA in cecum digesta compared with Group 0.00. SCFAs have a wide range of anti-inflammatory properties, including the ability to increase colonic regulatory T cells [26], alter dendritic cell and macrophage function [27], and production of pro-inflammatory cytokines [28]. Gut microbiota conversion of inulin-type fructose into propionate could inhibit malignant cell proliferation, lessens systemic inflammation in liver [29]. The reduction in intestinal pH due to increased SCFA production decreases the formation of pro-inflammatory and pro-oxidant uremic toxins from colonic bacteria [14].

0.20-fed piglets exhibited less microbial diversity than 0.6-fed piglets. More microbial diversity is generally associated with a healthier phenotype [30]. Both Ruminococcaceae_NK4A214_group and Anaerotruncus abundance in cecal chyme were higher in Group 0.20 than that in Group 0.60. It is well known that intestinal microbiota is a crucial factor in maintaining intestinal barrier and harmful metabolites’ transfer. They can affect the intestinal permeability, enhance the transfer of harmful substances in blood, and stimulate inflammatory response [31]. The regulation of gut microbiota such as Ruminococcaceae_NK4A214_group and Anaerotruncus could be developed to an increase in cecal SCFA such as iso-butyrate acid, iso-valeric acid and valeric acid concentration. The higher levels of iso-BCFAs may be associated with alteration in the metabolism of BCAAs, namely valine, leucine, and isoleucine, which can serve as precursors of BCFAs [32]. Thus, the increased production of iso-butyric acid and iso-valeric acid should indicate increased protein degradation during LPS stress in Group 0.20. LPS challenge increases the plasma-urea nitrogen level in piglets due to muscle proteolysis as result of increased inflammation [33]. Study found inverse correlations between circulating iso-branched-chain fatty acids and C-reactive protein (CRP, an inflammatory marker) in patients with morbid obesity [34]. Released amino acids due to inflammation may be channeled to the liver to synthesize acute phase proteins and/or to serve as an energy source [35]. Further examination of the these and other related markers of inflammation and oxidative stress are warranted in studies of longer duration. Claudin-1 and occluding are key components of the epithelial tight junction. LPS stress caused lower claudin mRNA expression in jejunal mucosa. As a result, intestinal barrier function was affected as claudin mRNA expression decreased in Group 0.20 compared with Group 0.60.

Conclusions

In conclusion, diarrhea was affected by dietary amylose/amylopectin ratio (DAR). Diarrhea rate and diarrhea degree were higher when DAR was 0.40 and lower when it was 0.20 or 0.80 during third week after weaning. Consumption of diet with DAR 0.20 leads to increase in cecal pH, iso- and valeric acid concentration and in claudin mRNA expression in mucosa of ileum. After diarrhea, microbial composition changed to restore intestinal health in piglets supplemented diet with DAR 0.40. This was confirmed by higher alpha diversity and higher cecal acetic acid and propionic acid concentration in this group (0.40).

Declarations

Ethics approval and consent to participate

Experimental procedure in this study was reviewed and approved by the Animal Care and Use Committee of the Hunan international joint laboratory of animal intestinal ecology and health, Hunan Normal University.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yin Yulong and Yang Huansheng organized the experiment and gave some advice on experiment idea. Yang Can conducted the experiment and was a major contributor in writing the manuscript. Wang Min, Tang Xiaowu and Wang Yancan conducted the experimental analysis. Li Fengna reviewed the manuscript and gave some advice on experiment idea. All authors read and approved the final manuscript.

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Authorship

Can Yang, postdoctor in Hunan Normal University, tutor is Dr. Yulong Yin.

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Table 1
Composition of experimental diets (as fed basis)

| Ingredients, %       | Pre-care period | Late-care period |
|----------------------|-----------------|------------------|
|                      | A  | B  | C  | D  | E  | A  | B  | C  | D  | E  |
| Waxy corn starch     | 54.80 | 45.21 | 38.36 | 33.43 | 29.32 | 53.54 | 44.17 | 37.48 | 32.66 | 28.64 |
| High-Maize 1043      | -   | 9.59 | 16.44 | 21.37 | 25.48 | -   | 9.37 | 16.06 | 20.88 | 24.90 |
| Soybean meal         | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 |
| Full fat expanded soybean meal | 9.00 | 9.00 | 9.00 | 9.00 | 9.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 |
| Fermented soybean meal | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 |
| Fish meal            | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.80 | 5.80 | 5.80 | 5.80 | 5.80 |
| Whey, dried          | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| Plasma protein powder | 4.84 | 4.84 | 4.84 | 4.84 | 4.84 | -   | -   | -   | -   | -   |
| Monocalcium Phosphate | 1.17 | 1.17 | 1.17 | 1.17 | 1.17 | 1.33 | 1.33 | 1.33 | 1.33 | 1.33 |
| Soybean oil          | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | -   | -   | -   | -   | -   |
| Premix<sup>1</sup>    | 0.92 | 0.92 | 0.92 | 0.92 | 0.92 | 0.92 | 0.92 | 0.92 | 0.92 | 0.92 |
| Choline chloride     | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 |
| Limestone            | 0.74 | 0.74 | 0.74 | 0.74 | 0.74 | 0.72 | 0.72 | 0.72 | 0.72 | 0.72 |
| DL-lysine            | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 | 0.21 | 0.21 | 0.21 | 0.21 | 0.21 |
| DL Methionine        | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.21 | 0.21 | 0.21 | 0.21 | 0.21 |
| Salt                 | -   | -   | -   | -   | -   | 0.19 | 0.19 | 0.19 | 0.19 | 0.19 |
| TOTAL                | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

Calculated nutrient content<sup>2</sup>

|                        | Pre-care period | Late-care period |
|------------------------|-----------------|------------------|
| Amylose/amylopectin ratio | 0.00 | 0.20 | 0.40 | 0.60 | 0.80 | 0.00 | 0.20 | 0.40 | 0.60 | 0.80 |
| Digestive energy, kcal/kg | 3500 | 3500 | 3500 | 3500 | 3500 | 3508 | 3508 | 3508 | 3508 | 3508 |
| Crude protein,%         | 18.50 | 18.50 | 18.50 | 18.50 | 18.50 | 18.00 | 18.00 | 18.00 | 18.00 | 18.00 |
| Ca,%                   | 0.85 | 0.85 | 0.85 | 0.85 | 0.85 | 0.92 | 0.92 | 0.92 | 0.92 | 0.92 |
| Av.P,%                 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.48 | 0.48 | 0.48 | 0.48 | 0.48 |
| Salt,%                 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Amino acids, %SID<sup>3</sup> |            |                  |
| Lys                    | 1.45 | 1.45 | 1.45 | 1.45 | 1.45 | 1.31 | 1.31 | 1.31 | 1.31 | 1.31 |
| TSAA                   | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 |
| Thr                    | 0.83 | 0.83 | 0.83 | 0.83 | 0.83 | 0.74 | 0.74 | 0.74 | 0.74 | 0.74 |
| Trp                    | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 |

<sup>1</sup>Vitamin-mineral premix supplied per kilogram of feed: 10,000 IU of Vitamin A, 1,000 IU of Vitamin D3, 80 IU of Vitamin E, 2.0 mg of Vitamin K3, 0.03 mg of Vitamin B12, 12 mg of riboflavin, 40 mg of niacin, 25 mg of d-pantothenic acid, 0.25 mg of biotin, 1.6 mg of folic acid, 3.0 mg of thiamine, 2.25 mg of pyridoxine, 300 mg of choline chloride, 150 mg of Fe (FeSO4), 100 mg of Zn (ZnSO4), 30 mg of Mn (MnSO4), 25 mg of Cu (CuSO4), 0.5 mg of I (KI03), 0.3 mg of Co (CoSO4), 0.3 mg of Se (Na2SeO3), and 4.0 mg of ethoxyquin.

<sup>2</sup>Nutrient content of diets based on estimated nutrient contents of ingredients according to NRC(2012).

<sup>3</sup>SID, Standardized ileal digestible.

Dietary amylose/amylopectin ratio of A, B, C, D, E was 0.00, 0.20, 0.40,0.60 and 0.80 respectively.
### Table 2
RT-PCR primers related to tight connection and inflammation

| Name      | F/R | Primer                                                                 |
|-----------|-----|------------------------------------------------------------------------|
| Claudin   | F   | TTTCCTCAATACAGGAGGGAAGC                                                |
|           | R   | CCCTCTCCCACATTCGAG                                                     |
| Occludin  | F   | CAGGTGCACCCTCCAGATTG                                                   |
|           | R   | GGACTTTCAAGGGCCTGGAT                                                   |
| ZO-1      | F   | CTGAGGGAATTGGGCAGGA                                                    |
|           | R   | TCACCAAAGGACTGAGCC                                                   |
| IL-1β     | F   | ACGTGCCTTCTGCTACTGC                                                   |
|           | R   | TCCCTCGGCTTTGACATT                                                   |
| 18S       | F   | GAGCGAAAGCATTTGCCAAG                                                  |
|           | R   | GGCATCGTTATGGGCTAGGA                                                  |

ZO-1: tight junction protein zonula occulden-1, IL-1β: interleukin 1 β, TNFα: tumor necrosis factor, alpha.

### Table 3
Effect of dietary amylose/amylopectin ratio on diarrhea occurrence of weaned piglets challenged with E.coli LPS

| Items         | A   | B     | C  | D   | E   | SEM | P value   |
|---------------|-----|-------|----|-----|-----|-----|-----------|
| Diarrhea rate |     |       |    |     |     |     |           |
| First week    | 19.64 | 17.86 | 9.52 | 11.90 | 13.69 | 2.08 | 0.517     |
| Second week   | 13.89 | 24.31 | 29.86 | 21.53 | 20.14 | 2.76 | 0.464     |
| Third week    | 23.21 | 11.31 | 29.17 | 18.45 | 7.74  | 2.25 | 0.024     |
| Fourth week²  | 19.64 | 8.33  | 24.40 | 19.05 | 9.52  | 2.79 | 0.293     |
| Total four weeks² | 19.29 | 15.12 | 22.99 | 17.59 | 12.50 | 1.51 | 0.238     |

Diarrhea degree

| Items         | A   | B     | C  | D   | E   | SEM | P value   |
|---------------|-----|-------|----|-----|-----|-----|-----------|
| First week    | 7.42 | 6.75  | 4.00 | 5.67 | 5.75 | 0.90 | 0.790     |
| Second week   | 5.83 | 10.42 | 13.25 | 10.50 | 8.33  | 1.31 | 0.466     |
| Third week    | 10.42 | 4.67  | 14.25 | 9.17  | 3.08  | 1.19 | 0.029     |
| Fourth week²  | 8.92 | 3.67  | 11.92 | 10.17 | 4.42  | 1.45 | 0.295     |
| Total four weeks² | 32.58 | 25.50 | 43.42 | 35.50 | 21.58 | 3.19 | 0.224     |

¹Statistical analysis ended in day 27. Dietary amylose/amylopectin ratio of A, B, C, D, E was 0.00, 0.20, 0.40, 0.60 and 0.80 respectively.

### Table 4
Effect of dietary amylose/amylopectin ratio on pH value of digest of weaned piglets challenged with E.coli LPS

| Items ¹ | A      | B      | C      | D      | E      | SEM    | P value |
|---------|--------|--------|--------|--------|--------|--------|---------|
|         | LPS    | SAL    | LPS    | SAL    | LPS    | SAL    | D*S     |
| Stomach | 2.77   | 3.15   | 3.40   | 3.54   | 3.22   | 3.79   | 2.62    | 3.39    | 2.79   | 0.11  | 0.91 | 0.35 | 0.69 |
| Jejunum | 5.85   | 5.57   | 5.76   | 6.35   | 5.79   | 5.54   | 6.16    | 5.28    | 6.13   | 0.08  | 0.92 | 0.33 | 0.57 |
| Ileum   | 6.84   | 6.50   | 6.21   | 7.17   | 6.49   | 7.20   | 6.92    | 6.66    | 6.84   | 7.10  | 0.05  | 0.83 | 0.15 | 0.11 |
| Cecum   | 6.14   | 6.13   | 6.71   | 6.34   | 6.43   | 6.15   | 6.55    | 6.06    | 6.03   | 6.10  | 0.04  | 0.02 | 0.02 | 0.22 |
| Colon   | 6.55   | 6.76   | 6.80   | 6.56   | 6.92   | 6.32   | 6.93    | 6.37    | 6.91   | 6.40  | 0.05  | 1.00 | 0.00 | 0.14 |
LPS: lipopolysaccharide, SAL: saline, DAR: dietary amylose/amylopectin ratio. Means within each row with different superscripts differ significantly ($P<0.05$).

Dietary amylose/amylopectin ratio of A, B, C, D, E was 0.00, 0.20, 0.40, 0.60 and 0.80 respectively.
| Items                  | A          | B          | C          | D          | E          | SEM | Pvalue |
|-----------------------|------------|------------|------------|------------|------------|-----|--------|
|                       | LPS        | SAL        | LPS        | SAL        | LPS        | SAL |        |
| Jejunum               |            |            |            |            |            |     |        |
| Acetic acid           | 41.84      | 30.39      | 300.98     | 56.52      | 58.57      | 17.84| 69.97  | 99.37  | 49.07    | 27.86    | 10.63 | 0.24  |
| Propionic acid        | 11.14      | 7.23       | 77.46      | 28.46      | 22.61      | 7.96 | 13.32  | 20.08  | 24.19    | 10.67    | 2.95  | 0.28  |
| Iso-Butyrate acid     | 0.00       | 0.00       | 12.42      | 0.00       | 0.00       | 0.00 | 3.88   | 0.00   | 0.00     | 0.00     | 0.45  | 0.55  |
| Butyrate acid         | 10.35B     | 0.00       | 62.50AB    | 0.00       | 0.00B      | 0.00 | 6.57   | 37.87A | 86.79    | 3.37     | 0.00  | 0.38  |
| Iso-Valeric acid      | 6.68       | 7.69       | 20.16      | 0.00       | 0.00       | 4.64 | 9.11   | 25.37  | 15.59    | 13.49    | 1.84  | 0.23  |
| Valeric acid          | 27.52      | 9.05       | 46.52      | 0.00       | 27.48      | 33.69| 7.90   | 18.08  | 37.25    | 23.59    | 1.88  | 0.23  |
| Total amount of SCFA  | 97.53      | 54.37      | 520.04     | 84.98      | 108.66     | 64.13| 100.30 | 173.35 | 163.97   | 162.40   | 13.92 | 0.25  |
| Ileum                 |            |            |            |            |            |     |        |
| Acetic acid           | 594.22     | 927.69     | 786.89     | 631.66     | 541.72     | 928.44| 634.98 | 544.22 | 942.19   | 848.87   | 37.54 | 0.18  |
| Propionic acid        | 44.18      | 58.87      | 91.71      | 41.11      | 81.28      | 73.66| 48.28  | 60.25  | 77.71    | 104.72   | 11.91 | 0.83  |
| Iso-Butyrate acid     | 0.00       | 0.00       | 0.00       | 0.00       | 0.00       | 4.25 | 0.00   | 0.00   | 0.00     | 0.00     | 0.47  | 0.55  |
| Butyrate acid         | 48.89      | 52.47      | 51.66      | 28.96      | 44.53      | 62.24| 27.83  | 30.76  | 78.89    | 68.53    | 5.35  | 0.14  |
| Iso-Valeric acid      | 0.00       | 0.00       | 0.00       | 10.03      | 0.00       | 17.68| 0.00   | 0.00   | 0.00     | 3.04     | 1.25  | 0.15  |
| Valeric acid          | 1.32       | 0.00       | 0.00       | 0.00       | 0.00       | 0.00 | 0.00   | 0.00   | 0.00     | 0.00     | 0.15  | 0.60  |
| Total amount of SCFA  | 688.62     | 1039.03    | 930.26     | 711.75     | 667.53     | 1086.28| 711.09 | 635.23 | 1098.79  | 1025.16  | 39.02 | 0.06  |
| Cecal                 |            |            |            |            |            |     |        |
| Acetic acid           | 2746.25B   | 3610.85    | 3433.77AB  | 4426.02    | 4198.72A   | 4643.30| 3093.49B| 3187.17| 2901.98B| 3329.84 | 148.66 | 0.03  |
| Propionic acid        | 1242.81B   | 1476.83    | 1631.64AB  | 1805.10    | 2204.71A   | 1844.70| 1391.95B| 1414.73| 1473.23B| 1279.86 | 69.77 | 0.02  |
| Iso-Butyrate acid     | 86.89BC    | 89.75      | 211.57A    | 135.97     | 124.75B    | 117.36| 132.89BC| 63.96  | 82.11C   | 70.14   | 5.42  | 0.001 |
| Butyrate acid         | 585.71     | 965.03     | 637.10     | 712.82     | 820.75     | 966.94| 802.65| 778.01| 668.31   | 758.83   | 45.61 | 0.65  |
| Iso-Valeric acid      | 113.68B    | 110.53     | 309.97A    | 184.45     | 201.58B    | 122.02| 255.10B| 80.98  | 117.09B  | 83.84   | 10.09 | 0.00  |
| Valeric acid          | 192.85B    | 200.68     | 379.11A    | 292.34     | 295.05AB   | 232.47| 245.74B| 142.09| 211.27B  | 159.77  | 14.57 | 0.02  |
| Total amount of SCFA  | 4968.18B   | 6453.67    | 6603.15AB  | 7556.70    | 7845.56A   | 7926.79| 5921.81B| 5666.94| 5453.97B| 5682.28 | 260.26 | 0.03  |
| Colon                 |            |            |            |            |            |     |        |
| Acetic                | 2862.32    | 3032.80    | 2292.78    | 3213.04    | 2378.01    | 3367.35| 2104.43| 3360.75| 1701.21  | 3347.96  | 65.92 | 0.40  |
Dietary amylose/amylopectin ratio of A, B, C, D, E was 0.00, 0.20, 0.40, 0.60 and 0.80 respectively.

**Table 6**

| Items          | A       | B     | C     | D     | E     | SEM | P-value |
|----------------|---------|-------|-------|-------|-------|-----|---------|
| Colon          |         |       |       |       |       |     |         |
| Cecal          | 429.98<sup>A</sup> | 424.59 | 401.36<sup>A</sup> | 429.59 | 396.38<sup>AB</sup> | 385.18 | 396.34<sup>A</sup> | 414.19 | 372.74<sup>B</sup> | 362.18 | 5.26 | 0.01 | 0.73 | 0.69 |
| Colon          | 436.03 | 475.67 | 470.36 | 461.69 | 450.36 | 474.08 | 446.81 | 477.60 | 464.20 | 434.81 | 7.12 | 0.96 | 0.45 | 0.55 |

Dietary amylose/amylopectin ratio of A, B, C, D, E was 0.00, 0.20, 0.40, 0.60 and 0.80 respectively.

**Table 7**

| Items          | A       | B     | C     | D     | E     | SEM | P-value |
|----------------|---------|-------|-------|-------|-------|-----|---------|
| Jejunal mucosa |         |       |       |       |       |     |         |
| Claudin        | 0.75<sup>B</sup> | 1.04 | 0.98<sup>B</sup> | 1.03 | 1.01<sup>B</sup> | 1.02 | 1.11<sup>A</sup> | 1.51 | 0.79<sup>B</sup> | 1.16 | 0.04 | 0.045 | 0.01 | 0.49 |
| Zo-1           | 0.92 | 1.03 | 0.96 | 1.04 | 0.96 | 1.00 | 0.82 | 0.71 | 0.61 | 0.04 | 0.15 | 0.22 | 0.46 |
| IL-1β          | 0.84 | 1.11 | 0.60 | 0.91 | 0.39 | 0.73 | 0.65 | 0.77 | 0.37 | 0.87 | 0.08 | 0.53 | 0.06 | 0.97 |
| TNFα           | 1.25 | 1.06 | 0.75 | 1.63 | 0.61 | 0.83 | 0.65 | 0.73 | 0.47 | 0.79 | 0.13 | 0.50 | 0.32 | 0.78 |
| Ileal mucosa   |         |       |       |       |       |     |         |
| Occludin       | 1.49 | 1.06 | 1.27 | 1.93 | 1.08 | 1.44 | 0.98 | 1.05 | 1.08 | 1.62 | 0.10 | 0.55 | 0.26 | 0.51 |
| Claudin        | 1.15<sup>B</sup> | 1.04 | 2.37<sup>A</sup> | 2.20 | 1.25<sup>B</sup> | 1.53 | 1.13<sup>B</sup> | 1.24 | 1.22<sup>B</sup> | 1.38 | 0.05 | <0.0001 | 0.60 | 0.60 |
| Zo-1           | 1.07 | 1.06 | 1.07 | 1.25 | 1.21 | 1.02 | 0.82 | 0.92 | 0.90 | 1.11 | 0.06 | 0.63 | 0.65 | 0.84 |
| IL-1β          | 0.80 | 1.14 | 1.12 | 1.60 | 0.79 | 1.16 | 0.62 | 2.45 | 1.00 | 1.16 | 0.18 | 0.82 | 0.09 | 0.60 |
| TNFα           | 0.76<sup>C</sup> | 1.02 | 0.97<sup>BC</sup> | 1.02 | 1.96<sup>A</sup> | 1.25 | 1.30<sup>A</sup> | 1.84 | 1.46<sup>AB</sup> | 1.42 | 0.07 | 0.01 | 0.87 | 0.10 |

1. LPS: lipopolysaccharide, SAL: saline, DAR: dietary amylose/amylopectin ratio. Means within each row with different superscripts differ significantly (P<0.05).

ZO-1: tight junction protein zonula occluden-1, IL-1β: interleukin 1β, TNFα: tumor necrosis factor, alpha. Dietary amylose/amylopectin ratio of A, B, C, D, E was 0.00, 0.20, 0.40, 0.60 and 0.80 respectively.

**Figures**
Figure 1

The Venn diagram of the shared and unique OTUs between different DAR groups Dietary amyllose/amylopectin ratio of A, B, C, D, E was 0.00, 0.20, 0.40, 0.60 and 0.80 respectively. 791 OUTs were shared by the five groups, and 169, 211, 194, 368, 247 OTUs were only found in ceca of group 0.00, 0.20, 0.40, 0.60, 0.80 respectively.
Figure 2

Alpha (Fig.2 A) and Beta (Fig.2 B) difference between different DAR groups. Dietary amylose/amylopectin ratio (DAR) of A, B, C, D, E was 0.00, 0.20, 0.40, 0.60, and 0.80 respectively. No significant differences were found on Shannon, Simpson, ACE, PD_whole tree indexes and beta diversity of bacteria between different DAR groups (Table S2, Fig S1, S2). Alpha diversity representative by chao1 of group 0.40 tended to be lower than group 0.60 (P=0.053), with no significant difference compared with other groups (P>0.05). Alpha diversity representative by goods coverage of groups 0.20 and 0.40 was lower than group 0.60 (P<0.05), with no significant difference compared with other groups (P>0.05).
Figure 3

3A Bar graph showing the phylum level composition of bacteria. Color-coded bar plot showing the relative abundance of bacterial phyla across the different samples (Fig. 3 A left) or different groups (Fig. 3 A right). One representative sequence from a set of related sequences belonging to a same OUT was selected for continuous species annotation with RDP classifier, and the bacterial composition at phylum level of each sample was stated and visualized with histogram. First C represented it comes from ceca chyme. Second C represented dietary amylose/amylopectin ratio (DAR), DAR of A, B, C, D, E was 0.00, 0.20, 0.40, 0.60 and 0.80 respectively. No significant difference were found in phylum between different DAR groups. 3B The first 100 genus composition of bacteria. At the genus level, the abundance of Ruminococcaceae_NK4A214_group and Anaerotruncus in cecal chyme of Group 0.20 was significantly higher than that in Group 0.60 pigs (P<0.05), with no significant difference compared with other groups (P>0.05). The abundance of Cetobacterium was significant lower in cecal chyme from Group 0.20 compared with pigs from Group 0.60 (P<0.01), with no significant difference compared with other groups (P>0.05).
Figure 4

Correlation of environment and abundance of bacteria at genus level. Species information are arranged in rows and environment factors are arranged vertically and are on the horizontal axis (x-axis). Different colors indicate the relative abundance between species and environment factors, r<0 represented negative correlation, r>0 represented positive correlation, * means P<0.05. A: Acetic acid, P: Propionic acid, IB: Iso-Butyrate acid, B: Butyrate acid, IV: Iso-Valeric acid, V: Valeric acid, TS: Total amount of SCFA, TG: Triglyceride, CHO: Cholesterol, DR: diarrhea rate during third week. Diarrhea rate during third week negative correlated with abundance of Rikenellaceae_RC9_gut_group and X.Eubacterium_coprostanoligenes_group (P<0.05). Abundance of Ruminococcaceae_UCG.002, Ruminococcaceae_NK4A214_group was positive correlated with cecal total SCFA, acetic acid, propionic acid, iso-butyrate acid, iso-valeric acid and valeric acid concentrations (P<0.05). Abundance of Anaerotruncus was positive correlated with cecal iso-valeric acid concentration (P<0.05). Abundance of Ruminococcaceae_UCG.005, Prevotellaceae_NK3B31_group, Leeia was positive correlated with serum cholesterol concentrations (P<0.05).