Identification of gut microbes associated with feed efficiency by daily-phase feeding strategy in growing-finishing pigs

Qin Jiang a,b,c, Chunlin Xie a,b,c, Lingli Chen a,b,c, Hongli Xiao a,b,c, Zhilian Xie a,b,c, Xiaoyan Zhu a,b,c, Libao Ma a,b,c, Xianghua Yan a,b,c,*

* Corresponding author. 
E-mail address: xyan@mail.hzau.edu.cn (X. Yan).
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1st Introduction

A stable pork supply plays a vital role in addressing how to feed the world and provide animal protein sources in the coming decades (Lassaletta et al., 2019). However, pork production has large environmental challenges, such as greenhouse gas emissions, feed demand, land pollution, and freshwater use (Rauw et al., 2020; Tilman et al., 2002). Methods of sustainable swine production include genetic modification, reproductive techniques, environmental stressors, and feeding strategies (Ernst and Steibel, 2013; Wu et al., 2020; O’Connor et al., 2010). Feed efficiency has high impact on the sustainable pig industry due to feed costing up to 60%
to 75% of total production cost (Godinho et al., 2018; Patience et al., 2015; Wu et al., 2020). Precision feeding is an integrated approach that allows for feed demands with consideration for changes in nutrient requirements of each pig in the herd to optimize performance at minimal feed costs (Pomar et al., 2014), and is also a way to improve feed efficiency. Of note, daily-phase feeding (DPF) is a form of precision feeding that can be widely applied in farms (Andretta et al., 2014).

The majority of gut microbiota contribute to nutrient digestion and absorption, cellular growth, and tissue homeostasis in the host, and also play an important role in maintaining gut health in mammals (David et al., 2014; Gentile and Weir 2018; Lynch and Pedersen 2016; Subramanian et al., 2015). The pig genome and gut microbiome have been reported (Chen et al., 2021a; Groenen et al., 2017; Wang et al., 2019; Yang et al., 2017). Daily-phase feeding strategies can largely alter feed efficiency during swine production. Meanwhile, most current feeding strategies in China such as three-phase feeding (TPF) or five-phase feeding cannot avoid imbalances between swine nutrient requirements and dietary nutrient supply because daily changes in body weight are ignored (Pomar et al., 2021). Given the close relationship between gut microbiota and host nutritional metabolism in pigs (Gardiner et al., 2020; Patil et al., 2020), we need to investigate which key microbes play vital roles in improving feed efficiency during a DPF program.

In this study, we firstly investigated how a DPF strategy affected feed efficiency by analyzing the growth performance, intestinal morphology, and the intestinal functions. We then identified those microbes related to feed efficiency in growing-finishing pigs. Using 16S rDNA gene sequencing at 155 and 180 d of age, we tested the effect of a DPF program in contrast to a TPF program on pig growth performance and on the composition and potential functions of the intestinal microbiome. Finally, we further assessed the plasma metabolome profiles and analyzed the relationship between the potential key microbes and metabolites, which may contribute to the understanding of the interaction of host and microbiota on feed efficiency of swine.

2. Materials and methods

2.1. Animal ethics

All experiments involving swine were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals Monitoring Committee of Hubei Province, China, and the protocols were approved by Institutional Animal Care and Use Committee of Huazhong Agricultural University, Wuhan, China, under permit number HZAUWS-2019-020.

2.2. Experimental design and animals

In this study, 204 Landrace × Yorkshire pigs were raised in a commercial farm (Wuhan, Hubei, China). All experimental pigs were moved to an environmentally controlled fattening house (randomly assigned to 13 pens of the age of 75 d, and 101 and 103 pigs in 8 pens were assigned to the TPF program and DPF program, respectively. Pigs were fed with a commercial nursing feed for 5 d before the experiment. The pigs had ad libitum access to water and feed throughout the trial. Each pig had an electronic chip placed in their ear that enabled them to access to the feeders and the feeding station identified the pig when its head entered the feeder and constantly provided 30 g of feed when the feeder hopper was emptied. The meal size was calculated by recording the amount of food delivered in the short intervals between 2 consecutive visits registered by Electronic Feed Intake Recording Equipment (FIRE, Osborne, USA). Individual feed intake and body weight data were collected by FIRE from 80 to 180 d of age, and used to calculate growth performance indicators, such as average daily feed intake (ADF), average daily gain (ADG),

### Table 1

**Experimental diet composition and nutrients (as-fed basis, %).**

| Item                  | Nursing feed, from 15 to 25 kg | Phase I feed, from 25 to 45 kg | Phase II feed, from 45 to 75 kg | Phase III feed, from 75 kg to market weight |
|-----------------------|-------------------------------|-------------------------------|---------------------------------|-------------------------------------------|
| **Ingredient composition** |                               |                               |                                 |                                           |
| Corn                  | 63.5                          | 64.4                          | 66.1                            | 67.1                                      |
| Flour                 | 4                             | 4                             | 4                               | 4                                         |
| Soybean meal (CP, 46%) | 23.7                          | 21                            | 17.5                            | 13.9                                      |
| Flaxseed              | –                             | 2.5                           | 2.5                             |                                           |
| Rice bran             | –                             | 1.6                           | 1.5                             | 1.8                                       |
| Wheat middlings       | 2.2                           | 4.7                           | 7                               |                                           |
| Fish meal             | 1.5                           | 6                             | –                               |                                           |
| Full fat soybean      | 6                             | 0.7                           | 0.3                             |                                           |
| Soybean oil           | 1.8                           | 0.7                           | 0.2                             |                                           |
| Lysine (98.0%)        | 0.4                           | 0.5                           | 0.4                             |                                           |
| Methionine (98.5%)    | 0.4                           | 0.2                           | 0.1                             |                                           |
| Limestone             | 0.6                           | 1.3                           | 1.5                             | 1.7                                       |
| Monocalcium phosphate | 1.5                           | 0.6                           | 0.8                             | 0.4                                       |
| Sodium chloride       | 0.4                           | 0.3                           | 0.3                             |                                           |
| Magnesium sulfate     | 0.3                           | 0.2                           | 0.1                             |                                           |
| Premix                | 0.5                           | 0.5                           | 0.5                             |                                           |
| **Calculated nutrient level** |                       |                                |                                 |                                           |
| NE, kcal/kg           | 2,543.67                      | 2,403.65                      | 2,408.83                        | 2,421.30                                  |
| ME, kcal/kg           | 3,400                         | 3,260                         | 3,260                           | 3,260                                     |
| Chemical analysis     |                               |                                |                                 |                                           |
| Dry matter            | 88.56                         | 86.61                         | 86.58                           | 86.48                                     |
| Crude protein         | 18.00                         | 16.20                         | 15.50                           | 14.00                                     |
| Ether extract         | 5.43                          | 4.62                          | 4.26                            | 4.23                                      |
| Crude fiber           | 2.73                          | 3.27                          | 3.29                            | 3.29                                      |
| Ash                   | 4.86                          | 4.81                          | 4.70                            | 4.49                                      |
| Calcium               | 0.60                          | 0.65                          | 0.60                            | 0.60                                      |
| Total phosphorus      | 0.53                          | 0.44                          | 0.50                            | 0.44                                      |
| Available phosphorus  | 0.30                          | 0.25                          | 0.30                            | 0.22                                      |
| Lysine                | 1.09                          | 1.08                          | 0.94                            | 0.84                                      |
| Methionine            | 0.36                          | 0.43                          | 0.32                            | 0.38                                      |
| Aspartic acid         | 1.80                          | 1.55                          | 1.36                            | 1.06                                      |
| Threonine             | 0.79                          | 0.78                          | 0.62                            | 0.49                                      |
| Serine                | 0.92                          | 0.88                          | 0.76                            | 0.63                                      |
| Glutamate             | 3.36                          | 3.21                          | 2.91                            | 2.37                                      |
| Glycine               | 0.75                          | 0.73                          | 0.65                            | 0.55                                      |
| Alanine               | 0.98                          | 0.87                          | 0.79                            | 0.67                                      |
| Cysteine              | Not detected                  | Not detected                  | Not detected                    | Not detected                              |
| Valine                | 0.82                          | 0.77                          | 0.71                            | 0.64                                      |
| Isoleucine            | 0.72                          | 0.63                          | 0.57                            | 0.54                                      |
| Leucine               | 1.73                          | 1.46                          | 1.39                            | 1.19                                      |
| Tyrosine              | 0.66                          | 0.67                          | 0.66                            | 0.68                                      |
| Phenylalanine         | 0.84                          | 0.87                          | 0.79                            | 0.76                                      |
| Histidine             | 0.97                          | 0.88                          | 0.82                            | 0.71                                      |
| Arginine              | 1.10                          | 1.05                          | 0.98                            | 0.84                                      |

**Notes:**

1. Premix provided vitamins and minerals per kilogram of nursing feed: iron 180 mg, copper 150 mg, manganese 20 mg, zinc 150 mg, iodine 0.15 mg, selenium 0.2 mg, vitamin A 12,000 IU, vitamin D3 3,000 IU, vitamin E 40 IU, vitamin K3 2 mg, vitamin B1 3 mg, vitamin B2 6 mg, niacinamide 30 mg, pantothenic acid 20 mg, pyridoxine 4 mg, biotin 0.15 mg, folic acid 0.65 mg, choline 1.2 g and cobalamin 30 μg.

1. CP = crude protein; NE = net energy; ME = metabolic energy.
The feeder calibration was checked weekly. At 155 and 180 d, we randomly chose 1 pig per pen to collect fresh fecal and blood samples. Fecal samples were collected during or within less than 30 s after defecation, and blood samples were collected via jugular venipuncture into vacuum tubes and kept frozen in liquid nitrogen for transportation, and then stored at −80 °C until use. At 180 d of age, 1 pig from each pen was sacrificed for further sample collection.

2.3. Feeding program and diets

The experiment used 4 commercial feeds supplied by COFCO Corporation. Diets were formulated to meet the nutrient requirements of swine (NRC, 2012) and the nutritional values are shown in Table 1. The schematic design of conventional phase feed and daily mixed feed delivery to the pig pens is shown in Fig. 1A. Compared with the single feed silo that supplied 1 feed directly to 1 pig pen in the TPF program, the DPF program applied mixed feed with a gradual daily change of 2 adjacent phase feeds from 2 feed silos to 2 pig pens. The TPF program was supplied with fixed feed for phase I, phase II, and phase III which started at 81, 101, and 132 d of age, respectively. We designed the DPF program using adjacent phase feed based on an empirical model which predicts pig growth and its nutrient requirements (Hauschild et al., 2010). Detailed programs were performed with a blend of nursing feed and phase I feed mixed with a varied composition from 81 to 90 d of age, and a mix of phase I feed and phase II feed from 91 to 115 d of age, and a mix of phase II feed and phase III feed from 116 to 155 d of age according to Table S1. The simulated changes in nutrient levels are shown in Fig. S1. The blend formula was changed every day at 08:00 as the unconsumed diets were recycled to non-experimental pens and the new mixed diets were supplied to the feeder from 81 to 155 d of age in DPF, and from 156 to 180 d of age, pigs in DPF were fed with phase III feed that was same as the TPF program. The experimental design and sample collection time points are illustrated in Fig. 1B.

2.4. Intestinal morphological analysis

To evaluate the intestinal morphology, duodenal, jejunal, and ileal tissues fixed in 4% paraformaldehyde were embedded in paraffin and sliced, 5 μm paraffin sections were dewaxed with xylene, hydrated, and then stained with hematoxylin and eosin (H&E). For each sample, 8 intact villi-crypt units were selected for morphology observation using a light microscope coupled with Image J software (Rasband, NIH, USA). Villus height (VH, the height shown in Table 1. The schematic design of conventional phase feed and daily mixed feed delivery to the pig pens is shown in Fig. 1A. Compared with the single feed silo that supplied 1 feed directly to 1 pig pen in the TPF program, the DPF program applied mixed feed with a gradual daily change of 2 adjacent phase feeds from 2 feed silos to 2 pig pens. The TPF program was supplied with fixed feed for phase I, phase II, and phase III which started at 81, 101, and 132 d of age, respectively. We designed the DPF program using adjacent phase feed based on an empirical model which predicts pig growth and its nutrient requirements (Hauschild et al., 2010). Detailed programs were performed with a blend of nursing feed and phase I feed mixed with a varied composition from 81 to 90 d of age, and a mix of phase I feed and phase II feed from 91 to 115 d of age, and a mix of phase II feed and phase III feed from 116 to 155 d of age according to Table S1. The simulated changes in nutrient levels are shown in Fig. S1. The blend formula was changed every day at 08:00 as the unconsumed diets were recycled to non-experimental pens and the new mixed diets were supplied to the feeder from 81 to 155 d of age in DPF, and from 156 to 180 d of age, pigs in DPF were fed with phase III feed that was same as the TPF program. The experimental design and sample collection time points are illustrated in Fig. 1B.

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from the tip of villus to the villus-crypt junction) and crypt depth (CD, the depth of invagination between adjacent villi) were measured. Villus height/crypt depth was calculated.

### 2.5. Chemical analysis

Blood samples were placed on ice immediately after collection and centrifuged at 3,000 × g for 15 min. Serum samples were separated and stored in plastic tubes frozen at liquid nitrogen until analysis. The concentrations of serum total protein, blood urea nitrogen (BUN), alkaline phosphatase (ALP), albumin, glutamic-pyruvic transaminase (ALT), glutamic-oxaloacetic transaminase (AST), creatinine, and triglyceride were measured by an autoanalyzer (Abbott Alcyon 300, Abbott Diagnostics, Lake Forest, IL) and Abbott reagents (Table 2). Serum endotoxin, diamine oxidase, D-lactic acid concentration were analyzed, using commercial kits (Nanjing Jiangchong Bioengineering Institute of China, Nanjing, China), following the protocols described by the manufacturers.

Approximately 0.1 g of jejunal tissue was mixed with 1 ml phosphate-buffered saline (PBS) and homogenized after milling at 12,000 × g for 10 min at 4 °C. Cytokine concentrations of supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiangchong Bioengineering Institute of China, Nanjing, China). The activities of amylase, maltase, lactase, sucrase, lipase, and pepsin of the duodenum, jejunum, and pancreas were determined by colorimetry using assay kits (Nanjing Jiangchong Bioengineering Institute of China, Nanjing, China).

### 2.6. The 16S sequencing and data analyses

The total genomic DNA of fecal bacteria was extracted using a commercial kit (QIAGEN QIAamp PowerFecalDNA Kit, Germany). The integrity of the DNA was assessed by agarose gel electrophoresis. Genomic DNA was used as a template for PCR amplification as previously described (Xu et al., 2021). Universal primers 338F and 806R were used for PCR amplification of the V3–V4 hypervariable regions of 16S rRNA genes (338F, 5′-ACTCTACGGGAGGCAGCA-3′; 806R, 5′-GGACTACHVGGGTWTCTAAT-3′). The generated DNA pool was then sequenced on the Illumina HiSeq system with the sequencing strategy PE 300. The sequencing data were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME2) software package. All 16S rDNA sequencing data are publicly available in the National Center for Biotechnology Information (NCBI, PRJNA816606). Then paired end reads with overlap were merged to tags. The high-quality paired-end reads were combined with tags based on overlaps with FLASH. The tags were clustered to amplicon sequence variant (ASV) by scripts of software Dadada2. After that, the ASV unique representative sequences were obtained. Chimeras were filtered out by UCHIME (v4.2.40); ASV representative sequences were taxonomically classified using Ribosomal Database Project 2 (RDP2), Classifier v2.2 trained on the database.

The alpha diversity and principal coordinate analyses (PCoA) were calculated by Mothur (v1.31.2) with the corresponding rarefaction curve being drawn by software R (v4.2.0). The statistical significance of gut microbiota among different groups was assessed by permutational multivariate analysis of variance (PERMANOVA; 9,999 permutations, P < 0.001) in R. PCoA and PERMANOVA were based on the data matrix of the Bray–Curtis distances. Differential enrichment of bacterial features between the TPF and DPF groups was determined using linear discriminant analysis (LDA) effect size (LEfSe), with the all-against-all multiclass analysis, P < 0.05, and a logarithmic LDA threshold of 4.0. Functional analysis of gut microbiota was predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) (Douglas et al., 2020).

### 2.7. Metabolomic profiling by liquid chromatography/time-of-flight/mass spectrometry (LC-TOF/MS)

Blood plasma samples were extracted to obtain the supernatants. An equivalent volume was aliquoted from each sample, mixed to prepare the QC sample, and dried in a vacuum concentrator. A high-resolution tandem mass spectrometer Xevo G2 XS QTOF (Waters, UK) was used to detect metabolites eluted from the column. The Q-TOF was operated in both positive and negative ion modes. For positive ion mode, the capillary and sampling cone voltages were set at 3.0 kV and 40.0 V, respectively. For negative ion mode, the capillary and sampling cone voltages were set at 2.0 kV and 40.0 V, respectively. The mass spectrometry data were gained in Centroid MS mode. The TOF mass range was from 50 to 1,200 Da and the scan time was 0.2 s. For the MS/MS detection, all precursors were fragmented using 20 to 40 eV, and the scan time was 0.2 s. During the acquisition, the LE signal was gained every 3 s to calibrate the mass accuracy. In order to test the stability of the LC-MS during the whole acquisition, a quality control sample (pool of all samples) was gained after every 10 samples. Peak extraction was mainly achieved through the commercial software Progenesis QI (version 2.2), including peak alignment, peak extraction, normalization, deconvolution, and compound identification. Based on QC sample information, local polynomial regression fitting signal correction (Quality control-based robust LOESS signal correction, QC-RSC) was performed on the real sample signal. The obtained data were applied for principal component analysis (PCA) and orthogonal
partial least squares discriminant analysis (OPLS-DA). R² and Q² were used to evaluate the quality of PCA and OPLS-DA models. Differential metabolites were identified with variable importance projection (VIP) > 1.0 and P < 0.05.

2.8. qPCR for target genes

Total RNA was extracted from the jejunum of pigs. The relative expression of genes associated with anti-oxidation was measured by quantitative polymerase chain reaction (qPCR). Oligonucleotide primers used to amplify target genes are shown in Table S2. The qPCR program consisted of an initial denaturation at 95 °C (10 min) followed by 40 cycles of denaturation at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The 2⁻ΔΔCT method with GAPDH used as the reference gene was used to calculate the relative expression.

2.9. Statistical analysis

Experimental data were analyzed by unpaired two-tailed t-test or Mann–Whitney U test with GraphPad 9.0 software (GraphPad Software Inc., San Diego, USA). The detailed descriptions of the
3. Results

3.1. A daily-phase feeding program improves feed efficiency in growing-finitning pigs

Growth performance from 81 d of age to the end of feed shift time (155 d of age) and the end of the experiment (180 d of age) was not influenced by the treatment \((P > 0.05)\) with regard to ADG and ADFI (Fig. 2A and B). However, FCR was significantly lower in DPF than that in TPF with a remarkable decrease from 2.58 to 2.43 \((P < 0.001)\) at 155 d of age, and the significant differences lasted to 180 d of age with the ratio of 2.73 to 2.63 \((P < 0.001)\) (Fig. 2C). In addition, the DPF program reduced the intake of crude protein \((P < 0.01)\) (Fig. 2D), lysine \((P < 0.01)\) (Fig. 2E), crude fiber \((P < 0.001)\) (Fig. 2F), ash \((P < 0.01)\) (Fig. 2G), ether extract \((P < 0.01)\) (Fig. 2H), net energy \((P < 0.01)\) (Fig. 2I), and total phosphorus \((P < 0.05)\) (Fig. 2J) at 155 and 180 d of age. Available phosphorus intake was significantly reduced at 180 d of age \((P < 0.05)\) (Fig. 2K), but not at 155 d of age \((P = 0.0578)\).

3.2. A daily-phase feeding program improves intestinal function in growing-finitning pigs

Jejunal and ileal villus height to crypt depth ratio were significantly enhanced in DPF compared to TPF \((P < 0.05)\), while no significant changes were observed in the duodenum (Fig. 3A and B).

Table 3

Digestive enzyme activities in TPF and DPF group at 180 d of age.

| Item                  | Treatments | P-value |
|-----------------------|------------|---------|
| Duodenum, U/mg prot   |            |         |
| Amylase               | 1.946      | 2.459   | 0.36   |
| Maltase               | 43.19      | 33.18   | 0.12   |
| Lactase               | 5.467      | 6.34    | 0.34   |
| Sucrase               | 1.798      | 3.04    | 0.12   |
| ALP                   | 94.21      | 107.2   | 0.33   |
| Jejunum, U/mg prot    |            |         |
| Amylase               | 1.779      | 1.923   | 0.69   |
| Maltase               | 116.4      | 100.9   | 0.27   |
| Lactase               | 8.676      | 12.18   | 0.23   |
| Sucrase               | 8.91       | 11.95   | 0.20   |
| Pepsin                | 214.7      | 130.8   | 0.16   |
| Lipase                | 83.1       | 97.08   | 0.45   |
| ALP                   | 359.1      | 426.9   | 0.16   |
| Pancreas, U/mg prot   |            |         |
| Pepsin                | 311.5      | 271.9   | 0.58   |
| Lipase                | 30.63      | 32.06   | 0.47   |

TPF = three-phase feeding; DPF = daily-phase feeding; ALP = alkaline phosphatase.

1 Data are means of 8 pigs per treatment, \(P < 0.05\) determined by unpaired two-tailed Student’s \(t\)-test.

Fig. 4. Measurements of fecal microbiome alpha and beta diversity at amplicon sequence variant (ASV) level. Measurement of alpha diversity at ASV level using the (A) Chao1, (B) Shannon, and (C) Simpson between TPF and DPF program at 155 and 180 d of age. Data are expressed as the mean ± SEM (\(n = 8\)). \(* P < 0.05\) by Student’s \(t\)-test. (D) Principal coordinate analysis (PCoA) based on Bray–Curtis distance of all the samples between TPF (red dots) and DPF (blue dots) cohorts. (E) Heatmap of Bray–Curtis distances between each program and permutational multivariate analysis of variance (PERMANOVA; 9,999 permutations) were shown separately on 155 and 180 d of age. \(* P < 0.05\), \(** P < 0.01\). TPF = three-phase feeding; DPF = daily-phase feeding.
sucrase and ALP in the duodenum and jejunum, while a decreasing trend in pepsin was detected in the pancreas (Table 3). The production of pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), and interleukin-6 (IL-6) were measured in the jejunum, with no significant changes observed (Fig. S2A). By measuring the intestinal permeability and anti-oxidative status, we found that pigs in DPF had lower plasma diamine oxidase concentration ($P < 0.05$) than pigs in TPF (Fig. 3E), whereas the endotoxin and D-lactic acid concentrations were similar between the 2 programs (Fig. 3C). The activities of total antioxidant capacity (T-AOC) and total superoxide dismutase (T-SOD) of the jejunum were significantly higher in DPF than the TPF program ($P < 0.05$) (Fig. 3F and G), and there was an increasing trend in glutathione peroxidase (GSH-PX) (Fig. 3I) in DPF compared to TPF, while there was no difference between DPF and TPF in malondialdehyde (Fig. 3H). The mRNA expression results also showed that SOD1, SOD3 and NOQ1 ($P < 0.01$) were significantly increased and KEAP1 was decreased ($P < 0.05$) in DPF compared to the TPF program (Fig. S2B).

3.3. Daily-phase feeding decreased relative abundance of Oscillibacter, while increasing the abundance of Paraprevotella and Prevotella compared with TPF

Daily-phase feeding had no significant effect on the alpha diversity and richness of the gut microbiota compared to TPF at both 155 and 180 d of age (Fig. 4A–C). PCoA based on Bray–Curtis distances revealed that the gut microbiota of pigs was altered under the different feeding patterns (Fig. 4D). The gut microbiota between TPF and DPF differed significantly at 155 d of age, and it was worth noting that TPF and DPF no longer differed significantly at 180 d of age, as shown by the comparison of distances (Fig. 4E).

At the phylum level, Firmicutes, Bacteroidetes, Spirochaetes, and Proteobacteria predominated in both the TPF and DPF programs at the phylum level, Firmicutes, Bacteroidetes, Spirochaetes, and Proteobacteria predominated in both the TPF and DPF programs at 155 and 180 d of age.
DPF decreased relative abundance of Oscillibacter, while increasing the abundance of Paraprevotella and Prevotella compared with TPF. (A) Comparison of the major microbes at the species level between TPF and DPF programs at 155 and 180 d of age. (B) Linear discriminant analysis Effect Size (LEfSe) analysis based on amplicon sequence variants (ASVs) characterized the microbiomes between TPF (red) and DPF (blue) on 155 d of the pig. Boxplot of relative abundance of (C) Oscillibacter valericigenes, (D) Paraprevotella clara YIT11840, and (E) Prevotella copri DSM18205 between the TPF and DPF programs at 155 and 180 d of age. Whiskers in the boxplot represent the range from minimum to maximum. The horizontal line in the boxplot is the median. Level of significance by Mann–Whitney U test, *P < 0.05. (F) Heatmap of Spearman’s correlations and level of significance between genus, average daily feed intake (ADFI), average daily gain (ADG) and feed conversion ratio (FCR) on 155 d of age. The color represents the correlation, *P < 0.05, **P < 0.01. (G) Heatmap of Spearman’s correlations and level of significance between species, ADFI, ADG and FCR on 155 d of age. The color represents the correlation. n = 8, *P < 0.05. TPF = three-phase feeding; DPF = daily-phase feeding.
155 and 180 d of age. In comparison to TPF, the profiling of microbially phyla in DPW was characterized by a high proportion of Bacteroidetes and a low proportion of Proteobacteria (Fig. 5A), and the Firmicutes to Bacteroidetes ratio was significantly lower in DPW than the TPF program at 155 d of age (Fig. 5B). At the family level, the 3 most abundant bacterial families both at 155 and 180 d of age primarily consisted of Prevotellaceae, Lactobacillaceae, and Lachnospiraceae. The average abundance of Prevotellaceae of DPW increased to 35.1%, while it was 22.9% in the TPF program (Fig. 5C). At the genus level, 20 taxa accounted for about 95% of the total assigned sequences across systems at 155 d of age (Fig. 5D). "Prevotella" (30.2%), "Lactobacillus" (11.7%), "Treponema" (3.6%), and "Lachnospiraceae" (2.9%) were the 4 most abundant genera in both programs. "Clostridium" (3.4%) was notably more abundant in DPW than in TPF (1.8%), while "Paraprevotella" (2.5%) was more abundant in TPF than in DPW (1.0%). At the species level, pig gut in DPW harbored more "Prevotella copri DSM18205 (P < 0.05), and "Paraprevotella clara YIT11840 (P < 0.05) but fewer "Oscillibacter valericigenes (P < 0.05) in DPW at 155 d of age than in the TPF program (Fig. 6A–E). We determined the correlation between signature gut microbes of the feeding strategy and feed conversion ratio using Spearman's rank correlation coefficient and significance test. The results showed that "Oscillibacter" (P < 0.05) was positively correlated with feed conversion ratio, and at species level, "O. valericigenes" was significantly related to FCR (Fig. 6F and G). Thus, we concluded that DPW decreases the relative abundance of Oscillibacter, while increasing the abundance of Paraprevotella and Prevotella compared with TPF.

3.4. Microbial functional prediction of the DPW microbiota

To better understand the functional roles of the microbiome, we used PICRUSt2 to investigate the functional profiles of the gut bacterial community (Fig. 7). The results demonstrated that the relative abundances of the genes involved in “Methane metabolism”, “Carbon metabolism”, “Ribosome”, and “Biosynthesis of cofactors” pathways were significantly changed in the DPW program. A total of 9 predicted function genes involved in “cell motility” and “signal transduction” were differentially represented between the 2 programs at 155 d of age. As such, DPW pigs had a higher relative abundance of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway genes involved in “Fructose and mannose metabolism”, “Biosynthesis of cofactors”, “Pyrimidine metabolism”, compared to TPF pigs (P < 0.05). In contrast, the predicted pathways “Pyruvate metabolism”, “Propanoate metabolism”, “Flagellar assembly” and “Phosphotransferase system” were lower in DPW pigs, compared to TPF pigs (P < 0.05). The altered “Digestive system” and “Carbon metabolism” functional pathways provide strong evidence that the DPW program regulates carbohydrate metabolism and amino acid metabolism functions through gut microbiota.

3.5. A daily-phase feeding program increased plasma metabolites in the amino acid metabolism pathways

A total of 2,515 plasma metabolite features were detected by the UPLC-QTOF/MS. The PCA of the metabolites between TPF and DPW programs at 155 and 180 d of age were similar to that of the fecal microbiota (Fig. 8A). However, we did not detect the significant relationships between plasma metabolites and the FCR values at the significance threshold of P < 0.05. Metabolites with VIP > 1 and bacterial genera significantly affected by feeding programs were used for the Pearson’s correlation analysis. We found that 24 metabolites were significantly differentially expressed between the 2 programs at 155 d of age, with 16 over-expressed in the TPF program and 8 over-expressed in the DPW program (Fig. 8B). Among these 24 metabolites, 13 metabolites had a tendency to positively correlate with the abundance of "Prevotella copri DSM18205, and "P. clara YIT11840 (P < 0.05), and one metabolite showed a tendency to positively correlate with "O. valericigenes" (Fig. 8C). The TPF program enriched pathways related to vitamin B6 metabolism and purine metabolism, and DPW enriched pathways related to amino acid metabolism, including phenylalanine, tyrosine and tryptophan biosynthesis and phenylalanine metabolism (Fig. 8D and E).

4. Discussion

We performed this study to better understand microbial differences in the pig gut microbiome between TPF and DPW programs related to feed efficiency. A daily-phase feeding program could have beneficial impacts on feed efficiency, with improved intestinal morphology, intestinal permeability, and the anti-oxidative ability of the intestine, as well as increased abundance of potentially beneficial microbes. Integrative analysis showed that DPW altered the microbiota and that P. clara and Prevotella copri were 2 key microbes related to high levels of phenylalanine metabolites in plasma that may improve feed efficiency.
Fig. 8. The DPF program increased plasma metabolites in the amino acid metabolism pathways. (A) Principal component analysis (PCA) plots of microbial and metabolic profiles of TPF and DPF programs at 155 and 180 d of the pigs. (B) The volcano plots of the significantly differential metabolites in plasma under both pos and neg mode. The red spots
Pigs were fed with 3 or 5 feeding phases, which meant nutrients could be under- or over-supplied during the whole feeding period (Pomar and Remus, 2019). Daily-phase feeding is a practical solution for addressing this imbalance problem by adjusting the whole herd daily and providing nutritional requirements daily, respectively (Andretta et al., 2014). By measuring feed intake, body weight, and carcass composition of each pig, DPF has proven to be more precise in terms of meeting the nutrient requirements of the herd compared with TPF, excretes less nitrogen and phosphorus into the environment, and saves costs. Some studies did not observe that FCR could be reduced in DPF, which may be due to insufficient amino acid supplies (Andretta et al., 2014; Remus et al., 2019). In our study, we found that the DPF program could significantly reduce the FCR and save nutrition compared to the TPF program during the daily mixed feed feeding phases; FCR and these effects persisted even after the DPF group was fed the same feed as the TPF program. The efficiency of nitrogen and phosphorus utilization, however, was not measured.

Feed efficiency is a complex phenotype that captures how effectively feed is turned into food products for human beings. A genome-wide association scan was conducted to identify the genetic markers and genes associated with feed efficiency (Do et al., 2014), and the intestinal microbiota of different breeds with feed efficiency traits (Bergamaschi et al., 2020; Do et al., 2014), and microbes that may be influenced by host genetics (Camarinha-Silva et al., 2017) were identified. The gut microbiota of pigs with extremely high and low RFI under the same feeding conditions were examined to find potential gut bacteria associated with feed efficiency (McCormack et al., 2017; Tan et al., 2017; Yang et al., 2017). Other studies focused on influencing feed efficiency via various feeding strategies, such as reducing meal frequency (Yan et al., 2021), dietary protein restriction feeding (Fan et al., 2017), and time-restricted feeding (Zeb et al., 2020), were also assubjected to comparative analysis of the gut microbiota. The gut microbes from fecal microbiota transplantation (FMT) (Siegertstetter et al., 2018) and dietary supplementation with feed additives that could improve feed efficiency were also investigated (Li et al., 2020).

Here, we compared the gut microbiota between TPF and DPF programs that differed in feed efficiency, to identify the key microbes that could promote feed efficiency.

The gut microbiota of DPF differed the most from those of TPF, whereas the difference was not detectable after the 2 groups were fed the same diet for 25 d. This is consistent with the theory that a long-term dietary pattern may be the most suitable factor for influencing the gut microbiota (David et al., 2014). The gut microbiota of the 2 feeding programs showed this effect, demonstrating the significance of feeding strategy and diet in determining feed efficiency in growing-finishing pigs. Firmicutes and Bacteroidetes, the predominant phyla in the gut microbiome of most mammals, have been closely correlated with energy partitioning and feed efficiency in pigs (Bergamaschi et al., 2020). Phylum Bacteroidetes has been shown to be more abundant in pigs with high feed efficiency or lean pigs compared to those with lower feed efficiency or obese pigs (Jami et al., 2014). While in this study, no significant difference in the abundance of these 2 taxa was observed between TPF and DPF groups, Firmicutes and Bacteroidetes predominated in the fecal microbiome of all pigs, and the relative abundance of Bacteroidetes in the fecal microbiome of DPF pigs with high feed efficiency was slightly higher than that of pigs in the TPF group. Although microbial diversity showed no difference between TPF and DPF, we further identified the potential key gut microbes related to FCR. Our analysis reveals that Paraprevotella, Prevotella and Oscilbacter are correlated with the FCR. Prevotella and P. clara belong to the Prevotellaceae. Prevotella, a core genus of pigs, produces enzymes that can degrade complex dietary polysaccharides, then improve fiber digestibility and feed efficiency (Chen et al., 2021b; Amat et al., 2020). This could explain why Prevotella might be a keystone microbe to increasing host feed intake (Yang et al., 2017). P. clara is one of the predominant cellulolytic bacterial species that is closely related to the production of short-chain fatty acids (Gao et al., 2018). The metabolic pathways of aromatic amino acids (e.g., phenylalanine metabolism), which are related to the synthesis of indole propionic acid, have also been associated with the species from P. clara.

Gut microbiota homeostasis influences intestinal metabolism (Lee et al., 2014). We hypothesized that DPF-induced alterations in microbiota composition might be followed by changes in the abundance of various metabolites in plasma. In this study, a comprehensive analysis of the plasma metabolome of TPF and DPF pigs was conducted. Most of the differentially enriched metabolites between TPF and DPF pigs were accounted for as amino acid metabolites. Functional analysis showed that the differential metabolites were involved in phenylalanine, tyrosine and tryptophan biosynthesis and phenylalanine metabolism. In animal models, phenylalanine could help to improve host metabolism by enhancing intestinal barrier function, showing anti-inflammatory properties and strengthening immune function (Leibhuber et al., 2015; Xu et al., 2020). In this study, the 2 main microbes were highly positively related to the increase in amino acid catabolites. Particularly, the metabolite of trans-cinnamic acid, a gut microbial metabolite produced by the deamination of phenylalanine, is known to exhibit a wide range of biological activities such as antioxidant and anti-inflammatory activities (Foti et al., 2004). These results showed that the altered phenylalanine profile in DPF plasma might contribute to feed efficiency in pigs.

5. Conclusion

In summary, we revealed that a DPF program could improve feed efficiency in growing-finishing pigs via the development of villi, permeability, and the anti-oxidative ability of the intestine, and by altering the abundance of Paraprevotella, Prevotella and Oscilbacter, thus affecting the plasma metabolites in phenylalanine metabolism. Together, the combined results of the present study may aid future research in identifying the 2 key microbes that promote feed efficiency in growing-finishing pigs.

Author contributions

Qin Jiang, Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization, Funding acquisition, Writing - Original Draft; Chunlin Xie, Investigation, Validation; Lingli Chen, Investigation, Formal analysis, Visualization; Hongli Xiao, Investigation, Validation; Zhilian Xie, Investigation, Validation; Xiaoyan Zhu, Investigation, Validation; Libao Ma, Supervision, Resources; Xianghua Yan, Conceptualization, Supervision, Project administration, Funding acquisition.
Declaration of competing interest

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

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.09.005.

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