**Comparative analysis of the ileal bacterial composition of post-weaned pigs fed different high-quality protein sources**

J. Ortman, S. M. Sinn, W. R. Gibbons, M. L. Brown, J. M. DeRouchey, B. St-Pierre, M. Saqui-Salces, C. L. Levesque

*Animal* journal

**Supplementary Methods**

*Tissue sample collection, preparation, and histological analyses*

On each of collection day 7 and 21 post-weaning, a total of twelve pigs were processed individually at 20 min intervals, beginning at 0900 h. Following euthanasia via captive bolt stunning and exsanguination, the entire gastrointestinal tract was carefully excised from the abdominal cavity. The stomach was segregated at both the cardiac and pyloric regions prior to its removal. Tissue samples from the pyloric and corpus regions were dissected, rinsed with saline, blotted dry, then placed in buffered formalin for a minimum of 24 h. These were then dehydrated and infiltrated with paraffin wax (South Dakota Animal Disease Research & Diagnostic Laboratory, Brookings, SD, USA) for histological staining. The duodenum, jejunum, and ileum were separated by hemostats, and the small intestine was detached from the large intestine marked by the cecum. The proximal duodenum was divided into two segments: one section was gently rinsed with saline, blotted dry, then snap frozen in liquid nitrogen for further analysis, while the other was fixed and paraffin-embedded as described above for the stomach tissue.

Histological and immunohistochemical analyses were conducted on the stomach and duodenal tissues. Periodic acid-Schiff (PAS) staining of stomach tissue was used to assess mucin type according to a 5-point scale, ranging from 1 (pink - neutral) to 5 (blue - acidic). A mean slide score was calculated from 3 sections/tissue per slide. Duodenal sections were stained with Haematoxylin and Eosin to measure villus height and crypt depth. For each pig, 10 villi and 10 crypts were measured per tissue and a mean value was calculated. To reduce assay variability all slides were assessed by a single trained technician blinded to experimental treatment. Data are presented as the mean of 8 pigs/treatment. For goblet cells, mucin staining was performed on duodenal sections using the Alcian blue/PAS stain kit (Newcomer Supply, Middleton, WI, USA) following the manufacturer's instructions. The area (μm2) occupied by goblet cells was calculated by dividing the mucin stained area (blue or dark purple color) by the total tissue area. Images were captured at 200X magnification using a BX53F Olympus microscope, then analyzed using the Olympus Cellsense software (Olympus America Inc., Center Valley, PA, USA). The goblet cell percentage was calculated based on the total slide coverage of the tissue that was populated by goblet cells per field, and the average was determined from all fields for a group comparison.

 For immune cell infiltration analysis, 4 μm tissue sections were mounted on charged slides, deparaffinized, then rehydrated by sequential immersion in xylene, 100% ethanol, 80% ethanol then phosphate buffer saline. Antigen retrieval was performed by boiling in 10 mM sodium citrate buffer (pH 6.0). The diaminobenzidine (DAB) Substrate Kit (Abcam, Cambridge, MA, USA) was used for immunohistochemistry staining following the manufacturer instructions, with an additional 1 h blocking step with goat serum (1:200) followed by incubation with an anti-CD45 primary antibody (1:500 Abcam, Cambridge, MA, USA) for 2 h at ambient temperature. Inflammatory cells clusters were counted in all fields occupied by tissue at 200X magnification. Scores were assigned to each field as: 1 (clusters only at the base of the gland), 2 (clusters displacing tissue up to the neck of the gland) or 3 (inflammatory infiltrate fully displacing the gland). Each field was evaluated by multiplying the number of clusters by the score per field. For intestinal samples, the same procedure was followed with another possible score of 4 given to fields where the inflammatory infiltrate was invading the submucosa. Field values were averaged per animal for final group comparisons.

*Microbial Analysis*

Microbiome analysis was performed on ileal samples collected at the end of Phase II (d21) from pigs selected from each pen fed non-acidified diets. Digesta samples were obtained from a 30 cm section proximal to the ileo-cecal junction, snap frozen in liquid nitrogen, then stored frozen (-80°C) until processed. Microbial genomic DNA was extracted and purified using a commercial kit (PowerSoil DNA Isolation Kit, MoBio Laboratory Inc, Carlsbad, CA, USA) according to the manufacturer’s recommended protocol, which included cell lysis by bead beating.

 The V1–V3 region of bacterial 16S rRNA gene sequences was PCR-amplified using the 27F forward (Edwards *et al.*, 1989) and 519R reverse (Lane *et al.*, 1985) primer pair. PCR reactions were performed with the Phusion *Taq* DNA polymerase (Thermo Scientific, Waltham, MA, USA) under the following conditions: hot start (4 min, 98 °C), followed by 35 cycles of denaturation (10 s, 98 °C), annealing (30 s, 50 °C) and extension (30 s, 72 °C), then ending with a final extension period (10 min, 72 °C). PCR products were separated by agarose gel electrophoresis, and amplicons of the expected size (~500 bp) were excised for gel purification using the QiaexII Gel extraction kit (QIAGEN, Hilden, Germany). For each sample, approximately 400 ng of amplified DNA were submitted to Molecular Research DNA (MRDNA, Shallowater, TX, USA) for sequencing with the MiSeq 2x300 platform (Illumina, San Diego, CA, USA) to generate overlapping paired-end reads.

Unless specified otherwise, computational analysis of PCR-generated 16S rRNA gene amplicon sequences were performed using custom written Perl scripts (available upon request). Raw bacterial 16S rRNA gene V1–V3 amplicon sequences were provided by Molecular Research DNA as assembled contigs from overlapping MiSeq 2x300 paired-end reads from the same flow cell clusters. Reads were selected to meet the following criteria: presence of both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nt, and a minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15. Following quality screens, sequence reads were aligned, then clustered into Operational Taxonomic Units (OTU) at a genetic distance cutoff of 5% sequence dissimilarity. It was previously assessed (Opdahl *et al.*, 2018) that a 5% dissimilarity cutoff for 16S rRNA gene was more representative of the genetic variation within the V1–V3 hypervariable regions, as determined by the meta-analysis of Kim *et al.* (2011).

It is common practice to use 97% as an OTU clustering cutoff for datasets consisting of full length 16S rRNA sequences; however, we argue 97% should not be used indiscriminately as a cutoff for any sub-region of the 16S rRNA. It has been well established that variation in sequence is not uniform across the 16S rRNA gene, and that certain hypervariable regions are more variable than others. Overall, for instance, V4 and V5 tend to be the least variable, while V1 is amongst the most variable. In seeking to identify more representative cutoffs for V1-V3, in silico testing using 16S rRNA sequences from validly described species of well populated genera, such as *Bacillus* and *Prevotella* was performed. The dissimilarity percentage amongst species of the same genus for the V1-V3 region was evaluated (note that datasets consisting of only the V1-V3 region were generated from the full length sequences of valid bacterial species that were retrieved from the NCBI database, then the resulting V1-V3 sequences were aligned and clustered as per our standard procedure). The idea was that species of the same genus would be expected to have a consistent dissimilarity percentage to each other, which would represent their genus-level cutoff for the 16S rRNA, from which a species-level cutoff could be assessed. For 47 species of *Prevotella* and 115 species of *Bacillus*, both the mean and median for the respective genera were approximately 13%, which is much higher than the value of 5% that is commonly used as a genus-level cutoff. Based on these considerations the following determinations for performing OTU clustering were made 1) if 97% is commonly used as a cutoff for clustering of V4 or V4-V5 regions, which are less variable than V1-V3, then a lower cutoff should be used for V1-V3 and 2) because the dissimilarity percentage between species of the same genus for the V1-V3 region is 13%, then the typical 3% cutoff can justifiably be relaxed. In this context, 5% remains more stringent than the observed genus-level cutoff of 13%.

The OTU were screened for DNA sequence artifacts using the following methods. Chimeric sequences were first identified with the chimera.uchime and chimera.slayer commands from the MOTHUR source software package (v.1.36.1, University of Michigan, Ann Arbor, USA) (Schloss *et al.*, 2009). Secondly, the integrity of the 5′ and 3′ ends of OTU was evaluated using a database alignment search-based approach. When compared to their closest match of equal or longer sequence length from the NCBI nt database, as determined by BLASTN (2.5.0) (Altschul *et al.*, 1997), OTU with more than five nucleotides missing from the 5′ or 3′ end of their respective alignments were discarded as artifacts. Single read OTU were subjected to an additional screening, where only sequences that had a perfect, or near perfect, match to a sequence in the NCBI nt database were kept for analysis. Thus, the alignment had to span the entire sequence of the OTU and a maximum of 1% nucleotide dissimilarity was tolerated.

After removal of sequence chimeras and artifacts, the bacterial composition of each sample was determined by calculating the relative abundance of valid OTU. This was defined as the number of sequence reads assigned to an OTU in a given sample, divided by the number of total reads in that sample. Taxonomic assignment of valid OTU was determined using a combination of RDP Classifier (Wang *et al.*, 2007) and BLAST (Altschul *et al.*, 1997). The List of Prokaryotic Names with Standing in Nomenclature (LPSN) was also consulted for information on valid species belonging to taxa of interest (Euzéby, 2013, Parte, 2014).

**References**

Euzéby J 2013. List of prokaryotic names with standing in nomenclature, genus Mycobacterium. Retrieved on 4 August 2018 from <http://www.bacterio.net/>.

Kim M, Morrison M and Yu Z 2011. Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. Journal of Microbiological Methods 84, 81-87.

Opdahl LJ, Gonda MG and St-Pierre B 2018. Identification of uncultured bacterial species from Firmicutes, Bacteroidetes and CANDIDATUS Saccharibacteria as candidate cellulose utilizers from the rumen of beef cows. Microorganisms 6, 17.

Parte AC 2014. LPSN--list of prokaryotic names with standing in nomenclature. Nucleic Acids Research 42, D613-616.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH and Robinson CJ 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75, 7537-7541.

**Supplemental Table S1***Composition and nutrient content of experimental diets (as-fed basis)1 fed to weaned pigs.*

|  |  |  |  |
| --- | --- | --- | --- |
| Item | Phase I |  | Phase II |
| NEG | POS | MSBM |  | NEG | POS | MSBM |
| Ingredient (%) |  |  |  |  |  |  |  |
| Corn | 33.60 | 39.8 | 38.3 |  | 44.8 | 50.6 | 49.6 |
| Soybean meal | 37.30 | 25.0 | 25.0 |  | 40.7 | 30.7 | 30.7 |
| Fish meal | - | 7.49 | - |  | - | 4.99 | - |
| MSBM | - | - | 7.48 |  | - | - | 4.99 |
| Whey powder | 25.00 | 25.0 | 25.0 |  | 9.98 | 9.98 | 9.98 |
| Soybean oil | 1.00 | 1.00 | 1.00 |  | 1.00 | 1.00 | 1.00 |
| Calcium phosphate | 1.22 | 0.35 | 1.25 |  | 1.55 | 0.95 | 1.55 |
| Limestone | 0.85 | 0.47 | 0.87 |  | 0.92 | 0.70 | 0.95 |
| Sodium chloride | 0.30 | 0.30 | 0.30 |  | 0.35 | 0.35 | 0.35 |
| Synthetic amino acids2 | 0.29 | 0.24 | 0.33 |  | 0.33 | 0.38 | 0.46 |
| Trace mineral premix3 | 0.15 | 0.15 | 0.15 |  | 0.15 | 0.15 | 0.15 |
| Vitamin premix4 | 0.05 | 0.05 | 0.05 |  | 0.05 | 0.05 | 0.05 |
| Titanium dioxide | 0.20 | 0.20 | 0.20 |  | 0.20 | 0.20 | 0.20 |
| Analyzed composition (%) |  |  |  |  |  |  |  |
| Dry matter | 90.90 | 91.4 | 90.8 |  | 89.9 | 89.9 | 89.8 |
| CP | 24.30 | 23.9 | 23.7 |  | 23.9 | 23.0 | 22.5 |
| Ash | 6.71 | 6.70 | 6.80 |  | 6.56 | 6.07 | 6.32 |
| Crude fat | 2.44 | 2.95 | 1.41 |  | 1.97 | 2.64 | 1.79 |
| Crude fiber | 1.71 | 1.65 | 1.83 |  | 2.61 | 2.59 | 2.70 |
| Lysine | 1.60 | 1.48 | 1.53 |  | 1.51 | 1.57 | 1.50 |
| Formulated content |  |  |  |  |  |  |  |
| Metabolizable energy (MJ/kg) | 13.90 | 14.2 | 14.3 |  | 13.8 | 14.0 | 14.1 |
| Lysine:metabolizable energy (g/MJ) | 0.97 | 0.95 | 0.94 |  | 0.98 | 0.96 | 0.96 |

1Experimental NEG, POS and MSBM represent conventional soybean meal, Menhaden fishmeal, and microbially-enhanced soybean meal diets, respectively. Fishmeal and MSBM were included at 7.5 and 5% in Phase I (7d) and II (14d), respectively. Diet formulation details were previously reported in Sinn et al., 2016.

2Synthetic amino acids were Lysine-HCl, DL-Methionine, L-Threonine, L-Tryptophan, and L-Valine.

3Provided per kg of the complete diet: 165 mg Zn as zinc sulfate, 165 mg Fe as ferrous sulfate, 43.5 mg Mn as manganese sulfate, 16.5 mg Cu as basic copper chloride, 0.36 mg I as ethylenediamine dihydroiodide, and 0.3 mg of Se as sodium selenite.

4Provided per kg of the complete diet: 11 002 IU vitamin A supplement, 1 651 IU vitamin D3 supplement, 55.1 IU vitamin E supplement, 0.044 mg vitamin B12 supplement, 4.4 mg menadione as menadione dimethylpyrimidinol bisulfite, 9.91 mg riboflavin supplement, 60.6 mg D-pantothenic acid as D-calcium pantothenate, 55.1 mg niacin supplement, 1.1 mg folic acid, 3.3 mg pyridoxine as pyridoxine hydrochloride, 3.3 mg thiamine as thiamine mononitrate, and 0.171 mg biotin.

**Supplemental Table S2** *Nutrient content of the microbially-enhanced soybean meal (MSBM) and Menhaden fishmeal (FM), as-fed basis included in weaned pig diets.*

|  |  |  |
| --- | --- | --- |
| Item | MSBM | Fishmeal |
| Gross energy (kcal/kg) | 4688 | 4577 |
| Dry matter (%) | 95.80 | 92.90 |
| CP (%) | 58.40 | 63.20 |
| Ash (%) | 7.60 | 20.90 |
| Crude fat (%) | 0 | 9.22 |
| Crude fiber (%) | 5.74 | 0.39 |
| Indispensable amino acids (%) |
| Arginine | 3.95 | 3.91 |
| Histidine | 1.53 | 1.53 |
| Isoleucine | 2.85 | 2.62 |
| Leucine | 4.74 | 4.56 |
| Lysine | 3.68 | 5.13 |
| Methionine | 0.83 | 1.79 |
| Phenylalanine | 2.95 | 2.35 |
| Threonine | 2.36 | 2.67 |
| Valine | 2.95 | 3.00 |
| Dispensable amino acids (%) |
| Alanine | 2.63 | 4.01 |
| Aspartate | 6.57 | 5.80 |
| Cysteine | 0.85 | 0.52 |
| Glutamate | 9.89 | 8.66 |
| Glycine | 2.57 | 4.56 |
| Proline | 3.04 | 3.04 |
| Serine | 2.68 | 2.47 |
| Tyrosine | 2.06 | 1.99 |
| Total amino acids | 56.10 |  58.60 |

1Ingredients provided SD Innovation Partners (Brookings, South Dakota, USA).

**Supplemental Table S3** *Number of high-quality (Q15) and non-chimeric 16S rRNA reads used to determine the bacterial composition of ileal samples collected from pigs fed diets containing conventional soybean meal (NEG), fish meal (POS), or microbially-enhanced soybean meal (MSBM). Accession numbers to each sample raw sequence data is also presented.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Diet | Sample ID | Q151 reads | Non-chimeric2 reads | SRA accession numbers3 |
| NEG | N1 | 4886 | 4011 | SRR7275043 |
| NEG | N2 | 19894 | 17922 | SRR7275044 |
| NEG | N3 | 16602 | 11717 | SRR7275041 |
| NEG | N4 | 42477 | 38177 | SRR7275042 |
| NEG | N5 | 74432 | 66157 | SRR7275039 |
| NEG | N6 | 21114 | 20007 | SRR7275040 |
| NEG | N7 | 2300 | 2232 | SRR7275037 |
| NEG | N8 | 17426 | 16774 | SRR7275038 |
|  |  |  |  |  |
| POS | P1 | 56888 | 50813 | SRR7275035 |
| POS | P2 | 63186 | 48374 | SRR7275036 |
| POS | P3 | 15085 | 12298 | SRR7275051 |
| POS | P4 | 30927 | 30392 | SRR7275052 |
| POS | P5 | 3329 | 2099 | SRR7275049 |
| POS | P6 | 38390 | 33369 | SRR7275050 |
| POS | P7 | 23571 | 21226 | SRR7275047 |
|  |  |  |  |  |
| MSBM | M1 | 24292 | 19244 | SRR7275048 |
| MSBM | M2 | 25680 | 16353 | SRR7275045 |
| MSBM | M3 | 3383 | 2833 | SRR7275046 |
| MSBM | M4 | 15836 | 14807 | SRR7275054 |
| MSBM | M5 | 12945 | 12219 | SRR7275055 |
| MSBM | M6 | 7186 | 6814 | SRR7275053 |

1Number of reads for each sample that had both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nt, and a minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15.

2Number of reads used for bacterial composition analysis (i.e. after chimera check, 5’ and 3’ end quality check, as well as single-read operational taxonomic unit check).

3Sequence read archive (SRA) accession numbers for raw, unprocessed sequence reads with barcode.

**Supplemental Table S4.** *Comparative analysis of sequence read yields on select samples from pigs using different quality filtering methods. Two truncation-based methods (truncation at the first low quality single nucleotide or truncation at the first low quality region - sliding window) at two quality threshold cutoffs (Q20 or Q25) were compared to the dataset analyzed in the current report (‘V1-V3 filtered’). For all truncation-based filtering conditions tested, reads were also screened for length, with a commonly used cutoff of 200 nt.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  |  | Single nt | Sliding window |
| Sample ID | Raw reads unfiltered | V1-V3 filtered | Q20\* | Q25\* | Q20\* | Q25\* |
| N1 | 47442 | 4886 | 17173 | 12037 | 25644 | 25217 |
| N7 | 11544 | 2300 | 5067 | 3392 | 7294 | 7212 |
| M3 | 17192 | 3383 | 8222 | 5532 | 12330 | 12134 |
| M6 | 20073 | 7186 | 12428 | 7875 | 19686 | 19390 |
| P5 | 31029 | 3329 | 9580 | 6353 | 15513 | 15258 |

\* Reads of length equal to or greater than 200 nt.

**Supplementary Table S5.** Relative ratio of operational taxonomic unit (OTU) percentages for each pig as determined by Next-generation sequencing. Each column represents an individual pig identified with a number (i.e. 1, 2, 3) and a letter (P, N, M) representing the respective dietary treatments conventional soybean meal, Menhaden fishmeal, and microbially-enhanced soybean meal diets, respectively. Fishmeal and MSBM were included at 7.5 and 5% in Phase I (7d) and II (14d), respectively.

See Ortman et al\_Supplementary Table S5-OTU table.xlsx

**Supplementary Table S6.** Comparison between qPCR and Next-Generation Sequencing in assessing the abundance of major operational taxonomic units (OTUs) SD\_Ssd-00001, SD\_Ssd-00002, and SD\_Ssd-00011 in ileal digesta samples from weaned pigs. The level of abundance was assessed using fold enrichment for both methods, i.e. that the sample with the highest abundance was compared to the sample with the lowest abundance. Fold enrichment by qPCR was determined as base 2 raised to the power of ΔΔCt (2ΔΔCt), while representation by Next-Generation Sequencing was determined as the ratio between the abundances of the sample pairs.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample Pairs** | **Target** | **Ct#** | **2Ct** | **NGS ratio\*** |
| **N8/N3** | *L. amylovorus* | 4.22 | 18.6 | 76.5 |
| **N8/N3** | SD\_Ssd-00001 | 5.82 | 56.5 | 76.5 |
| **M5/M4** | *L. delbrueckii* | 9.43 | 689.8 | 926.2 |
| **M5/M4** | SD\_Ssd-00011 | 12.69 | 6608 | 926.2 |
| **M1/M4** | SD\_Ssd-00002 | 4.00 | 16.0 | 53.9 |

Ct = [Sample1(Ct of target OTU/species) – Sample2(Ct of target OTU/species)] –

 [Sample1(Ct of total bacteria) – Sample2(Ct of total bacteria)].

\*Ratio of OTU percentages as determined by Next-Generation Sequencing (Supplementary Table 4)

*L. amylovorus* (Marti et al, 2010)

Forward: TTCTGCCTTTTTGGGATCAA

Reverse: CCTTGTTTATTCAAGTGGGTGA

*L. delbrueckii* (Haarman et al, 2006)

Forward: CACTTGTACGTTGAAAACTGAATATCTTAA

Reverse: CGAACTCTCTCGGTCGCTTT

SD\_Ssd-00001

Forward: GGAACCAACAGATTTACTTCGG

Reverse: GCTGATCATGCGATCTGC

SD\_Ssd-00002

Forward: CCTAGATGATTTTAGTGCTTGC

Reverse: GACATGCGTCTAGTGTTGTTA

SD\_Ssd-00011

Forward: CCTTCGGGATGATTTGTTGG

Reverse: TCAAACTTGAATCATGCGATTCATG

Marti R, Dabert P, Ziebal C, and Pourcher A-M 2010. Evaluation of Lactobacillus sobrius/L. amylovorus as a New Microbial Marker of Pig Manure. Applied Environmental Microbiology 76, 1456–1461.doi: 10.1128/AEM.01895-09

Haarman M and Knol J 2006. Quantitative Real-Time PCR Analysis of Fecal Lactobacillus Species in Infants Receiving a Prebiotic Infant Formula. Applied Environmental Microbiology 72, 2359–2365.

**Supplementary Table S7.** Gene annotation for three strains from each of three species of Lactobacillus in ileal digesta samples of weaned pigs. Genomes were retrieved from the National Center for Biotechnology Information database (NCBI - <https://www.ncbi.nlm.nih.gov>): L. amylovorus (strains DSM 20531, GRL 1112, and GRL 1118), L. delbrueckii (strains 2038, ND.02, and ATCC BAA-365) and L. johnsonii (strains FI9785, N6.2, FI9785, and NCC 533). Annotation was performed separately for each individual genome using the online tool Rapid Annotation using Subsystem Technology (RAST) (<http://rast.nmpdr.org>) (Aziz et al., 2008).

See Ortman et al\_Supplementary Table S7-RAST outputs.xlsx