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Genetically identical co-housed pigs as models for dietary studies of gut microbiomes

Abstract: The impact of diet on the microbial composition in the gastrointestinal tract (GIT) has been well documented. However, quantifying the role of the diet in shaping microbial composition in humans has been difficult due to the influence of host genetics and the environment. To test the influence of diets on the GIT microbiome independently of host genotype and environment, two genetically identical co-housed pigs were used in an A-B-A-B design across four 14-day periods using two distinct diets that differed in dietary fiber source, soybean hulls or wheat bran. Shifts in fecal microbiomes were assessed with respect to dietary changes by 454-pyrosequencing analysis using the V3 region of the 16S rRNA genes. Similarity analysis revealed that the GIT microbiome distinctly clustered by diets rather than by individual. Diversity analysis showed that the diet fed had an influence on GIT microbiome diversity, which was host specific. While many bacterial taxa and KEGG orthologs reacted similarly to switches in diet, some bacterial taxa and KEGG orthologs reacted differentially in each of the pigs. While diet changed the GIT microbiome composition of isogenic co-housed pigs, inter-individual variations from epigenetics were not entirely eliminated by the use of cloned pigs.

Keywords: diet, genetically identical pig, gastrointestinal tract microbiome, taxonomic composition, functional composition, environment

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1 Introduction

The gastrointestinal tract (GIT) microbiome is known to play a role in determining an individual's health. Commensal GIT microbes modulate nutrient uptake and utilization, promote GIT development and maturation, and extract energy from indigestible non-starch polysaccharides [1,2]. Disruptions in GIT microbiome composition have been associated with a variety of GIT-related diseases, such as obesity, inflammatory bowel disease (IBD), and irritable bowel syndrome (IBS). Studies have shown that the GIT microbiomes of obese mice and humans differ from those of lean mice and humans [3-7]. Frank *et al.* [8] has reported that IBD patients show different GIT microbial compositions in comparison with healthy individuals. Additionally, the GIT microbiome composition of IBS patients has been found to be significantly different from that of healthy controls [9,10]. Disease symptoms could be alleviated by treatments that altered the GIT microbiome toward that of healthy individuals. For example, studies have shown the beneficial effects of probiotics on the reduction of body weight [11-13] and the treatment of IBS [14-16].

Diets are regarded as one of the main factors contributing to the change in GIT microbiome as the diet provides nutrients supporting the growth of microbes in the GIT. The role of diet in shaping GIT microbiome begins in infancy. Differences in the GIT microbiome between breast- and formula-fed newborns have been shown to be the result of these different diets [17,18]. The most remarkable example of the effect of diets on GIT microbiome composition was the addition of table foods

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to an infant's diet. The introduction of table food induces the largest compositional shift in GIT microbiome over one's lifetime [19]. Although the adult GIT microbiome was thought to reach a stable state [19], diet still has a significant influence on GIT microbiome composition in adults. For example, Turnbaugh *et al.* [20] revealed that the dietary switch from low-fat to high-fat diet significantly changed the structure of humanized adult mice GIT microbiome within one day. Furthermore, Faith *et al.* [21] found that a monotonous low-calorie liquid diet significantly altered the GIT bacterial abundance in adult humans who lost 10% of body weight when compared to adult humans who did not consume the weight-loss diet and lose weight.

There is growing interest in studying what dietary component alters GIT microbiome and the dietary fiber portion of the diet has been of interest. Dietary fiber consists of non-digestible carbohydrates and lignin that are intrinsic and intact in plants [22]. Dietary fiber is known to aid against GIT-related diseases, including constipation, colon cancer, obesity and diabetes [23]. Generally, there are two types of dietary fiber: soluble and insoluble fiber. Soluble fiber dissolves in water forming a gel-like material and is rapidly fermented by the microbes in the GIT [23]. Insoluble fiber is water insoluble and cannot form a gel-like material in water [23]. Microorganisms in the GIT ferment the insoluble fiber very slowly [23]. Soluble dietary fiber is composed of resistant starch, β -glucans, pectins and gums. Insoluble fiber consists of hemicelluloses, cellulose and lignin/phenolics.

Given the importance of the GIT microbiome and diet in GIT-related diseases, dietary intervention is thought to be a potential treatment for GIT-related diseases [24]. In order to understand how dietary alterations affect the GIT microbiome, it is important to be able to isolate the effect of the dietary intervention. This can be difficult given that host genetics and the environment also impact microbiome structure. In order to minimize the impact of host genetics, one can use genetically identical twins. Identical twins studies have been used to control for host genetics, but controlling past epigenetic events that are the result of environmental exposures are still problematic. Although twins are regarded as one of the best models for studying non-genetic influences on the human GIT microbiome, cloned animal models offer an additional level of control. For example, cloned animals can be maintained in the same environment after birth to reduce the influence of environment. Pigs make for a particularly attractive model for studying the effect of diet on human GIT microbiome for several reasons [25-30]. First, pigs are omnivores and can be fed diets that mimic realistic human interventions.

Second, the pig's GIT is anatomically, metabolically, and physiologically similar to human beings [31]. Third, pigs grow quickly and are especially susceptible to nutrient modulation. Finally, pigs and humans share a similar GIT microbial community composition [32], and therefore, the results obtained from a porcine model may be considered a more reasonable reflection of the humans' GIT than the typical murine models.

The objective of this study was to evaluate the use of genetically identical hosts for determining the effects of different diets on fecal microbiome composition independent of the confounding effects of host genotype and environment. We used two genetically identical co-housed pigs in an A-B-A-B design across four two-week periods using two diets containing different sources of dietary fiber, soybean hulls or wheat bran, which are sources of soluble and insoluble fiber, respectively [33]. Each pig was fed the same diet in the each period and switched onto the next diet after the two-week period. The composition of the fecal microbiome was characterized by high-throughput 16S rRNA hypervariable tag 454-pyrosequencing. We hypothesized that genetically identical co-housed pigs could be used to determine the influence of diet on GIT microbiome independent of host genotype and environment.

2 Methods

2.1 Fecal sampling

All animal care procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois. Two 18-month-old female healthy cloned adult pigs created from Duroc sow (Duroc 2-14) [34] using somatic cell nuclear transfer were used in an A-B-A-B experimental design with four 14-d periods (Figure 1). Pigs were fed two different diets, one containing 20% soybean hulls and another containing 20% wheat bran. The two diets (Supplementary Table 1) were formulated to provide similar energy and nutrients, which met NRC (1998) requirements for adult pigs [35]. The two pigs were from the same litter and had the same genotype. The clones were born by vaginal delivery and allowed to suckle. They were weaned at 4 weeks of age and continuously housed together. Pigs were fed once daily in the morning and had free access to water. The two pigs were fed a diet containing 20% soybean hulls prior to the experimental period. Feces were collected in sterile polypropylene tubes on the last day of each feeding period and frozen at -80°C until DNA extraction.

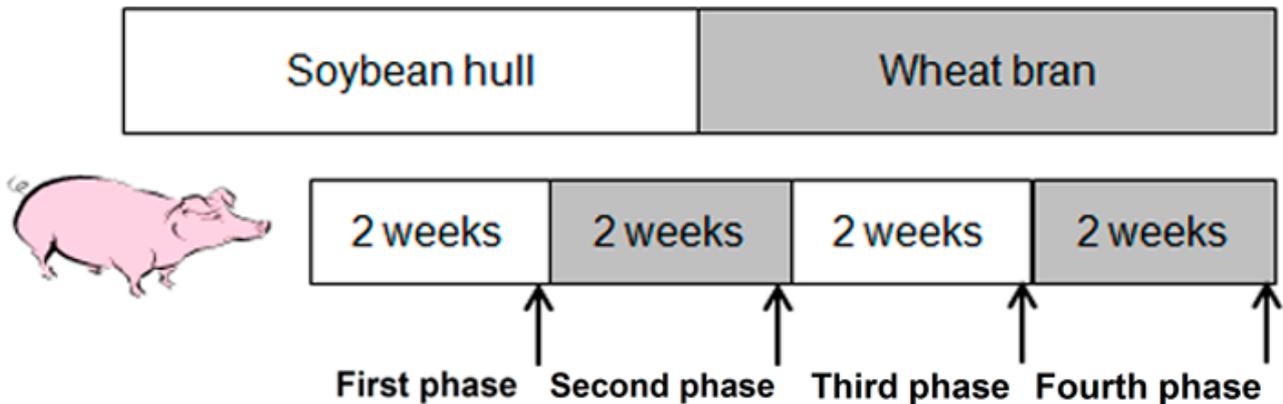


Figure 1: Experimental design. Dark arrows indicate fecal sample collection on the last day of each feeding phase.

2.2 DNA extraction

Genomic DNA was extracted using the RBB+C method [36] with minor modifications including the addition of 1000 μ l ASL buffer to the samples following the Qiagen DNA Stool Kit (Qiagen, Valencia, CA).

2.3 Pyrosequencing analysis

The hypervariable V3 region of the 16S rRNA gene was amplified in the PCR with specific primers for pyrosequencing analysis. For each sample, we used the fusion primer. The fusion forward primer (5'-GCCTCCCTCGCGCCATCAGNNNNNNNNNN**CCTACGGGAGGCAGCAG**-3') for pyrosequencing contained the 454 Life Sciences' primer A (underlined), a unique 10 base barcode used to tag each PCR product (NNNNNNNNNN), and the broadly conserved bacterial primer 341F (bold) [37]. Barcodes for each sample were ACGAGTGC GT, ACGCTCGACA, AGACGCACTC, AGCACTGTAG, ATCAGACACG, CGTGTCTCTA, CTCGCGTGTC and TAGTATCAGC, respectively. The fusion reverse primer (5'-GCCTTGCCAGCCC**GCTCAGATTACCGCGGCTGCTGG**-3') for pyrosequencing included the 454 Life Sciences' primer B (underlined) and the broadly conserved bacterial primer 534R (bold). The specific primers were used to amplify the 16S rRNA gene regions in a microbial community, which corresponded to positions 341 to 534 in *E. coli*.

PCRs were performed using 50 μ l volumes containing 5 μ l of 10X FastStart high fidelity reaction buffer with 18 mM MgCl₂, 1 μ l of 100 mM dNTP mixture, 1 μ l (10 μ M) of each fusion forward and reverse primer, 1 μ l of FastStart high fidelity enzyme blend (5 U/ μ l), 1 μ l of template DNA, 40 μ l of distilled water. The PCR reaction was conducted using a BIO-RAD MJ Mini Personal Thermal Cycler (BIO-RAD Laboratories) and the conditions were

94°C (5 min), followed by 20 cycles of 94°C (30 s), 60°C (30 s), 72°C (30 s) and a final extension of 72°C (7 min). The amplicon products were purified using QIAquick PCR Purification Kit and protocol. The product quality was assessed using a BioAnalyzer Agilent DNA 1000 LabChip. All of the amplified samples were sent to W. M. Keck Center for Biotechnology at the University of Illinois for pyrosequencing using a 454 Genome Sequencer FLX (Roche Applied Science, Indianapolis, IN).

2.4 Bioinformatics analysis

Primers and low quality sequences (quality score \leq 25) were removed by the W. M. Keck Center for Biotechnology at the University of Illinois. Sequences shorter than 100 nucleotides, with ambiguous characters, and containing homopolymers longer than 6 nucleotides were trimmed by mothur using trim.seqs command [38]. Chimeras were detected by mothur using chimera.uchime command and removed by Tornado using the splicer script [38,39]. A total of 172,545 high quality sequences from eight samples were generated with an average sequence length of 176 nt (Supplementary Table 2). The resulting clean sequences are in Supplementary Sequence Data. For taxonomic analysis, the resulting clean sequences were assigned to operational taxonomic units (OTUs) as previously described [40,41]. Briefly, the clean sequences were aligned against the Silva database and pre-clustered by mothur [38]. The remaining sequences were clustered at 97% sequence similarity by ModalClust (<https://bitbucket.org/msipos/modalclust>) using the complete linkage clustering method. To analyze samples in a comparable manner, we used sub.sample command in mothur to select an equal number of OTU sequences each sample (n=8,061 based on the sample with the fewest number of OTU sequences) (Supplementary Table 2) [38]. For functional predictions, the resulting

clean sequences were assigned to OTUs using QIIME (v 1.7) according to PICRUSt online ‘closed-reference’ OTU picking protocol against gg_13_5_otus database at 97% identity [42,43]. The PICRUSt compatible OTU table was further normalized and used to predict KEGG orthologs (KOs) using the online PICRUSt Galaxy version [42]. The pig GIT had a relatively high mean Nearest Sequenced Taxon Index (NSTI) value (mean NSTI = 0.20 ± 0.01 s.d.), which lowered the accuracy of the predicted metagenome [42]. The taxonomic and functional similarities among microbial communities were compared using Bray-Curtis dissimilarity statistics. The data was standardized by the total read depth and transformed by square root to reduce the effect of higher abundant over less abundant OTUs or functional genes. Resemblance matrices and principal coordinate analysis (PCoA) plots were created and visualized in Primer (Primer-E 2007). Permutational multivariate analysis of variance (PERMANOVA) in Primer (Primer-E 2007) was used to test the null-hypothesis that there was no difference between two cloned pigs and different diets. Heatmaps of OTUs and functional genes were generated using the gplots package in R. The dendrogram for hierarchical clustering (complete linking) were constructed from Bray-Curtis dissimilarity distances, which were calculated in the same way as before. Shannon diversity indices were calculated in *mothur* [38]. The representative sequence in each OTU was assigned using the RDP classifier v2.5 [44] with a bootstrap cutoff of 70% [45]. P-values < 0.05 were regarded significant unless specifically stated.

2.5 Network analysis

The partition of OTUs between samples in each cloned pig and phase was visualized in Cytoscape [46] as described in http://qiime.org/tutorials/making_cytoscape_networks.html. Briefly, samples and OTUs were two types of nodes. The OTU-nodes were connected to sample-nodes through edges that were weighted by the relative abundance value in an OTU. The edge-weighted spring embedded algorithm was used to cluster the OTUs and samples in these networks.

3 Results

3.1 Diet changed the GIT microbiome structure of genetically identical co-housed pigs

The two genetically identical co-housed pigs harbored distinctly different microbiomes at all stages of the experimental protocol. Bray-Curtis dissimilarity-based PCoA plots exhibited a disparate clustering by diet (PERMANOVA $p=0.037$ for taxonomy; $p=0.039$ for metabolic potential) rather than by individual (PERMANOVA $p=0.290$ for taxonomy; $p=0.478$ for metabolic potential) (Figure 2A and B). Heatmaps of OTUs and functional genes also showed that the GIT microbiomes were clustered by diet instead of by individual, similar to PCoA plots (Supplementary Figure 1A and B). Generally, the GIT microbiomes were not clustered according to

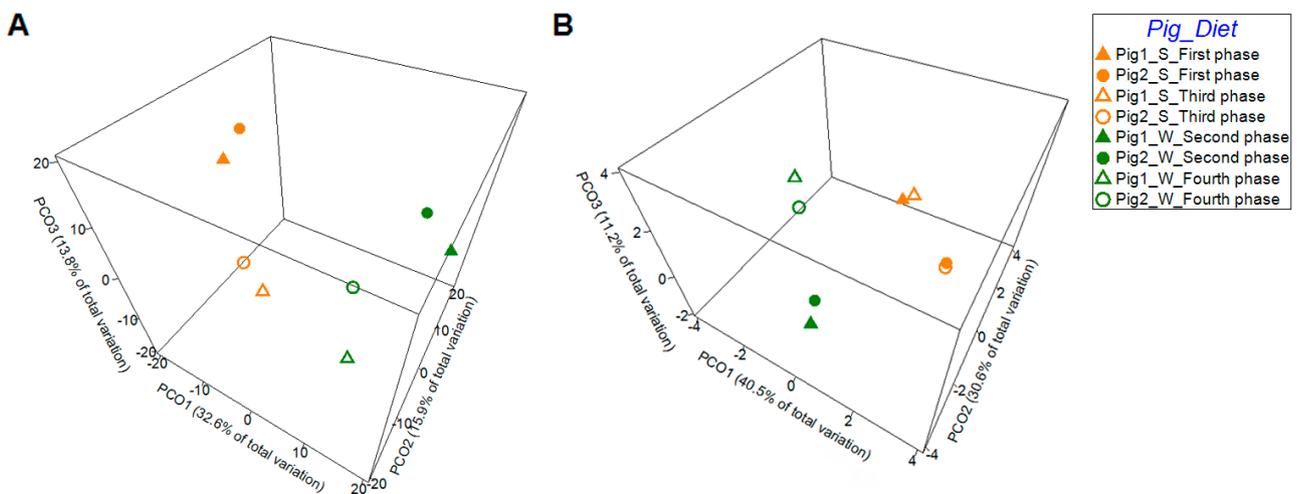


Figure 2: Bray-Curtis dissimilarity-based PCoA analysis showing clustering of GIT microbiome. (A) Comparison of the bacterial community structure in two pigs feed either of the two different diets, (B) Comparison of the functional KEGG orthologs structure in the two pigs feed either of the two different diets. Bray-Curtis dissimilarity-based PCoA was performed on OTUs from the fecal sample of an individual pig fed the soybean hulls or wheat bran diet in an A-B-A-B design across the four phases. The OTUs in the analysis are estimated based on 97% 16S rRNA sequence similarity. Orange or green color indicates the soybean hulls diet or the wheat bran diet, respectively. Triangles or circles indicate samples collected from pigs #1 or 2, respectively.

individual. However, closer inspection revealed that the two genetically identical co-housed pigs harbored similar taxonomic composition at all stages of the experimental protocol and functional composition at the second and fourth stage (Figure 2 and Supplementary Figure 1). The functional composition of the same pig at the first and third stage was more similarly to each other than that of the two cloned pigs at the same stage (Figure 2B and Supplementary Figure 1B). These suggest that diet alters the overall GIT microbiome of genetically identical pigs and there were differences in the overall microbiome composition between the cloned pigs.

3.2 The impact of diet on GIT microbiome diversity was host specific

Diversity in both pigs increased when diet was switched from soybean hulls in the first phase to wheat bran in the second phase (Figure 3). However, the effect of diet on diversity appeared to be host-dependent in the third and fourth phases. Pig #1 maintained a stable GIT microbiome diversity index while the GIT microbiome of pig #2 underwent a sharp decrease in diversity during the third phase (Figure 3). Interestingly, microbiome diversity in pig #2 increased when diet was changed to wheat bran diet in the fourth phase, while pig #1 showed only a slight decrease in diversity in the same time period (Figure 3).

3.3 Bacterial taxa responded to diet change in different ways in the two cloned pigs

The different response to diet in genetically identical co-housed pigs was also reflected in the microbiome community structure. Taxonomic assignment was carried

out at both the phylum and genus level. OTUs were assigned to 12 phyla (Supplementary Figure 2) and 62 genera (Supplementary Table 3) by the RDP classifier. At phyla level, we detected five taxa (at >0.1% abundance in at least one sample) which reacted similarly to diet switch in the two replicates (Supplementary Figure 3). Specifically, the relative abundance of Firmicutes decreased during the wheat bran diet, while there was an opposite effect on the relative abundance of Bacteroidetes, Tenericutes, Fibrobacteres and Synergistetes (Supplementary Figure 3). Phylogenetic analysis revealed that at the genus level, 11 genera (at > 0.1% abundance in at least one sample) were observed to respond similarly to diet change in the two replicates (Supplementary Figure 4). The relative abundance of *Sutterella* and *Gemmiger*, affiliated with the phylum Proteobacteria, and *Megasphaera*, *Turcibacter* and *Anaerofilum*, within the phylum Firmicutes, increased in pigs fed the soybean hulls diet compared with pigs fed the wheat bran diet (Supplementary Figure 4). *Fibrobacter* (phylum Fibrobacteres), *Bacteroides* (phylum Bacteroidetes), *Cloacibacillus* (phylum Synergistetes), *Erysipelotrichaceae_incertae_sedis*, *Fingoldia* and *Flavonifractor* (phylum Firmicutes) increased in the relative abundance in pigs fed the wheat bran diet when compared with pigs fed the soybean hulls diet (Supplementary Figure 4). *Butyricoccus* and *Parasporobacterium* (at > 0.1% abundance in at least one sample), within the phylum Firmicutes, were two genera that reacted oppositely to diet change in the two cloned pigs in each stage (Supplementary Figure 5). These suggest that diet can alter the GIT microbiome taxonomic composition in cloned pigs within two weeks and inter-individual differences existed in the genetically identical pigs.

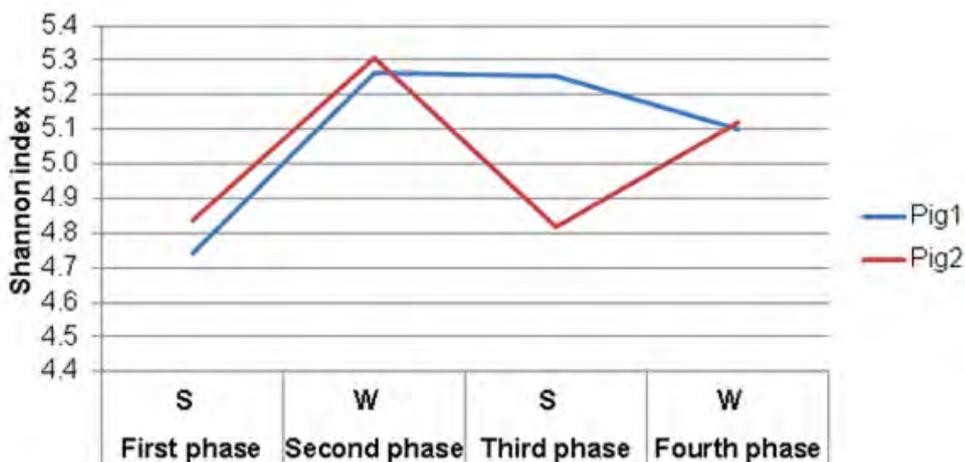


Figure 3: Diets and inter-individual differences in the pig's fecal microbiome. S: soybean hulls diet. W: wheat bran diet. The OTUs in the analysis are estimated based on 97% 16S rRNA sequence similarity.

3.4 Predicted functional gene content also responded differently to the diet in the two cloned pigs

In order to correlate microbiome community function to diet, we utilized PICRUST to predict KOs from the 16S rRNA gene composition data. This *in silico* approach resulted in the prediction of 1606 KOs from the two genetically identical co-housed pigs (Supplementary Table 4). Thirty-eight KOs (at > 0.1% abundance in at least one sample) were observed to react similarly to diet change in the two genetically identical pigs (Supplementary Figure 6). Specifically, the relative abundance of 16 KOs decreased during the wheat bran diet, while there was an opposite effect on the relative abundance of 22 KOs (Supplementary Figure 5). Twelve KOs (at > 0.1% abundance in at least one sample) reacted oppositely to the dietary switch in the two cloned pigs (Supplementary Figure 7). These KOs included (D-3-phosphoglycerate dehydrogenase (K00058), orotate phosphoribosyltransferase (K00762), undecaprenyl diphosphate synthase (K00806), aspartate kinase (K00928), glutamine synthetase (K01915), small subunit ribosomal protein S4 (K02986), RNA polymerase primary sigma factor (K03086), single-strand DNA-binding protein (K03111), peptidyl-prolyl cis-trans isomerase B (K03768), ATP-binding cassette (K06147), elongation factor Tu

(K02358), and an OmpR family two-component system, phosphate regulon sensor histidine kinase PhoR (K07636). While the KOs were associated with different metabolisms, but only those related to amino acid/protein metabolism were directly related to diet. These findings suggest diet can impact the GIT microbiome functional composition in cloned pigs within two weeks and inter-individual differences existed in the genetically identical pigs.

3.5 Network analysis of OTUs between the different samples from the two cloned pigs

Network analysis was used to visually display the shared and unique OTUs between the different samples. The nodes in the network were determined by the number of shared OTUs between samples and the relative abundance value within each OTU. The separation of the OTU networks showed that diet switches influenced the GIT microbiome taxonomic composition of two cloned pigs in different ways (Figure 4). The OTU networks of the two cloned pigs at each dietary phase revealed the inter-individual differences between the two cloned pigs with respect to the differences in number of shared and unique OTUs detected in each dietary phase and between two cloned pigs (Figure 4 and 5). The sample-nodes in the each phase in each pig were differently spaced suggesting

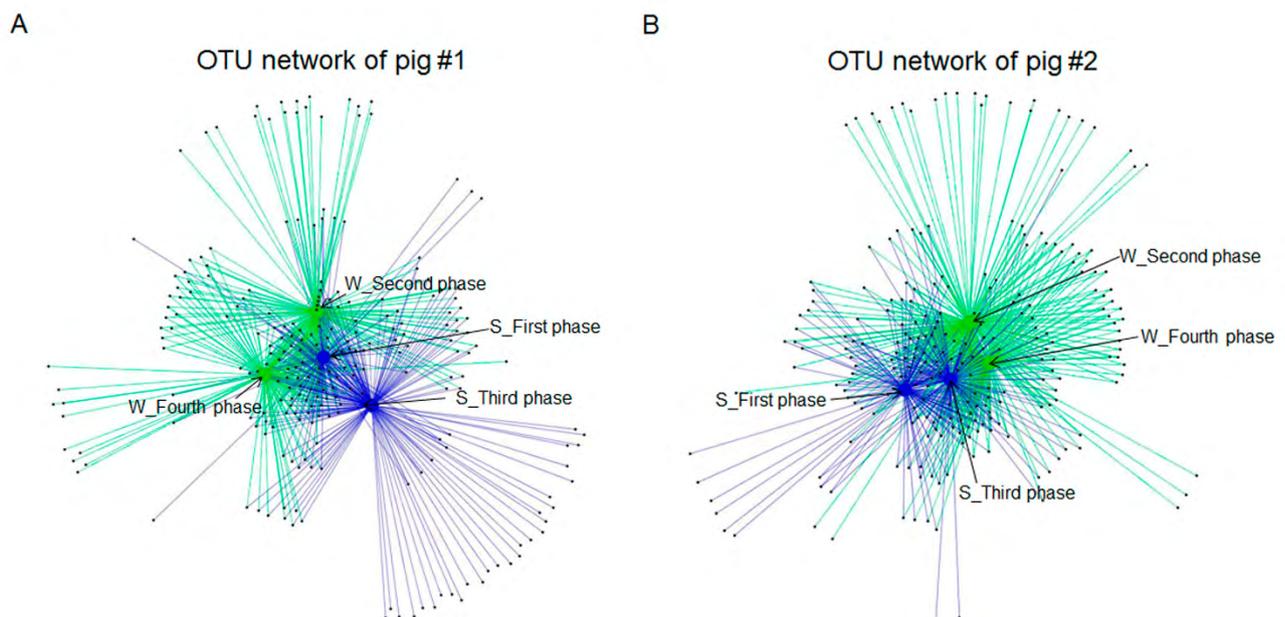


Figure 4: Network-based analysis of shared OTUs composition of each of the genetically identical pigs. (A) OTU network of pig #1, (B) OTU network of pig #2. Blue nodes represent samples collected from pigs fed the soybean hulls diet. Green nodes represent samples collected from pigs fed the wheat bran diet. Black nodes represent OTUs. Blue edges represent connection between samples collected from pigs fed the soybean hulls diet and OTUs. Green edges represent connection between samples collected from pigs fed the wheat bran diet and OTUs. Edges are weighted by the relative abundance value in each OTU. S: soybean hulls diet. W: wheat bran diet.

that the bacterial community responded differently to the dietary change in the two cloned pigs (Figures 4 and 5).

4 Discussion

The use of a longitudinal study design allows us to study how bacteria and potential functional genes responded to a dietary switch in the two genetically identical pigs. The use of genetically identical cloned pigs was expected to eliminate variability due to genetic background.

However, our study found that genetically identical co-housed cloned pigs did not respond in the same way to dietary changes. Since the cloned pigs were maintained in identical conditions, it is unlikely that the changes in GIT microbiome, which could not be explained by diet, resulted from an environmental factor; rather, this is likely the result of inter-individual differences in the clones. In this regard, clonal differences in metabolic phenotypes and GIT microbiome composition have been previously reported. For instance, equal inter-individual variation

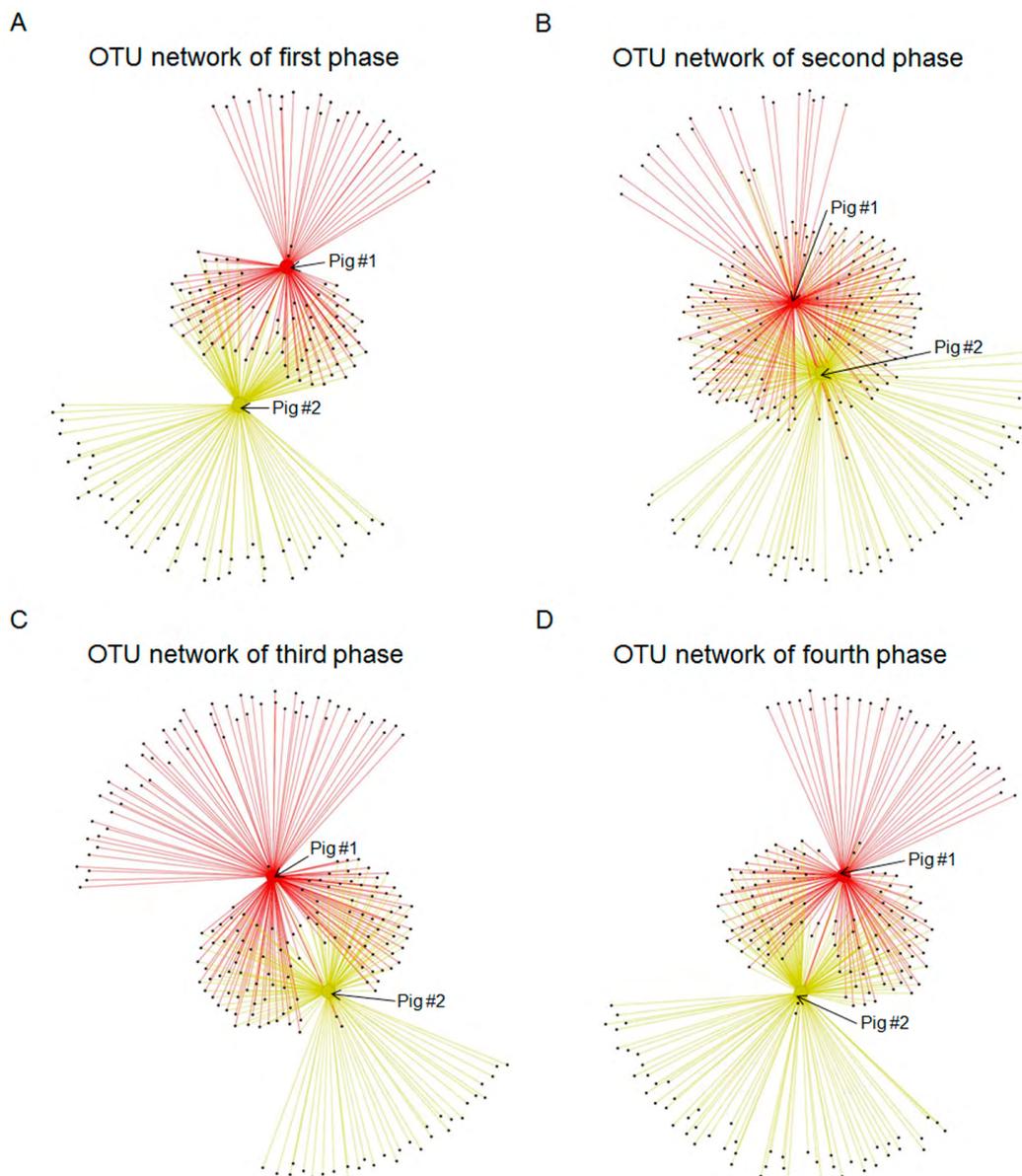


Figure 5: Network-based analysis of shared OTUs composition of each of the genetically identical pigs at each feeding phase. (A) OTU network of first phase, (B) OTU network of second phase, (C) OTU network of third phase, (D) OTU network of fourth phase. Red nodes represent samples collected from pig #1. Yellow nodes represent samples collected from pig #2. Black nodes represent OTUs. Red edges represent connection between samples collected from pig #1 and OTUs. Yellow edges represent connection between samples collected from pig #2 and OTUs. Edges are weighted by the relative abundance value in an OTU.

in cloned and non-cloned pigs was observed in studies that addressed how a variation of metabolic phenotypes and bacterial composition in the GIT was influenced by cloning [47-49]. One explanation for these inter-individual microbiomes is that cloned pigs created by somatic cell nuclear transfer may not have the same maternal mitochondrial DNA and epigenetic constitution, although their nuclear DNA is identical [47,49]. The differences in maternal mitochondrial DNA and epigenetic constitution may result in a distinct GIT microbiome composition in response to diet.

The changes in GIT microbial composition were very rapid and observed 14 days after dietary switch. This is consistent with previous studies that demonstrated that a 2-week dietary intervention period was sufficient to elicit changes in GIT microbiome composition [50-52]. The dietary sources used in our study exerted a differential influence on the GIT taxonomic and the predicted functional composition of these genetically identical co-housed pigs. These results agree with prior studies, which reported that different dietary fibers perceptibly change the GIT microbiome composition [52-58]. For example, in a recent study, Hooda *et al.* [53] revealed that polydextrose and soluble corn fiber consumption promoted the growth of beneficial bacteria in humans, including Veillonellaceae and *F. prausnitzii*. Martinez *et al.* [55] indicated that resistant starch type 2 and 4 induced different changes in the human fecal microbiome composition. Resistant starch type 4 raised the proportions of *Bifidobacterium adolescentis* and *Parabacteroides distasonis*, while resistant starch type 2 increased the relative abundance of *Ruminococcus bromii* and *Eubacterium rectale* [55]. Trompette *et al.* [58] has reported that pectin decreased the relative proportions of Firmicutes to Bacteroidetes when compared to cellulose. The relative abundance of two genera (*Butyricoccus* and *Parasporobacterium*) was observed to respond in different ways to the dietary switches in the two cloned pigs. *Butyricoccus* is a butyrate producer, a key metabolite for gut health, and has also been reported to be affected in the elderly [59]. While there were shared OTUs that were resistant to change in these pigs, there were also unique OTUs that were very different in their response to diet. These results are in agreement with prior studies reporting that inter-individual differences exerted more influence than differences within an individual in the response to a dietary intervention [60-62]. These inter-individual variations most probably resulting from epigenetics are not entirely eliminated by the use of genetically identical cloned pigs. Our analysis also showed that the influence of dietary change on GIT microbiome was dependent on the

individual; each individual being their own best control.

In summary, our results demonstrated that diet differentially affects the fecal microbiome composition in genetically identical cloned pigs over short time scales. Inter-individual variations were not totally eliminated in the genetically identical cloned pigs, which impacts the ability to study the influence of diet on GIT microbiome composition independent of the impact of host genotype and environment.

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