Profiling the gastrointestinal microbiota in response to *Salmonella*: Low versus high *Salmonella* shedding in the natural porcine host

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1 Introduction

*Salmonella* causes gastroenteritis in humans and is a leading cause of bacterial foodborne disease in the U.S. (Scallan et al., 2011). Incidence of human salmonellosis is estimated to be 1 million cases per year, resulting in approximately 350 deaths and a projected financial burden of $2.7 billion annually (ERS, 2011). Controlling *Salmonella* in the food chain is complicated by the ability of *Salmonella* to colonize livestock without causing clinical symptoms/disease. *Salmonella*-carrier animals are a significant reservoir for contamination of naive animals, the environment, and our food supply. *Salmonella* carriage and shedding in pigs varies greatly both experimentally and on-farm. To investigate the dynamics between the porcine intestinal microbiota and *Salmonella* shedding, we temporally profiled the microbiota of pigs retrospectively classified as low and high *Salmonella*-shedders. Fifty-four piglets were collectively housed, fed and challenged with 10⁶ *Salmonella enterica* serovar Typhimurium. Bacterial quantitation of *Salmonella* in swine feces was determined, and total fecal DNA was isolated for 16S rRNA gene sequencing from groups of high-shedder, low-shedder, and non-inoculated pigs (n = 5/group; 15 pigs total). Analyses of bacterial community structures revealed significant differences between the microbiota of high-shedder and low-shedder pigs before inoculation and at 2 and 7 days post-inoculation (d.p.i.); microbiota differences were not detected between low-shedder and non-inoculated pigs. Because the microbiota composition prior to *Salmonella* challenge may influence future shedding status, the “will-be” high and low shedder phylotypes were compared, revealing higher abundance of the Ruminococcaceae family in the “will-be” low shedders. At 2 d.p.i., a significant difference in evenness for the high shedder microbiota compared to the other two groups was driven by decreases in Prevotella abundance and increases in various genera (e.g. Catenibacterium, Xylanibacter). By 21 d.p.i., the microbial communities of high-shedder and low-shedder pigs were no longer significantly different from one another, but were both significantly different from non-inoculated pigs, suggesting a similar *Salmonella*-induced alteration in maturation of the swine intestinal microbiota regardless of shedding status. Our results correlate microbial shifts with *Salmonella* shedding status in pigs, further defining the complex interactions among the host, pathogen, and microbiota of this important public health issue and food safety concern.

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duced production performance and decreased economic returns due to a lower average daily gain and higher feed conversion rates in pigs with a higher prevalence of Salmonella (Farzan and Friendship, 2010; Funk and Gebreyes, 2004). It is estimated that the swine industry endures $100 million in annual production losses due to swine salmonellosis (Schwartz, 1999).

On the >50% of swine farms that test positive for Salmonella (NAHMS, 2009), the level of colonization, shedding and persistence of Salmonella in pigs can vary greatly throughout the various stages of production. Variation in Salmonella shedding has also been observed experimentally between pigs following inoculation with equivalent doses of the same Salmonella serovar (Boyen et al., 2009; Huang et al., 2011; Utthe et al., 2009). Porcine genetics and gene expression differences have shown an association with Salmonella shedding phenotypes in swine (Huang et al., 2011; Utthe et al., 2011a, b), but may not fully explain the observed variability. The microbiota of the porcine gastrointestinal tract could also contribute to the variation in Salmonella shedding from pigs.

Investigations of the porcine intestinal microbiota have largely focused on the effects of nutritional and dietary additives (Liu et al., 2012; Sauer et al., 2011). Investigations of antibiotic usage (Allen et al., 2011; Looft et al., 2012), production practices (Kim et al., 2012; Williams et al., 2008), and disease (Azcarate-Peril et al., 2011) have also characterized the swine microbiota in response to perturbation, including Salmonella in the ileum of newly-weaned piglets (Dowd et al., 2008). Investigations have shown that Salmonella colonization influences the gut microbiota (Jurcova et al., 2013), with most studies examining colitis in engineered murine models (inbred mice which may have genetic susceptibility to Salmonella infections with or without antibiotic pretreatment) to analyze the consequence of Salmonella infection on the host intestinal microbiota (Ahmer and Gunn, 2011). Such investigations have described the requirement of intestinal inflammation (a response of the host’s immune defense system) for Salmonella to circumvent colonization resistance, a protective barrier provided by the intestinal microbiota that impedes pathogen colonization and prevents disease in the host (Stecher et al., 2007; Winter et al., 2010). Beyond colonization resistance, the microbiota was shown to mediate Salmonella clearance from the gut of slgA-deficient mice (Endt et al., 2010). Furthermore, Salmonella-induced colitis (Ferreira et al., 2011) and high levels of Salmonella shedding (Lawley et al., 2008) were controlled by the murine intestinal microbiota. Taken together, these data suggest that the swine intestinal microbiota may influence the shedding status of Salmonella-carrier pigs.

The purpose of the current study was to investigate the dynamics between the porcine intestinal microbiota and Salmonella shedding in the natural swine host. The interaction of Salmonella with host commensal bacteria could affect the ability of Salmonella to express virulence genes, colonize swine, and establish a carrier state in the pig. This is the first evaluation of the composition of the porcine microbiota before and after the porcine microbiota was shown to mediate the response to perturbation, including Salmonella enterica serovar Typhimurium (S. Typhimurium) challenge to explore the relationship between the host microbiota and extreme (high versus low) Salmonella shedding phenotypes in swine. Our investigation of this intriguing interplay identified significant correlations between Salmonella shedding status and microbiota profiles during both the acute infection and long-term colonization.

2. Materials and methods

2.1. Swine study

Cross-bred piglets from 6 sows were farrowed at the National Animal Disease Center, Ames, IA, weaned at 12 days of age, and raised in isolation facilities. All pigs tested fecal-negative for Salmonella spp. thrice over a six week period using bacteriological culture techniques. One week prior to Salmonella challenge, one pig from each litter was placed in an isolation room to serve as the non-inoculated (NI) control group. The remaining 54 pigs were housed in two separate isolation rooms. All pigs received the same feed ad libitum. At 7–8 weeks of age (day 0), the 54 pigs were intranasally inoculated (a natural route of infection due to pigs’ rooting behavior (Fedorka-Cray et al., 1995)) with 1 ml PBS containing $10^9$ colony forming units (cfu) of Salmonella enterica serovar Typhimurium $\chi232$; the NI control group received an intranasal inoculation of 1 ml PBS. Fecal samples were obtained on 0, 1, 2, 7, 14 and 21 days post-inoculation (d.p.i.) for quantitative and qualitative Salmonella culture analyses as previously described (Bearsen and Bearson, 2008). To categorize the high Salmonella shedder pigs (HS) and the low Salmonella shedder pigs (LS), the cumulative area under the log curve (AULC) was calculated from the log normalized cfu data from each test day for each pig (Huang et al., 2011) and plotted in GraphPad Prism 5.01 (La Jolla, CA); the cumulative AULC accounts for shedding over all time points. Five HS and five LS pigs were selected for the subsequent microbiome analyses based on (1) the AULC data and (2) the inclusion of littermates in each group (in order to control for variability in the microbial community caused by host genetic differences); thus, some pigs that could have been classified and selected as HS or LS pigs were excluded from the analysis if one of their littermates was already classified in the same group. A diarrhea score for each pig was assessed visually by the same four evaluators at the time of collection and before shedding status was determined with a score from 1 to 5 (1 = dry feces, 2 = moist feces, 3 = mild diarrhea, 4 = severe diarrhea, and 5 = watery diarrhea) (Song et al., 2012). Body temperatures were assessed using a rectal thermometer. The number of Salmonella present in fecal samples (cfu/g) of the HS and LS pigs was $\log_{10}$ transformed. Statistical analyses were performed by GraphPad Prism 5.01 using two-way ANOVA (analysis of variance) followed by the Bonferroni multiple comparison post-test to determine all pair-wise differences. Procedures involving animals followed humane protocols as approved by the USDA, ARS, NADC Animal Care and Use Committee in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.2. 16S rRNA gene sequence analysis

DNA was isolated from pig feces at 0, 2, 7, and 21 d.p.i., and 16S rRNA genes were amplified as previously described (Allen et al., 2011). Briefly, 0.25 g of feces were bead-beaten and DNA was extracted using the PowerSoil DNA isolation kit (MoBio, Carlsbad, CA). DNA was quantitated using a Nanodrop ND-1000 spectrophotometer, and ten ng of fecal DNA were used in PCR reactions with the same barcoded primers and cycle conditions as reported previously (Allen et al., 2011). Amplified DNA was gel-purified (MinElute PCR purification kit, Qiagen, Valencia, CA), quantitated using a Quanti-IT PicoGreen dsDNA assay kit (Invitrogen), and sequenced on a 454 Genome Sequencer FLX using Titanium chemistry (Roche Diagnostics, Branford, CT). Sequence data were processed per Roche’s protocols, AmpliconNoise (Quince et al., 2011), mothur (versions 1.22–1.25, (Schloss et al., 2009, 2011)), and Uchime (Edgar et al., 2011) to remove barcodes and reduce sequence artifacts produced during PCR and sequencing. Uchime and barcode removal were implemented in mothur (Schloss et al., 2009). This pipeline yielded 13,834 unique sequences out of 1,247,796 total sequences across all samples. Sample coverages were all greater than 97% as measured by the Good’s coverage calculator in mothur. Rarefaction curves generated from each individual swine microbiome plateau and show tight 95% confidence intervals.
(Supplementary Fig. 1). Raw reads were deposited in the GenBank Sequence Read Archive under accession number SRP013958.

The Ribosomal Database Project (RDP version 7, (Cole et al., 2009)) (cutoff = 60) was implemented in mothur (version 1.23, pds training set) to assign the unique sequences to phylotypes. Additionally, distance matrices were generated in mothur (Schloss et al., 2009) at operational taxonomic unit (OTU) cutoffs of 0.03 (species level). OTU frequency counts were normalized to the sample with the lowest number of reads post-processing (~2500). These data were then analyzed and plotted in PAST (version 2.14, (Hammer et al., 2001)) using a canonical correspondence analysis, which is a way to analyze frequency count data from multiple samples in terms of ecological variables. Statistical analyses of both phylotype and OTU data included non-metric dimensional scaling (NMDS), analysis of similarity (ANOSIM), repeated measures analysis of variance (ANOVA), and pairwise t-tests to determine relationships between samples. Additionally, Metastats (White et al., 2009) was used to identify which members within a community were responsible for differences between communities. Kruskal–Wallis and Mann–Whitney tests were implemented in PAST to confirm the Metastats results. Mothur (Schloss et al., 2009) implemented all diversity calculations, including the parametric richness estimate via CatchAll (Bunge et al., 2012), the Simpson evenness index, and unifrac analyses (Lazourpine et al., 2011). Richness is the measure of the total number of unique species in a community, and evenness is the measure of the relative abundance of each of the members. All day 0 samples were considered non-inoculated in statistical tests. The following cutoffs were used for drawing conclusions: uncorrected p < 0.05, significant; p < 0.1, trend; R = 0–0.3, slight dissimilarity; R = 0.3–0.5, medium dissimilarity.

A quality control analysis was performed using technical replicates of one HS fecal DNA sample and one LS fecal DNA sample to gauge the variability of the 16S rRNA gene sequence analysis and confirm our pipeline. Each sample served as template in two parallel PCR assays, and the duplicate reactions were sequenced on every region of the Titanium plates. Both of these samples were therefore sequenced 12 times (six sequencing runs from two different PCRs). Multiple pairwise linear regression comparisons were made between the frequency count data from one 16S rRNA amplification reaction compared to the data from the parallel reaction sequenced on a different plate (Supplementary Fig. 2). The data were highly correlated (0.95 < r² < 0.99), supporting the value of our quality control pipeline and the reproducibility of the data.

2.3. Real-time PCR

Real-time PCR analyses were performed in duplicate on Salmonella and Prevotella populations in fecal DNA samples from the five HS, five LS, and five NI control pigs using primers that targeted the Salmonella hiaA gene (F: 5’CGCCTGCGAGAATTGCTACT and R: 5’AGCCCGCTAATCTCAAGCTTGG) (Brunelle et al., 2011), and the Prevotella 16S rRNA gene (F: 5’GGGTGTTGAACCTTGTGTTAAGG and R: 5’GGTCCTCTTTAAAACCCCATAAA) (Okabe et al., 2007). To create a standard curve for the Salmonella samples, a late-log culture of Salmonella was serially diluted 1:10, and each dilution was spiked with feces from Salmonella-negative control pigs prior to DNA extraction; an aliquot from each culture was reserved prior to spiking for enumeration. DNA extracted from Salmonella-negative control pig feces was used to test the specificity of the real-time primers, and was also used as a negative control in every test. A fecal-spiked standard curve could not be created for the Prevotella samples as there are no Prevotella-negative feces due to its ubiquitous nature in the gut. Prevotella control DNA was obtained by extracting DNA from a culture of Prevotella intermedia, strain VPI 4197. Each 20 µl PCR reaction (SYBR® Green PCR Master Mix, Applied Biosystems) containing 20 ng of DNA template was amplified in a Chromo4 Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA): 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 58°C (Prevotella) or 62°C (Salmonella) for 30 s; at the 58°C/62°C step, fluorescence data was acquired. A melting curve was also run for every real-time assay. The threshold cycle (Ct) values were determined by LinReg PCR 11.0 software (Ruijter et al., 2009). Quantitation of Salmonella from the samples was performed by interpolation from the standard curve (R² = 0.991). Because the input DNA was quantitated and standardized for each reaction, the ΔCt method for relative quantification normalized against unit mass was used to assess changes in Prevotella between the NI, HS, and LS pigs at 2 d.p.i. (Ct values at 0 d.p.i. were used as the calibrators for each pig at 2 d.p.i). Differences in Salmonella cfu and relative changes in Prevotella were compared respectively by the unpaired t-test and repeated measure ANOVA with Tukey post-test using GraphPad Prism 5.01.

3. Results

3.1. Variation of Salmonella shedding in swine

Variation in the levels of Salmonella fecal shedding among pigs experimentally inoculated with the same dose of S. Typhimurium and housed in the same environment observed in this study is congruent with previous reports (Huang et al., 2011). Salmonella shedding counts from the 54 inoculated pigs ranged by seven orders of magnitude (10⁷) over the course of the 21 day study. To identify high shedder (HS) and low shedder (LS) pigs to temporarily profile the intestinal microbial communities in response to S. Typhimurium challenge, the fecal counts at days 0, 1, 2, 7, 14, and 21 p.i. were log transformed, and the cumulative AULC was determined (Fig. 1). The five HS and five LS pigs chosen to study were selected based on their presence within the upper and lower quartile ranges of the AULC data, respectively, as well as on the inclusion of littermates with opposite shedding status in order to minimize the effects of genetic variation within the crossbred pigs.

A significant difference in the average fecal shedding of Salmonella was observed between the HS and LS groups (n = 5/group) at 1, 2 and 7 d.p.i. (Fig. 2A). Using a subjective measurement of diarrhea (1–5 scoring system, with increasing moisture content), the HS pigs at 2 d.p.i. had a significantly greater diarrhea score compared to day 1 (p < 0.01) and 7 p.i. (p < 0.001) (Fig. 2B). The HS pigs also had a significantly higher diarrhea score than the LS pigs at 2 d.p.i. (p < 0.01); no significant difference in diarrhea scores were observed in the LS pigs comparing days 0, 1, 2 and 7 p.i. The average body temperature was not significantly different between the two shedding groups over the course of the study, although both the LS and HS pigs had a significant (p < 0.001) increase in body temperature at 1 d.p.i. compared to day 0 (Fig. 2C).

3.2. Changes in microbial community structure associated with S. Typhimurium challenge

To compare the effects of differential Salmonella disease status on the gastrointestinal bacterial communities of the HS, LS and non-inoculated (NI) pigs over the 21-day study, fecal 16S rRNA gene sequences were analyzed by OTUs at the 0.03 dissimilarity cutoff. Comparisons of the data by day revealed a significant difference in community structure between the LS and HS pig groups prior to S. Typhimurium challenge (ANOSIM; p < 0.05), but neither group was different from the NI pigs (p > 0.1, Fig. 3A). Inoculation with S. Typhimurium caused dramatic changes in the bacterial community structure of the microbiota in the HS pigs at 2 and 7 d.p.i., demonstrating significant differences compared to the NI and LS pigs (p < 0.05, 0.35 < R < 0.5, Fig. 3B and C). However, over
the first week, the community structure of the LS pigs was not significantly altered by S. Typhimurium challenge compared to the NI pigs. Further evidence for these shifts was revealed by examining the phylotype assignments, which resolved the changing microbiota in the HS pigs even at the low resolution of phylum-level classification (Fig. 4). Interestingly, by 21 d.p.i., both S. Typhimurium-inoculated communities were more similar to each other \((p > 0.1, \text{LS versus HS})\) than to the NI communities \((p < 0.05, \text{each inoculated community versus non-inoculated, Fig. 3D})\), suggesting that S. Typhimurium induced comparable changes over time in the swine GI microbiota regardless of shedding status.

Two metrics of diversity, richness and evenness, were analyzed to assess the ecological underpinnings of the observed changes in community structure. The difference in parametric richness estimates \((\text{the total number of unique species in a community})\) between treatment groups within a timepoint was not significant \((\text{Supplementary Table 1}; \text{richness ranged from 392} \pm 50 \text{ to 616} \pm 27 \text{ bacterial species per treatment group per time; all} \ p > 0.1 \text{ by repeated measures ANOVA})\). Evenness, a measure of how numerically equivalent the members are within a community, was examined via the Simpson evenness index. The microbiota of the HS pigs at 2 d.p.i. had significantly different evenness compared to either HS pigs at 0 d.p.i. \((p < 0.01)\) or NI pigs at 2 d.p.i. \((p < 0.01)\), indicating that S. Typhimurium challenge greatly affected the evenness of the microbiota in HS pigs but not the LS pigs. This result can be viewed empirically at the genus level classification in Fig. 4. Another comparison of interest was the significant difference \((p < 0.01)\) in evenness of NI pigs at day 0 versus NI pigs at 21 d.p.i., suggesting a shift in the evenness of the microbial community as non-inoculated pigs mature. The results indicate that changes in community structure were due to changes in the relative abundance, not richness, of the swine intestinal bacteria, and that S. Typhimurium challenge influences these microbiota changes.

### 3.3. Specific phylotypes are affected by S. Typhimurium challenge

As can be seen in the NI swine microbiotas throughout the study, the swine microbiota phylotype analysis showed comparable membership to what has been reported previously for mammals \((\text{Ley et al., 2008})\), with the majority of genera belonging to the Firmicutes, Bacteroides, and Proteobacteria phyla (Fig. 4). The distribution of genera was consistent with a swine gut microbial community \((\text{Allen et al., 2011})\), with Prevotella and Succinivibrio dominating the phylotype data (Fig. 4). Maturation of the microbiota was measured by comparing the NI microbiotas at day 0 to day 21, revealing the Planctomycetes, Spirochaetes, and Fibrobacteria phyla with increased abundance at day 21 \((\text{Metastats} \ p < 0.05)\). At the genus level, 23 significantly different taxa were detected with maturation, including Prevotella \((\text{Metastats} \ p < 0.05)\).

Phylotype profiles of the “will-be” LS and HS pig groups at day 0 were compared because the differences in microbial community structure prior to S. Typhimurium challenge observed in Fig. 3A suggested that specific resident bacteria could be a factor in subsequent Salmonella-shedding status. Although no significant differences were detected at the phylum level, comparing the microbiota at day 0 in HS and LS pigs at the family level detected one family, the Ruminococcaceae, that was significantly different between these groups, with their relative abundance being highest in the “will-be” LS pigs \((\text{Metastats} \ p < 0.05; \text{Kruskal–Wallis} \ p < 0.05 \text{and} \ \chi^2 = 6.02; \text{Fig. 4})\). Examination of the data at the genus level revealed two somewhat rare genera \((\text{Coprobacillus} \text{and Phascolarctobacterium})\) of greater relative abundance in the “will-be” HS pigs \((\text{Metastats} \ p < 0.05; \text{Kruskal–Wallis} \ p < 0.05 \text{and} \ \chi^2 = 3.38 \text{and} 7.22, \text{respectively})\). Taken together, these data suggest that the Ruminococcaceae family might be important in preventing the future high-shedding status of pigs, or that the presence of certain genera prior to Salmonella challenge might promote colonization, exacerbate shedding, or a complex combination of multiple scenarios.

The community structure analyses \((\text{Fig. 3, Supplementary Table 1})\) showed significant changes in the HS pig microbiotas at 2 d.p.i., prompting an interest in discovering which genera were responsible for the shifts. Genera were compared across treatment groups at 2 d.p.i., and several genera showed significant differences \((\text{Fig. 5})\). The most abundant of the differentially present genera was Prevotella, which was lower in the HS pigs at day 2 p.i. \((p < 0.001, \text{Fig. 5})\).

### 3.4. Real-time PCR confirms the differences in Salmonella and Prevotella levels in the LS and HS pigs

To verify the shift in the relative abundance of Prevotella by 2 d.p.i. in the HS pigs, real-time PCR was employed. From day 0 to 2 d.p.i., Prevotella decreased 0.40-fold \((\log_2)\) in the NI pigs, 0.76-fold \((\log_2)\) in the LS pigs, and 5.95-fold \((\log_2)\) in the HS pigs; the magnitude of the decrease was different between the NI and HS pigs \((p < 0.05)\). Real-time PCR was also performed to assess
the presence and quantity of *Salmonella* in the fecal DNA from the HS, LS, and NI pigs at 0 and 2 d.p.i. As observed in the bacteriological analysis, *Salmonella* DNA was neither detected by PCR in any day 0 sample nor in the NI pigs at 2 d.p.i. In contrast, a significantly higher level (p < 0.01) of *Salmonella* was observed in the HS pigs (8.16 x 10^6 cfu/g feces) compared to the LS pigs (7.17 x 10^4 cfu/g feces) at 2 d.p.i. These data confirm the cfu data determined by bacteriological methods for the HS pigs (5.13 x 10^6 cfu/g feces), but not for the LS pigs (2.69 x 10^4 cfu/g feces). The latter can be explained by our observation from serial dilutions of *Salmonella* (10^6–10^1) that the limit of accurate quantitation for *Salmonella* in our qPCR assay is ~10^4 cfu/g feces.

**Fig. 2.** *Salmonella* fecal shedding, diarrhea scores and rectal temperatures. Red squares represent HS pigs (n = 5), green triangles denote LS pigs (n = 5), gray diamonds identify all other *Salmonella*-inoculated pigs (n = 44), and blue circles symbolize NI pigs (n = 5). (A) Average *Salmonella* fecal shedding from pigs. (B) Average diarrhea score of each pig category. (C) Average rectal temperature of each pig category. Significant differences (p < 0.05) between HS and LS pigs in 2A and 2B, as well as between day 0 and day 1 in 2C for both LS and HS pigs, are denoted by the number symbol (#).

4. Discussion

Swine are a natural host for *Salmonella* and multiple carrier states (active, passive, and latent) exist depending upon the extent of pathogen exposure and the history of clinical or sub-clinical disease (Stevens et al., 2009). Variability in the level of *Salmonella* colonization, carriage, and shedding in pigs has been described with naturally-infected swine in production facilities as well as during controlled experimental infections (Huang et al., 2011; Stevens et al., 2009). Even following a high dose challenge of swine with *S. Typhimurium*, *Salmonella* shedding varies widely among pigs, clinical symptoms can be mild and transient, and *Salmonella* often establishes a commensal-like state in the host, emphasizing differences in disease manifestation for *Salmonella* between natural hosts and model systems. Multiple factors influence the dynamics of *Salmonella* in swine, including features of the pathogen (virus-like mechanisms, exposure dosage), responses from the pig (health and age of the pig, innate and adaptive immunity, variable intrinsic disease resistance controlled by genetic variation), and contributions from the gastrointestinal microbiota (competition for niche colonization, microbial by-products, bacteriocins, nutrient limitation, bacteriophages, interaction with the host immune system). To evaluate the porcine intestinal microbiota with and without *Salmonella* challenge, cohabitating pigs from multiple litters were intranasally-inoculated with an equal dose of *Salmonella* enterica serovar Typhimurium, and non-inoculated littermates were housed separately. Temporal profiling of the microbial communities during the 3-week study permitted various comparisons, including: (1) the microbiotas prior to *S. Typhimurium* inoculation between pigs ultimately categorized as high and low *Salmonella* shedders; (2) changes in the microbiota profiles of the high-shedder (HS) and low-shedder (LS) pigs following challenge with *S. Typhimurium*, and in comparison to the non-inoculated (NI) pigs; and (3) early development of the swine microbiota in the NI pigs.

Our hypothesis was that the swine microbiota contains members that affect *Salmonella*-shedding status because the swine gut harbors bacterial antagonists of *S. Typhimurium* (Casey et al., 2004; Levine et al., 2012). Potentially, the microbiota of pigs classified as high *Salmonella* shedders could lack certain bacteria that impede (or possess bacteria that promote) colonization and invasion by *S. Typhimurium*. Thus, the variation in *Salmonella*-shedder status in the HS and LS pigs may be a consequence of their microbial composition at the time of *S. Typhimurium* challenge. The results showed a significant difference in the microbial community structure of the “will-be” HS and “will-be” LS groups at day 0, and despite the contributions of multiple phylotypes, the *Ruminococcaceae* family was more abundant in the “will-be” LS group. Previous work has shown that the *Ruminococcaceae* are enriched in the mucosal environment (Nava and Stappenbeck, 2011), an environment that *Salmonella* penetrates to establish a carrier state. *Ruminococcaceae* have shown decreased abundance in other animals with gut disturbance, such as in dogs with acute diarrhea (Suchodolski et al., 2012). Hallmarks of many members of the *Ruminococcaceae* include cellulolytic activity and the production of short-chain fatty acids (SCFAs). This latter function is of interest because of the benefits of SCFAs on gut health, the inhibition of *Salmonella* in an acidic environment (Bearson et al., 2006), and the anti-inflammatory properties of SCFAs (Tedelind et al., 2007). In outbred Swiss mice, theecal contents of streptomycin treated mice had an increased pH and decreased SCFAs (acetic, propionic, butyric, and valeric acid) compared to untreated mice; consequently, streptomycin-pretreated mice had increased susceptibility to *Salmonella* colonization (Que et al., 1986). Thus, a possible explanation for the dramatic *Salmonella* shedding phenotypes (HS vs. LS) in swine may be due to differences between the
Salmonella is known to induce inflammation. The increased moisture (diarrhea) in the HS pigs at 2 d.p.i. demonstrates the gut inflammation triggered by the host’s immune response to Salmonella. Studies in the engineered streptomycin-treated murine model suggest that the pathogenicity of Salmonella benefits from intestinal inflammation. Gut inflammation triggered shifts in the murine intestinal microbiota that reduced commensal growth while supporting Salmonella growth (Stecher et al., 2007). One potential mechanism of this modulation of the microbiota is neutrophil recruitment in response to Salmonella infection via the production of the neutrophil enzyme elastase (Gill et al., 2012). Also, Salmonella can utilize alternate electron acceptors or nutrients generated as a consequence of gut inflammation, thereby conferring a competitive advantage to Salmonella in the murine inflamed gut.
Fig. 4. The effect of S. Typhimurium challenge on swine intestinal bacterial phylotypes. Assignable reads were normalized (n = 4503 per microbiome) and the average of five pigs in each treatment group are shown at the phylum, family, and genus classification levels. Only the 25 most abundant families (out of 91 detected) and 30 most abundant genera (out of 233 detected) are plotted for clarity.
Thiennimitr et al., 2011; Winter et al., 2010). Given that inflammation is an important part of Salmonella’s pathogenesis strategy and that members of the Ruminococcaceae contribute to decreased inflammation, our results suggest the hypothesis that “will-be” high shedders may have an elevated gut inflammation status that more readily yields to Salmonella colonization and shedding.

In addition to potential protective bacteria in the “will-be” low shedders, the “will-be” high shedders had increased abundance of two genera, Phascolarctobacterium and Coprobacillus, although little is known about either genus beyond basic characterization studies of these gut commensals (Kageyama and Benno, 2000; Watanabe et al., 2012). The potentiation of microbial invaders by autochthonous bacteria is an important yet understudied area of research (Venturi and da Silva, 2012). Future studies are needed to define the role of these bacteria in Salmonella colonization and shedding in swine.

Upon challenge with S. Typhimurium, the changes in the microbiota associated with the highest Salmonella shedding levels could be associated with an inflammatory response. The HS pigs exhibited a significant shift in their microbial communities at 2 d.p.i. compared to the LS pigs and NI pigs; the microbiota of the LS pigs did not change compared to the NI pigs following the initial S. Typhimurium challenge. The HS pigs at 2 d.p.i. showed a dramatic decrease in Prevotella, one of the most prevalent genera in the swine gastrointestinal tract (Poroyko et al., 2010). Previous analysis of mouse colonic bacteria showed that the phylum Bacteroidetes (of which Prevotella is a member) decreased in abundance 7 days after either Citrobacter rodentium infection or chemical induction of inflammation (Lupp et al., 2007). Our phylum-level results are consistent with the model of an inflammation event causing a decrease in Bacteroidetes. Further support for the role of inflammation in the microbial changes is that at 2 d.p.i. in the HS pigs, a significant increase was observed in the relative abundance of members of the family Coriobacteriaceae (Slackia and Collinsella), which have been associated with inflammatory disease in mice and humans (Clavel et al., 2010).

Clearly the host’s inflammation status, both before and during Salmonella challenge, can impact the gut microbiota. However, it is equally important to consider the effect of the gut microbiota on the host. The “Superorganism,” referring to the host with its immuno-bacterial interaction, illustrates the beneficial nature of the host microbiota because it induces the production of host immune factors (such as IL-10) that maintain homeostasis, especially during pathogenic invasion (Round et al., 2010). Thus, the microbiota influences the host response, and Salmonella affects the host response when it is a member of that microbiota. Research within our collaborative group (using a different pig population) has demonstrated unique immune gene expression profiles in persistent Salmonella-shedding pigs compared to low Salmonella-shedding pigs at 2 d.p.i. with S. Typhimurium (Huang et al., 2011), as well as the positive association of Salmonella shedding status with serum levels of interferon-γ (IFNγ), a pro-inflammatory cytokine that plays a pivotal role in the cell-mediated, Th1-dependent immune response during the early stages of Salmonella infection (Uthe et al., 2009). Transcriptional profiling of the current HS/LS pig population is in progress.
and association analyses will be performed to determine if the microbiota composition correlates with host gene expression for a given Salmonella shedding phenotype.

Defining the factors that control (1) the shifts in the microbial community and (2) the variation in Salmonella shedding following challenge are complicated by pre-existing differences between the HS and LS pigs prior to challenge as well as the natural shift in the community as a consequence of maturation. Although cause and effect is difficult, if not impossible, to define, two likely drivers of community shifts were observed in this study. (Fig. 3D), one by the introduction of Salmonella and the other by time. At day 2 p.i., Salmonella was the strongest driver of change and dissimilarity in the microbial community, but at day 21 p.i., time (age of pigs) was the strongest. In the non-inoculated pigs, a population shift in the community structure occurred between the first week (day 0, 2 and 7) and the third week (day 21) of the study, and this was driven by multiple genera including Prevotella. These changes are presumably due to progressive maturation of the intestinal microbiota and establishment of its core functionality (Kim et al., 2011). Interestingly, the microbiotas of the LS pigs were similar to the NI pigs during the early stages of S. Typhimurium infection (1–7 d.p.i.) but were different from the HS pigs. However, at 21 d.p.i., the S. Typhimurium-inoculated pigs (LS and HS) were no longer different from one another, but were both different from the NI pigs, suggesting that the introduction of S. Typhimurium into the porcine gastrointestinal tract altered the progression of maturation of the intestinal microbiota. Furthermore, the microbiota profiles at 21 d.p.i. suggested that the S. Typhimurium challenge changed the microbiota in a common manner despite the differences in the degree of shedding or clinical symptoms. This finding may be applicable to swine production because an association between Salmonella prevalence and herd health status impacts economic returns (Farzan and Friendship, 2010; Funk and Gebreyes, 2004).

As this study employed three different methods of Salmonella detection in swine feces (media-based culture analysis, qPCR amplification, and high throughput pyrosequencing), a comparison can be made between the Salmonella quantitative bacteriology and the molecular detection of Salmonella DNA. Real-time PCR with Salmonella-specific primers confirmed the results of Salmonella quantitative bacteriology when Salmonella levels were >10^6 cfu/g feces, such as in the HS pigs at 2 d.p.i. (>10^6 cfu/g feces). Pyrosequencing of 16S rRNA gene amplicons only detected an average relative abundance of three Salmonella sequences in the HS pigs at 2 d.p.i.; no Salmonella sequences were detected in the LS pigs. Others have shown a similar molecular limit of detection of Salmonella (Fukushima et al., 2003) and for bacteria in general (Lagier et al., 2012). This suggests that microorganisms of lower abundance (<10^6 cfu/g feces) were potentially missed in the 16S-based analysis. On the other hand, considering that a gram of pig feces contains ~10^10 bacteria (Allison et al., 1979; Stanton and Humphrey, 2003), it is notable that pyrosequencing detected Salmonella at 4 orders of magnitude less than the overall bacterial load in the fecal samples. Furthermore, at a ratio of merely 1:1000 (Salmonella:fecal bacteria), introduction of Salmonella into the gastrointestinal tract of the HS pigs resulted in significant shifts in the microbiota community. In brief, our investigation underscores the value of using complementary microbiological and molecular biology techniques to capture microbial diversity and abundance.

In summary, Salmonella fecal shedding has food safety implications because Salmonella-carrier pigs can contaminate the environment at numerous points along the farm-to-fork continuum. This study revealed significant differences in the porcine intestinal microbiota between pigs categorized as high and low Salmonella shedders prior to and following inoculation with S. Typhimurium. These differences were due to variation in abundance, not richness, of the microbiota members. Furthermore, following recovery from the acute phase of infection, the microbiotas of the LS and HS pigs became similar to one another, but were significantly different from the NI pigs, suggesting that S. Typhimurium induced comparable changes in the swine GI microbiota regardless of shedding status. The data indicate that during swine production, the gastrointestinal microbiota could be influenced by sub-clinical Salmonella infections with potential impacts on animal health and food safety.

Disclosure statements
Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendations or endorsement by the U.S. Department of Agriculture.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.03.022.

References


