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

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ABSTRACT

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 naïve 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 10^9 *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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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 our food chain is impeded by the existence of over 2500 serovars (subtypes), its broad host range and ubiquitous nature, and its ability to sub-clinically colonize food-producing animals (Bearson and Bearson, 2011; Boyen et al., 2008; Van Parys et al., 2012; Wigley, 2004). *Salmonella*-carrier animals are a serious food safety issue because they shed *Salmonella* in their feces, thereby contaminating: (1) neighboring naïve animals; (2) slaughter plants and meat products during processing; (3) edible crops when manure is used as a soil amendment; and (4) water supplies when fertilizer (manure) runs off into waterways. *Salmonella* colonization in swine may also impact herd health status with

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reduced 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; Uthe et al., 2009). Porcine genetics and gene expression differences have shown an association with *Salmonella* shedding phenotypes in swine (Huang et al., 2011; Uthe 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 (Juricova 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 sIgA-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 *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 1×10^9 colony forming units (cfu) of *Salmonella enterica* serovar Typhimurium χ4232; 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 (Bearson 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 Quant-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