

ORIGINAL ARTICLE

Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations

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Disturbance of the beneficial gut microbial community is a potential collateral effect of antibiotics, which have many uses in animal agriculture (disease treatment or prevention and feed efficiency improvement). Understanding antibiotic effects on bacterial communities at different intestinal locations is essential to realize the full benefits and consequences of in-feed antibiotics. In this study, we defined the lumenal and mucosal bacterial communities from the small intestine (ileum) and large intestine (cecum and colon) plus feces, and characterized the effects of in-feed antibiotics (chlortetracycline, sulfamethazine and penicillin (ASP250)) on these communities. 16S rRNA gene sequence and metagenomic analyses of bacterial membership and functions revealed dramatic differences between small and large intestinal locations, including enrichment of *Firmicutes* and phage-encoding genes in the ileum. The large intestinal microbiota encoded numerous genes to degrade plant cell wall components, and these genes were lacking in the ileum. The mucosa-associated ileal microbiota harbored greater bacterial diversity than the lumen but similar membership to the mucosa of the large intestine, suggesting that most gut microbes can associate with the mucosa and might serve as an inoculum for the lumen. The collateral effects on the microbiota of antibiotic-fed animals caused divergence from that of control animals, with notable changes being increases in *Escherichia coli* populations in the ileum, *Lachnobacterium* spp. in all gut locations, and resistance genes to antibiotics not administered. Characterizing the differential metabolic capacities and response to perturbation at distinct intestinal locations will inform strategies to improve gut health and food safety.

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Introduction

The mammalian gastrointestinal tract is a heterogeneous ecosystem with distinct, stratified environments. Resident bacteria benefit from nutrient-rich digesta while assisting the host via nutrient digestion, vitamin synthesis, pathogen displacement and immune system maturation (Green *et al.*, 2006; Bird

et al., 2010). Factors such as nutrient availability, pH, redox potential and peristalsis strongly influence the composition of bacterial communities in the intestinal tract (Hao and Lee, 2004). The nutrient and bacterial distribution is additionally influenced by the spatial organization of the intestine, which can be thought to exist along two axes, a longitudinal axis (proximal to distal) and a radial axis (lumen to the mucosa) (Takahashi and Sakaguchi, 2006).

Because bacteria along the mucosa are in close contact with the host, it is important to define these bacteria functionally and phylogenetically. Mucosal bacteria thrive on host-provided nutrients, such as mucin and dead epithelial cells, liberating mucosal carbohydrates that can be utilized by enteric pathogens (Ashida *et al.*, 2012; Ng *et al.*, 2013).

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Mucosa-associated bacterial communities differ from those in the intestinal contents (Zoetendal *et al.*, 2002), and those differences vary across the intestinal tract (Hill *et al.*, 2010). Common human commensal bacteria produce colonization factors that lead to long, stable colonization of mucosal crypts (Lee *et al.*, 2013). Studies in mice show an enrichment of *Firmicutes* spp. on the mucosa, and an enrichment of *Bacteroidaceae*, *Enterococcaceae* and *Lactobacillaceae* spp. in the lumen (Nava *et al.*, 2011). Despite these reported patterns, other studies have shown significant heterogeneity of mucosal bacterial communities within individuals (Zhang *et al.*, 2014). Defining mucosa-associated bacteria across time and space will inform their role in health and disease.

Longitudinal studies along the intestinal tract also suggest location-specific microbial communities, and the significance of these differences remains unclear. The human colon may harbor as many as 1000 bacterial species, with 10^{11} cells per gram of feces (Whitman *et al.*, 1998), whereas small intestinal bacterial communities show much lower diversity (species richness and abundance) and cell densities (Booijink *et al.*, 2010). This reduced microbiota is no less important to health or disease. In pigs with necrotizing enterocolitis, more *Clostridium* spp. were associated with the ileal mucosa than in healthy pigs (Azcarate-Peril *et al.*, 2011). These observations illustrate the value of considering spatial distribution when describing gut bacteria to better understand their niches and their potential to impact host health and performance.

Antibiotics are used in livestock to treat disease, prevent disease and improve feed efficiency (Cromwell, 2002; Dibner and Richards, 2005). Studies of changes in microbial communities with in-feed antibiotics have detected shifts in bacterial membership and functions in swine feces (Allen *et al.*, 2011; Kim *et al.*, 2012; Looft *et al.*, 2012), but have not addressed localized communities in the gut. Feces often serve as a proxy for the gut microbiota because they can be harvested readily and frequently, but fecal bacteria are a composite of potentially discrete upstream populations (Suchodolski *et al.*, 2005). Direct sampling of specific gut locations allows for differentiation of communities of the gut microbiota, potentially revealing how performance-enhancing antibiotics affect bacterial taxa in these microhabitats. Spatial changes in the bacteria along the ileal, cecal and colonic epithelium have been shown in chicks receiving in-feed antibiotics (Chichlowski *et al.*, 2007). Understanding how in-feed antibiotics affect the membership and metabolic potential of spatially distributed bacteria may inform alternatives to antibiotics that mimic these changes.

In this study, we used phylogeny and metagenomic analyses to define the longitudinal and radial distribution of bacterial membership and functions in the swine intestine. The results show dramatic

differences in the microbiome of the small intestine (ileum) compared with the large intestine (cecum, colon, feces), including luminal versus mucosal communities and the encoded functions of the ileal microbiota. The effect of the in-feed antibiotic ASP250 was also examined, with the results showing some changes in bacterial diversity specific to the mucosa and others that are consistent across gut locations. In addition, antibiotic exposure led to increased abundance of certain antibiotic resistance and bacterial metabolic genes.

Materials and methods

Swine

Piglets were acquired and managed as previously described (Allen *et al.*, 2011). Briefly, piglets were divided into two groups of six at ~3 months of age, with equal representation of littermates and sex. All pigs were fed the same diet (TechStart 17-25, Kent Feeds, Muscatine, IA, USA) until the start of the experiment, at which point six control pigs continued to receive TechStart, whereas the other group received TechStart containing ASP250 (chlortetracycline 100 g per ton, sulfamethazine 100 g per ton, penicillin 50 g per ton) *ad libitum* until euthanization.

Two pigs (one from each treatment group) were euthanized on six necropsy days between 12 January 2010 and 28 January 2010 at ~3 months of age. Euthanization was by intracardiac injection of sodium pentobarbital (26% solution) (1 ml per 4.5 kg Sleepaway; Fort Dodge Laboratories, Overland Park, KS, USA) after being anesthetized with an intramuscular injection of a cocktail of ketamine (8 mg per kg), xylazine (4 mg per kg) and Telazol (6 mg per kg, Fort Dodge Animal Health, Fort Dodge, IA, USA), in accordance with National Animal Disease Center Animal Care and Use Committee guidelines. Three sections of the GI tract (the ileum, the cecum and mid-colon) were harvested immediately after euthanasia. The sections were tied off by string ligatures and placed on ice for transport to the lab. With each intestinal sample, both a tissue and a lumen-content sample were taken for DNA extraction. Tissues were washed by gently flowing sterile phosphate-buffered saline across the tissue until no contents were visible. A sterile glass microscope slide was used to scrape any attached bacteria off of the surface for DNA extractions. These tissue scrapings plus corresponding gut contents and freshly-voided feces were collected from each pig, and DNAs were extracted using the manufacturer's protocol with the UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA, USA).

16S rRNA gene sequence analysis.

Amplification of the V1–V3 region of bacterial 16S rRNA genes was carried out as previously described (Allen *et al.*, 2011). Briefly, the conserved primers 8F (5'-AGAGTTTGATCCTGGCTCAG) (Weisburg

et al., 1991) and 518R (5'-ATTACCGCGGCTGCTGG) (Muyzer *et al.*, 1993) were designed with attached eight-nucleotide unique sequence barcodes (Hamady *et al.*, 2008; Allen *et al.*, 2011). PCRs were performed with 22 cycles (Allen *et al.*, 2011), and products were separated by gel electrophoresis and purified using the MinElute kit (Qiagen, Valencia, CA, USA). They were then sequenced on a 454 Genome Sequencer FLX using the manufacturer's protocol for Titanium chemistry (Roche Diagnostics, Branford, CT, USA).

Phylotype analysis

Sequence data were processed per Roche's protocols, followed by AmpliconNoise (Quince *et al.*, 2011), mothur (Schloss *et al.*, 2009, 2011) and Uchime (Edgar *et al.*, 2011) to remove barcodes and reduce sequence artifacts produced during PCR and sequencing. Uchime was implemented in mothur (Schloss *et al.*, 2009). Operational taxonomic unit (OTU)-based phylogenetic analysis (97% similarity cutoff) and hypothesis testing were performed with normalized data in mothur (Schloss *et al.*, 2009). To visualize changes in bacterial diversity, a nonmetric multidimensional scaling plot and dendrogram of these data using the Bray–Curtis index of OTUs were calculated in PAST (Hammer *et al.*, 2001). The estimated total richness of operational taxonomic units was calculated by CatchAll (Bunge, 2011) within mothur. The bacterial richness estimate resulting from the best parametric model was reported for each sample. Taxonomic assignments of the 16S rRNA gene sequences were made using the Ribosomal Database project web tools (Cole *et al.*, 2009). The Metastats statistical software was used for making comparisons between samples and identifying trends (White *et al.*, 2009).

Metagenomic analysis

DNAs from feces and gut contents (of the ileum, cecum and colon) were sequenced on Roche's Genome Sequencer FLX instrument (Roche Diagnostics) and processed as described previously (Allen *et al.*, 2011). Four samples from each of 12 pigs, minus samples from four pigs that were missing either ileal or fecal material, yielded 44 total DNA samples. These samples were divided across four regions of two Titanium plates, and the resulting DNA sequences were de-replicated (Gomez-Alvarez *et al.*, 2009). Assemblies and mapping experiments were carried out in CLC Genomics Workbench v. 5.5.1 (Prismet, Aarhus, Denmark) using default settings, and these assemblies were used only for phage analysis. Unassembled metagenomes were analyzed using the metagenomic and BLASTx pipelines in CAMERA (<https://portal.camera.calit2.net>, (Seshadri *et al.*, 2007)), searched against sequence libraries derived from the Carbohydrate-Active Enzyme (CAZy; www.cazy.org) database (Cantarel *et al.*, 2009) and blasted in-house

against the antibiotic resistance genes database (Liu and Pop, 2009; Looft *et al.*, 2012). Frequency counts of all functional data were normalized to the lowest total number of assigned reads (SEED subsystems) or of bacterial reads (resistance genes) among the 44 metagenomes. Normalized datasets were tabulated and analyzed for patterns (for example, cluster analyses and ANOSIM) in PAST (Hammer *et al.*, 2001). In addition, STAMP (Parks and Beiko, 2010) was used to make statistical comparisons between samples. Because the metagenomic samples might not be independent (for example, the ileum contents move downstream to become the cecal contents), the Kruskal–Wallis H-test was used rather than analysis of variation, followed by the Games–Howell *post-hoc* test and Storey's false discovery rate multiple comparison correction (Parks and Beiko, 2010).

Data presentation

A *P*-value less than 0.05 with a *q*-value (false discovery rate) less than 0.05 was considered significant ($q < 0.1$, trend), and *R* between 0–0.3 was considered a slight correlation, whereas *R* greater than 0.3 was considered a correlation for ANOSIM. The data are deposited in NCBI's Short Read Archive under Biosamples SAMN02215176–SAMN02215219 (metagenomes) and SAMN02215220–SAMN02215300 (16S survey), both of which affiliate with Bioproject PRJNA72355.

Results

Swine intestinal bacteria exhibit longitudinal and radial specificity

The overall bacterial diversity (mean) included common intestinal bacterial groups, dominated by *Firmicutes* (35%), *Bacteroidetes* (21%), *Proteobacteria* (3%) and *Spirochaetes* (2%), as shown previously in pigs (Allen *et al.*, 2011; Lamendella *et al.*, 2011; Looft *et al.*, 2012). However, the ileum lumen samples harbored almost exclusively *Firmicutes* and *Proteobacteria*, whereas the phylum-level profiles of the cecum and mid-colon were highly congruent (Figure 1a; Supplementary Table 1). Only one phylum, the *Synergistetes*, showed significant differences, with incremental increases in each subsequent gut section: ileum < cecum ($q = 0.001$), cecum < colon ($q = 0.003$). In addition, species-level analyses of OTUs revealed significant differences related to gut location ($P < 0.01$, $R = 0.65$). The ileal contents (both non-medicated and medicated) showed reduced diversity (richness and abundance) when compared with the rest of the intestinal tract communities (Figure 1b). Differences were driven by the dominance of members of the *Firmicutes* phylum (*Anaerobacter* and *Turcibacter*) in the ileum and various genera in the colon (*Prevotella*, *Oscillibacter* and *Succinivibrio*).

Bacterial communities of surfaces were different than those associated with the lumen, with

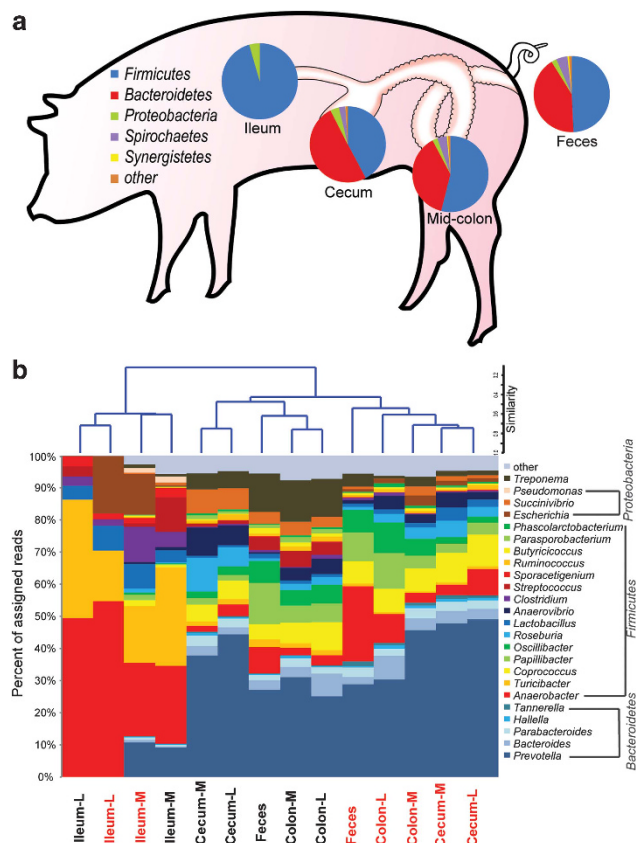


Figure 1 Swine intestinal bacterial communities differentiate along longitudinal and radial axes. **(a)** Phylum-level distribution of luminal bacteria through the intestinal tract. The mean per gut location is plotted. **(b)** Spatial distribution of bacteria in the swine intestinal tract based on assigned 16S rRNA sequences. The mean percent of assigned reads per treatment group is plotted. A dendrogram shows clustering of OTU data (Bray–Curtis dissimilarity measure), averaged by treatment and location. M, mucosa samples; L, lumen contents; red, medicated; black, non-medicated.

genus-level classifications revealing specific longitudinal versus radial differences (Figure 1b). Only the ileum revealed differences that were statistically significant between the mucosal luminal communities. One genus was found at a higher level in the lumen (*Anaerobacter*; $q=0.01$) than on the mucosa (Supplementary Table 2). Although most lumen-associated bacteria were also found on the mucosa, 33 genera were found in greater abundance or exclusively associated on the ileal mucosa (for example, *Prevotella*, *Pseudomonas*, *Campylobacter*, *Treponema*, *Coprococcus* and *Papillibacter*; $q<0.05$) (Supplementary Table 2).

The ileum in particular harbored striking differences between the bacterial community structure of the lumen and mucosa. Only 13 OTUs (standard error (s.e.)=1.5) were detected in the lumen compared to 299 OTUs (s.e.=38) associated with the host tissue at the same ileal location. The difference between the richness of the tissue and lumen communities at the other gut locations

was not significant, ranging from 580–968 OTUs (Supplementary Figure 1). Although the ileum had reduced diversity (Supplementary Table 3), the bacterial membership of the mucosa reflected that seen in the other intestinal sections (Figure 1b).

Bacterial physiological functions are specialized to gut locations

Forty-four metagenomes from the available gut contents of the twelve pigs (10 ileums, 12 ceca, 12 mid-colons and 10 feces) were sequenced, annotated with SEED subsystems and compared. The functional potential of the metagenomes, as annotated by SEED subsystems, corresponded to the abundance of bacterial phyla as detected by phylotype analysis (Supplementary Figure 2). Vectors for additional variables showed gut location to have the largest effect on functional membership, followed by antibiotic treatment, sibling status and gender (Supplementary Figure 2). This suggests that limited bacterial richness of the ileum relative to other intestinal locations is driving the functional differences. Indeed, more ileum lumen metagenomic sequences were annotated as eukaryota ($37 \pm 19\%$) and viruses ($0.67 \pm 0.77\%$) than in any of the other locations (remaining samples were $<1\%$ and $<0.05\%$, respectively) (Supplementary Figure 3). The SEED subsystem functional profiles of all non-medicated samples were compared in STAMP to distinguish location-specific functionality. Most of the significant differences could be attributed to the ileum, with some subsystems being exclusively present (for example, NADH peroxidase Npx) or absent (for example, cellobiose phosphorylase) in the ileum ($q<0.05$; Supplementary Table 4).

The lack of cellobiose phosphorylase homologs in the ileum suggested the differential carbohydrate utilization of discrete gut microbiotas, which was investigated further by analyzing the metagenomes against the carbohydrate-active enzyme database (CAZy; www.cazy.org). The precise substrate specificity of a gene assigned to a CAZy family cannot be readily predicted because CAZy families have long been found to group together enzymes of different substrate specificity. However, some CAZy families are strongly associated with the digestion of plant cell wall (PCW) components such as pectin (the most notable of these families include GH28, GH43, GH105, GH106, PL1 and PL11 (Cantarel *et al.*, 2012) or hemicellulose (exemplified by families GH10, GH26, GH43, GH115). Starch breakdown is catalyzed by other CAZy families, including GH13, GH77 and GT35. Our analysis revealed that unlike samples from the large intestine, the ileum is completely devoid of enzymes for the breakdown of pectin and hemicelluloses (Figure 2). By contrast, all sites investigated here encode starch-degrading enzymes (Figure 2) belonging to bacterial rather than eukaryotic families, supporting that the results are not due to the varying presence of swine DNA in the datasets.

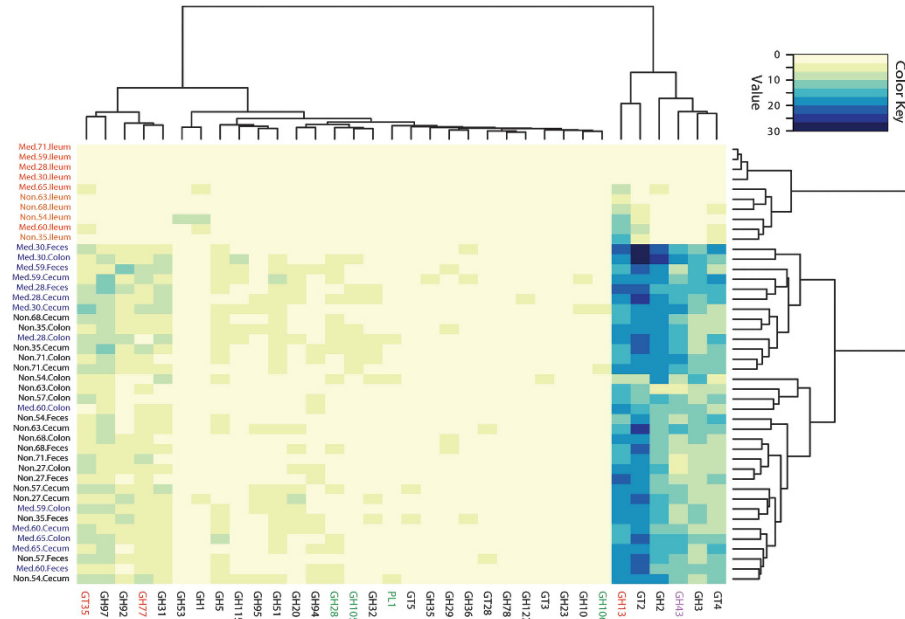


Figure 2 CAZy family abundances per sample. Heatmap depicts the relative abundances of several CAZy families (x-axis) by sample (y-axis). Sample names are coded by treatment status (Med, medicated; Non, non-medicated), pig number and sampling site. Relative abundance was calculated as the fraction of reads with statistically significant sequence similarity to sequences in a particular CAZy gene family, multiplied by a scaling factor (11 000). CAZy families are colored for functions highlighted in the results: starch breakdown (red), and the digestion of PCW components such as pectin (green) and hemicellulose (purple).

Certain differences between the functional potential of the ileum versus the other sampled gut locations suggested important ecological traits in the microbiome of the small intestine. The ileum showed increased abundance of some sporulation subsystems (Supplementary Table 4), and sporulation is a key aspect of bacterial dormancy. Detection of dormancy traits in the ileal microbiota could be an indication of survival in a hostile environment, either from passage through the upper intestinal tract, existing in the ileum habitat with intermittent food boluses, or enduring the downstream and external environments. Further examination of all 'spore' and 'sporulation' genes from the SEED subsystem annotation of the metagenomes revealed that the ileum harbored significantly more dormancy-encoding genes than most of the large intestinal samples and all of the medicated samples (Figure 3). The ileum is additionally enriched in numerous bacterial ABC transporters for monosaccharide and amino-acid uptake, and in bacterial carbohydrate transport phosphotransferase systems (Supplementary Table 4). Taken together, these results suggest characteristics that are important for bacterial fitness and community ecology in the ileal habitat.

Dynamic phages in the swine gut ecosystem

Phage-encoding subsystems were analyzed as both the taxonomic (Supplementary Figure 3) and statistical (Supplementary Table 4) analyses suggested their elevated abundance in the ileum. As with

dormancy, increased abundance of subsystems annotated with 'phage' were detected in the ileum (Figure 3). Metagenomes from the ileums of the non-medicated pigs ($n = 4$) were assembled, yielding two phage-related contigs of greater than 10 Kb in length and $\times 10$ sequencing coverage. The 12-Kb contig contains 16 putative open-reading frames, many of which have full-length homologs in Gram-positive gut bacteria (for example, *Clostridium* and *Lactobacillus* sp.; Supplementary Figure 4). The other phage-like contig is nearly 21 Kb with a mere seven open-reading frames, only one of which has a full-length homolog (34% amino-acid identity to a metallophosphoesterase in *Bacillus* phage G; Supplementary Figure 4). A putative Gram-positive host for these phages is consistent with the 16S results. Ileal metagenomes from each of the four pigs contributed sequences to these contigs, with three of the four pigs contributing nearly equal numbers of sequences, demonstrating that these phages might be consistent members of this environment. In addition, the assembly of these partial phage genomes from whole-cell metagenomes (rather than viral metagenomes) suggests that these phages are actively replicating in the ileum. To examine the ubiquity of the ileal phages, we mined fecal phage metagenomes that were previously prepared from these pigs and 12 others in a time-course aspect of this study (Allen *et al.*, 2011). Hundreds of fecal phage sequences mapped onto the two ileal phage genomes (511 reads mapped to the 21-Kb contig, and 262 reads mapped to the 12-Kb contig), suggesting continuity of these phages across swine gut samples

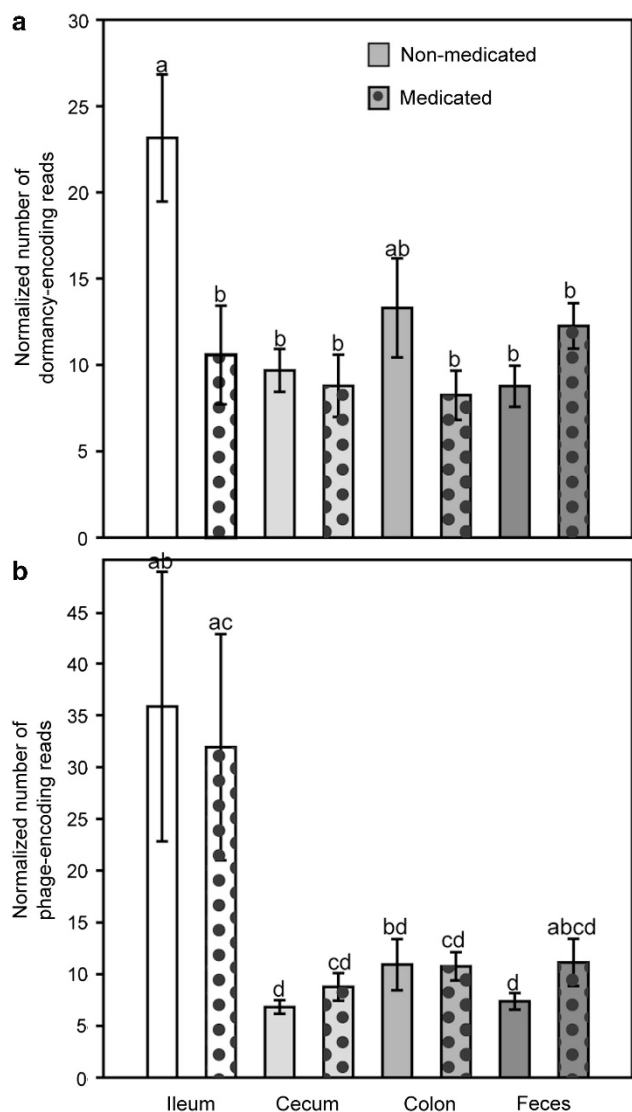


Figure 3 Relative abundance of dormancy (a) and phage (b) functional genes in swine gut metagenomes. (a) Biological replicates are plotted showing the standard error (s.e.) around the mean. The results of multiple pairwise comparisons (Mann-Whitney *t*-test) are shown, with *a* being significantly different from *b*, *b* from *c*, and so on, at $P < 0.05$.

and over time. In contrast, virtually no sequences belonging to these phage contigs were detected in the ASP250-treated ileal metagenomes (only five reads greater than 100 bp), indicating that ASP250 treatment greatly reduced these phages in the ileal microbiota.

The effect of ASP250 on bacterial community structure and function

Many changes that were detected in the membership and functions of the bacterial communities with antibiotic treatment reflected those seen in our previous study (Looft *et al.*, 2012), with the added resolution of different intestinal locations. Analysis of the community structure at the 0.03 OTU level

indicated divergence of the medicated microbiota from the non-medicated microbiota ($P < 0.01$, $R = 0.21$) (Figure 4). Several non-abundant phyla decreased after exposure to antibiotics and one (*Tenericutes*) increased in the cecum after treatment ($q < 0.01$). There were many gut compartment-specific effects with exposure to antibiotics (Figure 5, Supplementary Table 1), including *Streptococcus* decreasing in the ileum, cecum and colon ($q < 0.1$), and *Treponema* decreasing in the cecum and colon after ASP250 treatment ($q < 0.04$). Certain genera increased with antibiotic treatment, such as *Escherichia*, *Lachnobacterium* and *Salsuginibacillus* spp. in the cecum and colon ($q < 0.04$) (Figure 5, Supplementary Table 1). Some antibiotic effects were specific to one gut location, such as reduced *Helicobacter* spp. in the cecum ($q = 0.03$), reduced *Turicibacter* in the colon ($q = 0.04$) and increased *Anaeroplasma* and *Paraprevotella* spp. in the cecum ($q = 0.03$).

Comparison of the effect of in-feed antibiotics on functions encoded by the metagenomes confirmed our findings (Looft *et al.*, 2012) and revealed new information. SEED subsystems were grouped and analyzed at classification level 2 because analysis at level 4 (the finest resolution) showed no significant differences across all samples after multiple comparison correction. Twelve SEED subsystems were differentially abundant with antibiotic treatment ($q < 0.01$; Table 1). Many of these categories are likely attributed to increased *E. coli* abundance (for example, Gram-negative cell wall components and unknown carbohydrate utilization (cluster yeg)) and confirm our previous results based on analyses of clusters of orthologous groups (Looft *et al.*, 2012). Location-specific treatment analyses showed trends, such as a decrease in the sporulation-associated protein category in the ileum and an increase in the aromatic amino-acid category in the cecum (Table 2). Genes encoding the muconate and lactonizing enzyme family increased in two locations (cecum and colon) with ASP250 treatment. Enzymes in this family participate in aromatic-acid catabolism and are found in Proteobacteria and Actinobacteria. Importantly, the feces alone did not report the sum of the antibiotic effects on the upstream gut locations, showing increased abundance of two SEED subsystem and two categories (Nitrate and nitrite ammonification, and Quorum sensing and biofilm formation, respectively) that were not differentially detected elsewhere.

Antibiotic resistance genes were specifically annotated using the ARDB (Liu and Pop, 2009). Diverse and abundant antibiotic resistance genes were detected in both medicated and non-medicated swine metagenomes, with 213 different resistance genes detected across all metagenomes. Among the antibiotic resistance genes differentially detected with ASP250 treatment, 12 were higher in the medicated metagenomes and eight were higher in the non-medicated metagenomes (Supplementary Table 5) ($P < 0.05$). Some of these confirm previous findings

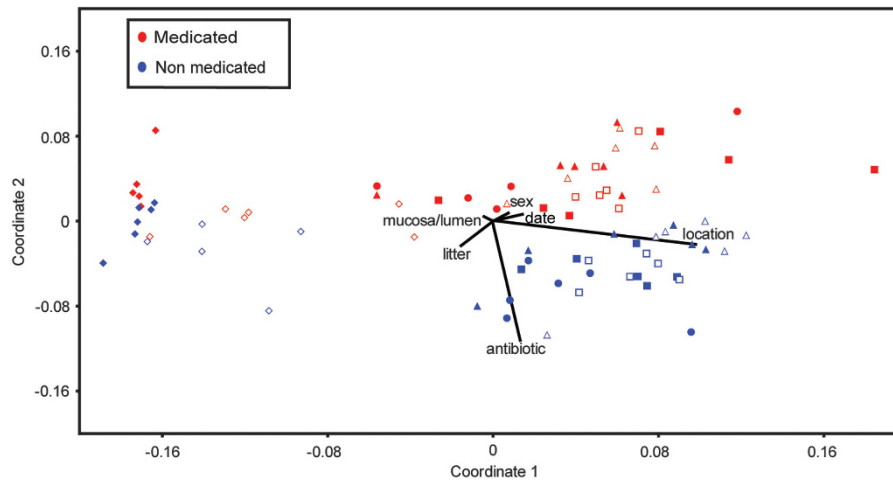


Figure 4 Nonmetric multidimensional scaling analysis of OTU-based bacterial 16S rRNA gene sequence abundances in individual pig intestinal samples. Environmental variables are plotted as vectors. The length of each vector is arbitrarily scaled, so only their directions and relative lengths should be considered. Lumen samples are solid and mucosal samples are outlined. Diamond, ileum; triangle, cecum; square, colon; and circle, feces.

(for example, aminoglycoside O-phosphotransferases) whereas others are newly resolved (for example, AmpC β -lactamases) as increasing in abundance with ASP250 treatment. Many of the swine metagenomic resistance genes have been reported to reside on mobile genetic elements (Supplementary Table 5).

Discussion

Previous research has analyzed the culturable members of various gut sections (Allison *et al.*, 1979), the as-yet-uncultured members (Leser *et al.*, 2002) or both (Pryde *et al.*, 1999), but this is the first study to apply 16S phylotyping with metagenomics to capture a broad view of swine gut microbial ecology. We also analyzed the disturbance of this microbiota by an in-feed antibiotic cocktail, ASP250, which is a continuation of our previous work describing the effect of these antibiotics on the swine fecal microbiota (Looft *et al.*, 2012). For practical reasons, feces are usually the samples of choice to study intestinal microbial communities. However, studying the bacteria at discrete gut locations improves the power of making inferences about coevolved bacterial partners in a complex mammalian ecosystem. Our results show that feces alone are not informative of the ecology of specific intestinal habitats, and location-specific differences possibly reveal microbial targets that could be manipulated to influence gut health.

Our hypothesis was that the mucosa would harbor bacteria that are specialized to thrive in the mucosal environment, and so its microbial community would be less diverse than that in the lumen. Surprisingly, community structure analyses revealed high bacterial richness at all mucosal sites. In fact, the distal small intestine (ileum) harbored a greater richness of

bacteria on the mucosa compared with the lumen. (Savage, 1987) proposed that the mucosal bacterial populations in the gut both stabilize the intestinal community and prevent pathogens by providing a constant, established inoculum for the lumen to complete the breakdown of non-digested food substrates (Williams *et al.*, 2011). Perhaps the mucosal bacteria of the ileum serve as an inoculum for the large intestine. In contrast to the ileum, the mucosal and luminal microbiotas of the large intestine contained similar genera with similar community structures, suggesting that occupation of the mucosa is a trait shared by most large intestinal microbes. Recent studies have highlighted the importance of several beneficial and mucosa-associated bacteria in swine, such as butyrate-producing and mucin-degrading bacteria (Looft *et al.*, 2012; Levine *et al.*, 2013). Investigations of mucosal microbial ecology upstream of the ileum combined with physiological characterizations of mucosal commensals will further inform Savage's hypothesis and our understanding of the gut ecosystem.

Many of the mucosal colonizers are not dominant members of the gut community, but rather are part of a milieu of specialized intestinal bacteria that have adapted to colonize the mucosa. *Pseudomonas* spp., for example, are well adapted to colonize the mucosa (Tsang *et al.*, 1994; Aristoteli and Willcox, 2003) and have been identified in human intestinal and mucosal studies (Aristoteli and Willcox, 2003; Shinohara *et al.*, 2010). Many of the other bacteria enriched on the mucosa, such as *Campylobacter* spp., *Weissella* spp, and *Sutterella* spp., are known mucosal colonizers or pathogens and are likely mucosal specialists (On, 1996). These genera may be broadly specific to mucosal surfaces across mammals, having been identified on the mucosal surfaces of multiple host species (On, 1996; Heilig *et al.*, 2002; Green *et al.*, 2006).

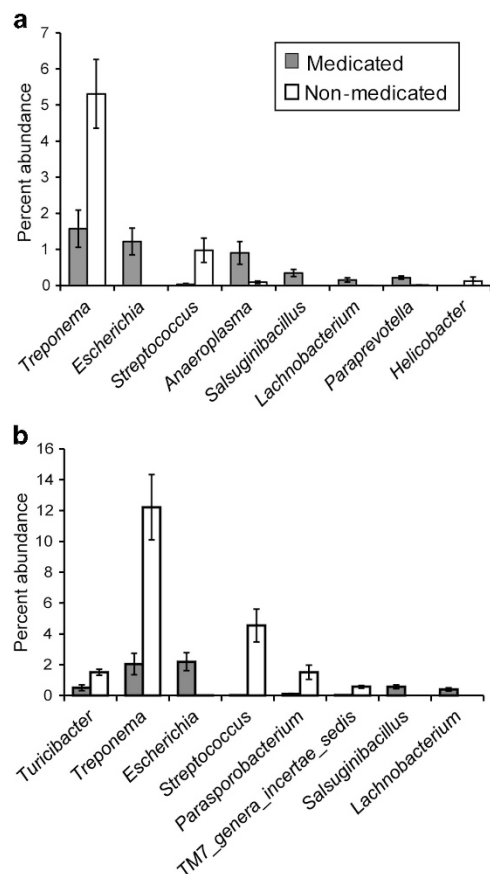


Figure 5 Bacterial genera that are differentially present due to antibiotic treatment, based on taxonomic inference of bacteria (16S rRNA sequences) from (a) cecal and (b) colonic intestinal samples ($P < 0.01$, $q < 0.05$). *Acholeplasma* and *Holdemania* (higher in medicated cecum); *Rhodopirellula* and *Hydrogenoanaerobacterium* (higher in non-medicated colon) was not shown because of low abundance.

The predominance of two genera in the ileum, *Turicibacter* and *Anerobacter*, was striking when compared with the large intestine. Both genera are spore formers and likely aerotolerant (Siunov *et al.*, 1999; Cuiv *et al.*, 2011). This is supported by the observation of the ileum harboring more dormancy-encoding genes than other gut compartments. The digesta passes quickly through the small intestines and is perhaps a harsh environment for commensal organisms. With oxygen levels in the ileum reaching 5% (Hillman *et al.*, 1993), dormant, aerotolerant organisms would be well adapted to surviving this environment.

Analyzing the luminal metagenomes for function revealed numerous traits that contribute to bacterial fitness in the discrete environments of the small and large intestines of swine. These intestinal regions have considerably different physiological functions (mechanical and chemical digestion and nutrient absorption in the former, water absorption and microbial fermentation in the latter), and their respective microbiotas reflect these differences. Notably, the ileal metagenome lacked the ability to

degrade PCW components. This function is frequently encoded by *Bacteroidetes*, a phylum that was essentially absent from the ileal lumen, but represented 50% of the luminal community in the cecum and colon. Pigs are hindgut fermenters and rely on these beneficial microbes in their large intestine to ferment complex carbohydrates that remain in the food to extract more energy. The lack of PCW degradation was complemented by enrichment for carbohydrate transport phosphotransferase systems in the ileum, as has been shown in human ileostomy samples (Zoetendal *et al.*, 2012). Comparative genome analyses of human intestinal *Firmicutes* versus *Bacteroidetes* also showed carbohydrate transport phosphotransferase systems system and ABC transporter enrichment in *Firmicutes*, and PCW-degradation enzyme enrichment in *Bacteroidetes* (Mahowald *et al.*, 2009). Our data connect these functional traits of bacterial phyla to environmental fitness—ileal *Firmicutes* lack microbial partners to degrade complex molecules, but the host's small intestinal effort releases small polysaccharides, proteins and fats. The ileal metagenomes encode the machinery to take up simple molecules, exhibiting the nutritional fitness of the *Firmicutes*-dominated microbiota in this environment.

Interestingly, the ileal metagenomes showed enrichment for phage-related genes compared with the other gut locations. Phages have an important role in the gut ecosystem as evidenced by the diversity of phages in swine fecal viromes (Allen *et al.*, 2011), phage ubiquity across more than 100 human fecal metagenomes (Qin *et al.*, 2010), and their enrichment in human ileostomy samples compared with fecal metagenomes (Zoetendal *et al.*, 2012). The assembly of two partial phage genomes from the bacterial metagenomes of the ileum suggests that they are present in greater copy number than that expected for lysogens. The mapping of fecal virome sequences from these pigs and others over time suggests that they are consistent members of the swine gut ecosystem. Like the explanation for differential substrate utilization in different gut compartments, perhaps one explanation for the greater proportion of phages in the ileal metagenomes compared with those of the large intestine is connected to gut physiology: the ileal microbiota could be subject to cyclic feast or famine conditions, and due to the role of phages in nutrient release (Abedon, 2009), there could be a fitness cost of phage resistance in ileal bacteria. In addition, pseudolysogeny is an unstable state of phage replication that could promote viral survival in nutrient-limited environments (Ripp and Miller, 1997) and yield-increased copies of stalled-phage genomes in bacterial cells. Studies of phage ecology in a host-associated environment of limited bacterial richness, such as the ileum, will inform the complex inter-kingdom dynamics in host-associated environments.

Disturbing the swine gut microbiome with continuous ASP250 for 2 weeks caused many noteworthy

Table 1 Mean relative frequency (%) of metagenomic functions differentially represented with ASP250 antibiotic treatment ($q < 0.01$)

<i>SEED level 2 subsystem</i>	<i>Non-medicated</i>	<i>Medicated</i>
<i>Of greater abundance in metagenomes of medicated swine</i>		
Gram-negative cell wall components	0.638591	1.205382
Nitrate and nitrite ammonification	0.022103	0.215753
Protein secretion system, Type VII (Chaperone/Usher pathway, CU)	0.004336	0.096187
DNA-binding regulatory proteins, strays	0.010018	0.089637
Glutathione-regulated potassium-efflux system and associated functions	0.009764	0.085247
Unknown carbohydrate utilization (cluster Yeg)	0.002651	0.054405
Uncharacterized sugar kinase cluster (ygc)	0.000165	0.053897
MukBEF chromosome condensation	0.000268	0.035143
Dessication stress	2.83E-05	0.033518
Protein of unknown function (CBSS-214092.1.peg.3450)	0	0.015746
Bacteriophage P4 cluster	0	0.016394
<i>Of greater abundance in metagenomes of non-medicated swine</i>		
Protein biosynthesis	7.332998	5.600991

Table 2 Trending differences ($P < 0.01$, $q > 0.1$) between medicated and non-medicated metagenomes annotated by SEED subsystem classification level 2 in specific swine gut locations

<i>SEED subsystem (level 2)</i>	<i>Mean relative frequency (%)</i>	
	<i>Medicated</i>	<i>Non-medicated</i>
<i>Ileum</i>		
DNA uptake, competence	0.15	0.40
Transport of iron	0.14	0.09
Heme, hemin uptake and utilization systems in Gram positives	0.07	0.25
CBSS-160492.1.peg.550	0.06	0.02
Sporulation-associated proteins with broader functions	0.02	0.08
<i>Cecum</i>		
Aromatic amino acids and derivatives	1.61	1.28
Heat shock	0.34	0.52
Pyridoxine	0.27	0.40
Muconate lactonizing enzyme family	0.02	0.01
<i>Colon</i>		
Protein biosynthesis	5.48	7.53
CBSS-269801.1.peg.1715	0.02	0.06
Muconate lactonizing enzyme family	0.02	0.01
<i>Feces</i>		
Quorum sensing and biofilm formation	0.15	0.08
Nitrate and nitrite ammonification	0.04	0.01

changes in bacterial membership and functions. *Lachnobacterium* spp. increased in both the lumen and mucosa with antibiotics and could represent a beneficial effect of ASP250. Members of this genus are known to produce bacteriocins (antimicrobial compounds) and butyrate, are associated with the colonic mucosa of mice (Nava *et al.*, 2011), and have been considered as a potential direct-fed microbial (that is, probiotic) in livestock production (McAllister *et al.*, 2011). In contrast, a collateral effect of ASP250 was on *E. coli*, populations of which are known to increase with ASP250 and certain other

gut disturbances (Allen *et al.*, 2011; Looft *et al.*, 2012). Examination of various gut locations further resolved this phenomenon, showing a dramatic increase in *E. coli* abundance in the ileum (lumen and mucosa) relative to other gut locations and feces. Many of the antibiotic-induced functional changes observed in the metagenomes are attributable to *E. coli*. Others are relevant to the function of specific compartments, such as the relative increase of genes encoding the aromatic amino acids and derivatives subsystem in the cecum with ASP250 treatment. These seemingly relevant changes in genetic potential are noted, and the effect of this changing genetic potential on gene expression (metatranscriptomics) is an active area of research in our lab.

Analysis of antibiotic resistance genes revealed both expected and unexpected results. Penicillin is part of the ASP250 cocktail, and the direct selection for a penicillin resistance gene (β -lactamase) is an expected outcome that was detected in this study and previously by qPCR (Looft *et al.*, 2012). In contrast, aminoglycoside O-phosphotransferases consistently show increased abundance with ASP250 treatment, in the present as well as previous studies (Rosengren *et al.*, 2007; Looft *et al.*, 2012), despite that this class of resistance gene confers resistance to an antibiotic that is not administered in ASP250. These results suggest that increased abundance of this resistance gene is due to either co-selection with another resistance gene or to an increase in the abundance of the bacteria harboring this gene. Our two studies were conducted 3 years apart and on pigs from different farms, thereby emphasizing the ubiquity of this resistance gene in the swine intestinal ecosystem.

Conclusions

Part of discovering and implementing alternatives to in-feed antibiotics is understanding the effects of antibiotics on the membership and functions of the gut microbiota. We defined the spatial distribution

of bacterial communities in the mucosa and lumen of the intestinal tract, and identified numerous effects of antibiotics on bacterial membership and functions. Future work should move beyond genetic changes and toward effects on gene expression or indeed on proteins and metabolites. In addition, systems biology will be essential to integrate microbial changes with host alterations. The controversy over the use of antibiotics in agriculture is likely to continue, but understanding how antibiotics affect intestinal bacterial communities, both along the mucosa and in the lumen, may inform alternative strategies that preserve the performance benefits while reducing potential risks to human and animal health.

Conflict of Interest

The authors declare no conflict of interest.

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