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Antimicrobial use in swine production and its effect on the swine gut microbiota and antimicrobial resistance

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Abstract

Antimicrobials have been used in swine production at subtherapeutic levels since the early 1950s to increase feed efficiency and promote growth. In North America, a number of antimicrobials are available for use in swine. However, the continuous administration of subtherapeutic, low concentrations of antimicrobials to pigs also provides selective pressure for antimicrobial-resistant bacteria and resistance determinants. For this reason, subtherapeutic antimicrobial use in livestock remains a source of controversy and concern. The swine gut microbiota demonstrates a number of changes in response to antimicrobial administration depending on the dosage, duration of treatment, age of the pigs, and gut location that is sampled. Both culture-independent and dependent studies have also shown that the swine gut microbiota contains a large number of antimicrobial resistance determinants even in the absence of antimicrobial exposure. Heavy metals, such as zinc and copper, which are often added at relatively high doses to swine feed, may also play a role in maintaining antimicrobial resistance and on the stability of the swine gut microbiota. This review focuses on the use of antimicrobials in swine production, with an emphasis on the North American regulatory context, and their effect on the swine gut microbiota and on antimicrobial resistance determinants in the gut microbiota.

Key Words: swine production, antimicrobials, gut microbiota, phylogenetic biodiversity, antimicrobial resistance

Introduction

Pork remains the most widely consumed meat with an estimated 114.5 million tonnes of pork eaten worldwide in 2013 (Food and Agriculture Organization 2014). Globally, swine (*Sus scrofa domesticus*) production has steadily increased over the past 50 years with a total inventory of 941 million pigs and over 106 million kg of pig meat produced in 2009 (Food and Agriculture Organization 2012), totals higher than those for all other meat sources. Modern swine operations are much larger with increasingly greater numbers of pigs raised per farm (United States Department of Agriculture 2008). Antimicrobials have been used for over 60 years at all stages of swine production (Jukes et al. 1950) and larger swine operations are more likely to use antimicrobials for disease prevention and growth promotion (MacDonald and McBride 2009).

The four main reasons for antimicrobial administration are: 1) the treatment of digestive, skin, respiratory, and other infections, 2) as a prophylactic measure to prevent infectious disease at specific stages of swine rearing, for example to prevent diarrhea at weaning, 3) as a metaphylactic treatment to treat large numbers of animals when disease is observed in a proportion of the herd, and 4) as growth promoters to stimulate growth rates and improve feed conversion efficiency (Viola and DeVincent 2006). Antimicrobials administered for the purpose of growth promotion are given to all animals at subtherapeutic levels while individual or small numbers of pigs (prophylaxis) or the entire herd (metaphylaxis) may be treated with higher levels of antimicrobials (therapeutic) to treat infectious disease (McEwen and Fedorka-Cray 2002). Subtherapeutic is defined as a dosage that is lower than what would be required for disease treatment (therapeutic) (Allen et al. 2013). The administration route of antimicrobials is usually via feed, although antimicrobials may also be given through intramuscular injection or in drinking water (Public Health Agency of Canada 2014).

Worldwide, it is estimated that greater than 50% of antimicrobials produced are consumed by food-producing animals (World Health Organization 2012). China, the world's biggest swine-producing country (Food and Agriculture Organization 2012), is also the largest consumer of antimicrobials in livestock, with nearly 100 million kg added to feed each year (Hvistendahl 2012). In the United States, over 50% of all antimicrobials sold (excluding ionophores) are used in food-producing animals (United States Food and Drug Administration 2011) and over 90% of nursery and grower/finishing pigs receive antimicrobials in their feed at some point (United States Department of Agriculture 2007). In Canada, over 85% of all antimicrobials sold (excluding ionophores) are used in food-producing animals and 90 to 95% of pigs are exposed to antimicrobials during production (Deckert et al. 2010). The objective of this review is to summarize the use of antimicrobials in swine production, with an emphasis on the North American industry, and to evaluate the effect these antimicrobials have on the cultivable and uncultivable bacteria in the swine gut microbiota and on antimicrobial resistance determinants in the gut microbiota. The literature search was conducted from January to March 2015 using Google Scholar (scholar.google.ca), PubMed (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>), and Scopus (<http://www.scopus.com>). Studies that investigated the effect of antimicrobials on the swine gut microbiota and/or on antimicrobial resistance in commensal bacteria were included. Search terms included combinations of: antibiotics OR antimicrobials, swine OR pigs, microbiota OR microbiome, resistance OR resistant, anaerobic bacteria OR commensal bacteria.

Therapeutic use of antimicrobials

Modern animal husbandry often involves having a large number of animals kept in close proximity with one another. As a result, it provides conditions that are optimal for the spread of

infectious disease. These conditions can necessitate the use of antimicrobials to control disease and prevent disease in other animals, i.e. metaphylaxis (Fluit et al. 2006). The duration of antimicrobial disease treatment in swine typically ranges from 20-40 days (Stone et al. 2009). The treatment of individual pigs via intramuscular injection is generally only used in cases of acute, severe infection (Friendship and Prescott 2006). For prophylactic and metaphylactic indications in Canadian swine herds, 10 antimicrobial products may be administered to pigs at concentrations between 13.8 and 400 mg kg⁻¹ feed for either a specific duration (two to six weeks) or for an undefined period of time (generally in weeks; Canadian Food Inspection Agency 2015). For a specific type of illness, pigs can be treated with one of eight antimicrobial agents at concentrations of 27.5 to 500 mg kg⁻¹ feed for a period of five days to seven weeks (Canadian Food Inspection Agency 2015).

Subtherapeutic use of antimicrobials

Subtherapeutic use of antimicrobials in particular remains a source of controversy since its purpose is usually one that is unrelated to disease treatment and instead focused on the growth performance of the animal. Due to the fact that the subtherapeutic use of antimicrobials usually results in the long-term, continuous exposure of large numbers of pigs to antimicrobials, it plays a larger role in the persistence and dissemination of antimicrobial resistance than therapeutic use, which is much shorter in duration, at higher doses, and usually involves fewer pigs being treated (Marshall and Levy 2011). This effect has been observed in pigs where swine farms using subtherapeutic antimicrobials had higher levels of antimicrobial-resistant Gram-negative fecal bacteria than swine farms where antimicrobials were only used for therapeutic purposes (Gellin et al. 1989).

The mechanism(s) responsible for the growth-promoting effects of in-feed antimicrobials remains poorly characterized but several potential modes of action have been proposed. These include: 1) reduction of harmful metabolites produced by intestinal bacteria, 2) increased availability of nutrients to the pig that would otherwise be consumed by the gut microbiota, 3) increased absorption of dietary nutrients due to a thinner intestinal wall, and 4) control of endemic subclinical disease (Butaye et al. 2003; Cromwell 2002; Dibner and Richards 2005). This last mechanism, the control of non-specific subclinical disease through antimicrobial administration, is generally the most popular explanation. This is supported by the fact that herds with a higher disease load and those raised in unsanitary conditions respond better to antimicrobials used for growth promotion than do healthy pigs in clean environments (Jin et al. 2008; Holman and Chénier 2013). It is notable that antimicrobials also tend to be more efficacious in terms of growth promotion in younger pigs, which are more susceptible to disease, than in older pigs (Bosi et al. 2011; Cromwell 2002). As such, in-feed antimicrobials for the purpose of growth promotion are most effective in the nursery phase with little to no response seen in older pigs (Holt et al. 2011; Skinner et al. 2014). Alternatively, it has also been proposed that antimicrobials have anti-inflammatory effects that are independent of any interaction with the gut microbiota (Niewold 2007).

The most commonly used antimicrobials for growth promotion in North American swine operations are tylosin, chlortetracycline, bacitracin, and lincomycin (Deckert et al. 2010; United States Department of Agriculture 2007). The average duration of treatment with these antimicrobials for any purpose in growing-finishing pigs ranges from 22.7 to 76.8 days, with the entire herd being treated at the same time (United States Department of Agriculture 2007). In Canada, nine antimicrobial products may be added to feed at concentrations between 4.4 and 110

mg⁻¹ kg feed to improve growth, usually for an undefined duration (weeks to months) during the starting, growing or finishing periods, or during periods of stress (Canadian Food Inspection Agency 2015).

Veterinary and public health concerns

Antimicrobial resistance has been referred to as one of the greatest threats to public health (Marshall and Levy 2011; World Health Organization 2014). Antimicrobial-resistant infections increase morbidity and mortality, as well as the costs associated with treating these infections (Mauldin et al. 2010; Roberts et al. 2009). Meanwhile, the situation is exacerbated by the lack of new antimicrobial drugs in the discovery pipeline and clinical setting (Livermore et al. 2011; Piddock 2012). The use of antimicrobial agents selects for both antimicrobial-resistant bacteria and antimicrobial resistance determinants (Levy and Marshall 2004). In swine production, associations between antimicrobial use and antimicrobial resistance in swine have been well documented (Akwar et al. 2008; Dunlop et al. 1998; Holman and Chénier 2013; Rollo et al. 2010; Rosengren et al. 2007). Resistant bacteria, both pathogenic and commensal, may be passed to humans through food, direct contact with animals, or release of animal waste into the environment (Silbergeld et al. 2008). Importantly, antimicrobial resistance determinants often reside on mobile genetic elements (MGE) such as plasmids, transposons, insertion sequences, integrons, and bacteriophages (reviewed in Frost et al. 2005). These MGEs can be transferred and exchanged among bacteria via horizontal gene transfer processes such as cell-to cell conjugation, bacteriophage-mediated transduction, and transformation (reviewed in Thomas and Nielsen 2005).

The threat to human health posed by the agricultural use of antimicrobials has been a source of concern dating back over 45 years when the Swann report in the UK first

recommended that antibiotics added in animal feed should be of no therapeutic value in human medicine (Swann et al. 1969). In swine, resistance to tetracyclines, macrolides, lincosamides, and bacitracin has been observed most frequently, and not surprisingly these antimicrobials are used most often in swine production (Public Health Agency of Canada 2014; Frye et al. 2011). The World Health Organization (2011) has classified macrolides (tylosin), tetracyclines (chlortetracycline), and lincomycin (lincosamides) as highly important or critically important for human medicine. As a result, the use of these classes of drugs in swine can potentially provide selective pressure for the maintenance or emergence of resistant bacteria in humans (Marshall and Levy 2011).

Although making a direct link between antimicrobial use in swine production and antimicrobial-resistant infections in the human population is challenging, some studies have demonstrated such a link. For example, outbreaks involving strains of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 in Denmark (Mølbak et al. 1999), *S. enterica* serotype Typhimurium DT104 in England (Maguire et al. 1993), and *Enterococcus faecium* in China (Lu et al. 2002) originated from swine herds.

Since 2006, the European Union has banned the use of antibiotics in food-producing animals for the purpose of growth promotion (European Union 2005). In both Canada and the United States, the government has recently implemented plans for a voluntary reduction in antimicrobial use for the purpose of growth promotion in food-producing animals over an expected three-year period (Health Canada 2014; United States Food and Drug Administration 2013). In the US, this is also intended to put in-feed antimicrobials under the veterinary feed directive. Until these changes, however, a veterinarian's prescription was not generally required to purchase antimicrobials for use in swine in most areas of North America.

Heavy metals

Other agents with potential antimicrobial activity are also included in feeds given to swine. In particular, heavy metals, namely zinc and copper, are frequently added to swine feeds at levels (Cu: 10 to 250 mg kg⁻¹ feed, Zn: 125 to 3000 mg kg⁻¹ feed) which exceed dietary requirements (Cu: 5 to 10 mg kg⁻¹ feed; Zn: 50 to 125 mg kg⁻¹ feed; Jacela et al. 2010; Jondreville et al. 2003). While zinc and copper are essential dietary elements that are required by swine for a number of metabolic functions, higher concentrations are added to feed for their ability to promote growth and control colibacillosis (Fairbrother et al. 2005; Hojberg et al. 2005).

Not only do zinc and copper mimic some of the growth promoting and disease controlling effects of antibiotics, but they can also increase the abundance of antibiotic-resistance determinants and resistant bacteria in the swine gut (Bednorz et al. 2013; Hölzel et al. 2012). The reason for this phenomenon may be due to co-selection and co-resistance where antibiotic resistance determinants are located on the same mobile genetic element as heavy metals resistance genes (Baker-Austin et al. 2006; Frye et al. 2011). Cross-resistance, in the form of efflux pumps that can export structurally different molecules from the bacterial cell may also be a factor (Baker-Austin et al. 2006). Therefore, the extensive use of zinc and copper in swine feeds may also help explain why antimicrobial resistance persists even in the absence of antimicrobial exposure (Mathew et al. 2001; Pakpour et al. 2012; Stanton et al. 2011). An association between the isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) in swine and the use of relatively high levels of zinc in swine diets has also been recently observed (Amachawadi et al. 2015; Slifierz et al. 2015).

Swine gut microbiota

The mammalian gut is home to approximately 10^{14} bacterial cells and 500-1000 species of bacteria, the majority of which are anaerobic and not easily cultured in the laboratory (Lamendella et al. 2011; Sommer and Bäckhed 2013). It is difficult to reproduce not only the environmental conditions but also the symbiotic relationships among microbes found in the gut (Nocker et al. 2007). As a result, perhaps as little as 1% of intestinal bacteria are cultivable (Dowd et al. 2008). All bacteria found in the swine intestinal tract, however, are potentially affected upon consumption of an antimicrobial agent. Antimicrobial growth promoters in particular, tend not to be well absorbed in the gut and can act directly on the microbiota, mainly on Gram-positive bacteria (Erik and Knudsen 2001).

The gastrointestinal tract of the pig contains a large and diverse number of microorganisms, which are collectively termed the gut microbiota when referring solely to microorganisms, or the gut microbiome when referring to the genomes of microorganisms in this location, although sometimes these terms are used interchangeably (Lamendella et al. 2011; Turnbaugh et al. 2007). The swine intestinal microbiota is not homogenous; rather it differs based on the sample location, with significantly more microbial diversity and higher concentrations of bacteria in the colon (10^{10} to 10^{12} CFU g⁻¹ digesta) compared to the ileum (10^8 to 10^9 CFU g⁻¹ digesta), and in the ileum compared to the stomach and proximal small intestine (10^3 to 10^5 CFU g⁻¹ digesta) (Dibner and Richards 2005; Leser et al. 2000).

The gastrointestinal tract of swine is generally thought to be sterile prior to birth, but it rapidly becomes colonized with bacteria from the environment, diet, and sow (Dibner and Richards 2005; Kim and Isaacson 2015). The age of the pigs is also an important factor, as nursing piglets have a very different gut microbiota from post-weaned pigs (Holman and Chénier 2014; Mach et al. 2015). For example, the phylum *Proteobacteria* is more abundant prior to

weaning (Holman and Chénier 2014; Zhao et al. 2015). The differences in the gut microbiota of pre- vs. post-weaned piglets are driven largely by the shift in diet from an easily digestible sow's milk to grain-based feed that occurs at weaning. The immune system of the pig is also relatively immature at this time which may allow for the colonization of pathogenic bacteria (Lalles et al. 2007). Over time, the swine gut microbiota becomes increasingly more stable and as a result the gut microbiota becomes more resistant to dietary perturbations (Holman and Chénier 2014; Kim et al. 2012). The texture of the feed and granulometry (Mølbak et al. 2008), as well as the sanitary conditions of the housing environment can also alter the gut microbiota, with pigs raised under poor sanitation having been shown to shed more *Lactobacillus* (increase of 0.9 log₁₀ CFU g⁻¹ feces) and *Enterobacteriaceae* (increase of 1.0 log₁₀ CFU g⁻¹ feces) in their feces (Montagne et al. 2010).

The gut microbiota is a vital component of a healthy animal. Bacteria in the colon metabolize undigested carbohydrates into short-chain fatty acids which are then absorbed by the host. In this way, intestinal bacteria help recover nutrients which would otherwise be lost through excretion (Cummings and Macfarlane 1997; Laparra and Sanz 2010). Commensal gut microorganisms also provide resistance against the colonization of pathogens through competition for nutrients and binding sites on the host intestinal epithelium, as well as altering the local intestinal environment with the production of volatile fatty acids, modified bile acids and antimicrobial compounds (Allen and Stanton 2014; Gaskins 2001; Lalles et al. 2007). The host immune system largely tolerates these commensal gut bacteria which also help stimulate normal immunological development and homeostasis (Kelly et al. 2005; Sommer and Bäckhed 2013; Zoetendal et al. 2004).

The mammalian gut microbiota is comprised largely of *Bacteria*, but *Archaea*, *Protozoa*, viruses, and fungi are also present (Leser and Mølbak 2009; Sommer and Bäckhed 2013). The majority of the swine gut microbiota is comprised of relatively few different phyla, namely the *Bacteroidetes* and *Firmicutes* (Holman and Chénier 2014; Kim et al. 2011). At the genus level, *Prevotella*, *Lactobacillus*, *Treponema*, *Roseburia*, and *Streptococcus* are among the most abundant genera (Holman and Chénier 2014; Kim et al. 2011; Lamendella et al. 2011; Park et al. 2014). In the swine ileum, the microbiota is dominated almost entirely by *Firmicutes* at the phylum level and *Anaerobacter*, *Turicibacter*, and *Escherichia* at the genus level (Looft et al. 2014a).

Effect of antimicrobials on antimicrobial resistance in the cultivable swine gut microbiota

Traditionally, the effect of antimicrobials on the emergence of antimicrobial-resistant bacterial strains has focused on pathogenic organisms such as *Campylobacter* spp., enterococci, *E. coli*, and *Salmonella*. Antimicrobial resistance in these organisms in swine production has recently been reviewed in detail elsewhere and therefore will not be discussed further (Barton 2014). However, antimicrobials do not only target pathogenic bacteria.

Dawson et al. (1984) conducted one of the first studies on the effect of antimicrobials on antimicrobial resistance in the total anaerobic bacterial population in the swine gut. Pigs in this study were fed either subtherapeutic (27.5 mg kg⁻¹ feed) or therapeutic (220 mg kg⁻¹ feed) levels of chlortetracycline for 85 and 14 days respectively. Only the pigs fed therapeutic levels of chlortetracycline had a significantly higher percentage of chlortetracycline-resistant anaerobes (at 14 days), however, after 85 days all pigs, including the controls, excreted high levels of chlortetracycline-resistant anaerobes (62-72%; Dawson et al. 1984).

Kalmokoff et al. (2011) also evaluated antimicrobial resistance in total anaerobes isolated from the feces of pigs fed either tylosin (44 or 88 mg kg⁻¹ feed) or virginiamycin (11 or 22 mg kg⁻¹ feed) and recorded that all anaerobes were resistant to both antibiotics, even in the absence of exposure. In addition to the potential co-selection from heavy metals in the diet or even the use of disinfectants during cleaning, this likely reflects the long period of time that antimicrobials have been used in swine production. As well, the fitness costs of carrying antimicrobial resistance determinants may be variable or mitigated in some circumstances (Andersson 2006).

Previously, we published a study examining the effect of the continuous administration of subtherapeutic concentrations of tylosin (11 to 44 mg kg⁻¹ feed) and chlortetracycline (5.5 mg kg⁻¹ feed) in farrow-to-finish pigs on the level of resistance to these agents in total anaerobic bacterial populations and on the detection and abundance of selected resistance genes (Holman and Chénier 2013). Pigs that were fed tylosin-supplemented feed exhibited a rapid increase in tylosin-resistant fecal anaerobes; 11.8% tylosin-resistant prior to and 89.6% after treatment. The concentration of the macrolide resistant gene *erm(B)* was similarly affected, with a 10-fold increase observed in tylosin-supplemented pigs. Despite a progressive decrease in tylosin concentration (44 mg kg⁻¹ feed to 22 mg kg⁻¹ feed to 11 mg kg⁻¹ feed) and a two-week withdrawal period prior to shipping, neither tylosin-resistant anaerobes nor the concentration of *erm(B)* was significantly affected. Notably, the total number of anaerobes was not affected by either antibiotic, only the resistant proportion was altered. Conversely, chlortetracycline, which was administered at a lower dosage, had no measurable effect on either chlortetracycline resistance in total anaerobes or on the abundance of selected *tet* genes (Holman and Chénier 2013).

Effect of antimicrobials on the swine gut microbiota using culture-independent methods

The challenges associated with culturing gut-associated microorganisms have led to the use of both single gene sequencing and metagenomic analysis. Typically, single gene sequencing focuses on the 16S rRNA gene; one or more of its nine hypervariable regions is amplified via PCR and then sequenced. Other highly conserved genes with regions of variability, such as *cpn60*, may also be used (Hill et al. 2004). Metagenomics refers to the analysis of all genetic material from a specific environment, rather than a single gene (Thomas et al. 2012). This technology has the advantage of not requiring knowledge about the DNA sequences in question and reduces some of the biases associated with PCR, although other limitations may be associated with this technology (Poretsky et al. 2014).

16S rRNA gene sequencing-based methods

Hence, the effect of in-feed antimicrobials on the swine gut microbiota has been investigated by several researchers using a number of culture-independent methods. Kalmokoff et al. (2011) reported no impact on the swine fecal microbiota in commercial pigs fed either tylosin (44 or 88 mg kg feed⁻¹) or virginiamycin (11 or 22 kg feed⁻¹) over a 15-week period as measured using both 16S rRNA gene DGGE (denaturing gradient gel electrophoresis; V3 region) and near-full length 16S rRNA gene clone library analysis. Also using 16S rRNA gene DGGE (V3 region), Collier et al. (2003) examined the effect of in-feed tylosin at 44 mg kg⁻¹ feed on the ileal microbiota of swine and observed a decrease in the number of DGGE bands following 14 days of treatment, however, no differences were reported at day 21, 28, or 35. The authors also noted a decrease in total bacterial 16S rRNA gene abundance at days 14 and 21, although not after 28 or 35 days. Of interest, was their finding that the relative proportion of

312 *Lactobacillus* 16S rRNA gene sequences was more prevalent after tylosin treatment (Collier et
313 al. 2003).

314 Namkung et al. (2004) and Gong et al. (2008) also used 16S rRNA gene DGGE (V3
315 region) and reported that lincomycin (110 mg kg⁻¹ feed) decreased microbial diversity in the ileal
316 microbiota compared with a basal diet over a four-week period, although not significantly so.
317 Similarly, Bosi et al. (2011) found that amoxicillin (600 mg kg⁻¹ feed), doxycycline (300 mg kg⁻¹
318 feed), or tilmicosin (400 mg kg⁻¹ feed), did not have any effect on the jejunal microbiota of three-
319 week old pigs as measured using the Shannon index and DGGE band number. Total
320 enterobacteria and lactobacilli were also not affected by any of the three antibiotics. The average
321 daily gain of the pigs was improved by antibiotic supplementation in this study, although only in
322 the 0 to 7 day and 0 to 21 day periods (Bosi et al. 2011).

323 A DGGE-based study (V6-V8 regions) by Janczyk et al. (2007) observed a decrease in
324 both diversity and richness (Shannon index: 0.79 in antibiotic-treated pigs vs. 1.03 in untreated
325 pigs) in the ileal microbiota of 39-day old piglets that had been administered a single dose of
326 amoxicillin (15 mg kg⁻¹ body weight) intramuscularly at birth. In piglets fed chlortetracycline
327 for two weeks at 50 mg kg⁻¹ feed following weaning, a significant change in the ileal microbiota
328 was recorded by Rettedal et al. (2009) in a study using 2050 sequences generated from the near-
329 full length 16S rRNA gene. These changes were largely associated with a decrease in
330 *Lactobacillus johnsonii*, *Turicibacter*, and an increase in *Lactobacillus amylovorus*. Through the
331 use of surrogate sows, these authors concluded that the antenatal environment had a much
332 greater impact on the ileal lumen microbiota compared to the postnatal environment (Rettedal et
333 al. 2009). Terminal restriction fragment length polymorphism was used by Thymann et al.
334 (2007) to determine that weaned pigs treated with amoxicillin (20 mg kg⁻¹ feed and

intramuscularly 8.75 mg kg⁻¹ body weight per 12 h) and 2500 mg ZnO kg⁻¹ feed had a lower microbial diversity in the small intestine than untreated pigs (Thymann et al. 2007).

The effect of feeding high levels of Zn (3000 mg kg⁻¹ feed) or Cu (250 mg kg⁻¹ feed) for two weeks post-weaning has also been found to reduce diversity of the ileal bacterial microbiota while only Cu was reported to decrease the colonic microbiota diversity when evaluated by DGGE (Namkung et al. 2006). In contrast, Perez and others (2011) observed no change in the fecal microbiota of pigs fed these same concentrations of Zn or Cu as measured by DGGE band numbers. The inconsistency among DGGE-based studies of the effect in-feed Zn on the swine gut microbiota was further demonstrated by Pieper et al. (2012) who found that higher doses of Zn (up to 2500 mg kg⁻¹ feed) actually increased the number of DGGE bands in samples taken from the ileal microbiota of post-weaned swine.

Quantification of 12 bacterial groups and five *Lactobacillus* spp. was carried out by Starke et al. (2014) using real-time PCR to determine the effect of supplementing the diets of post-weaned swine with high Zn concentrations (2425 mg kg⁻¹ feed) for 54 days, sampled at four different gastrointestinal locations. None of the bacterial groups, with the exception of the reduction of certain *Lactobacillus* spp. in the small intestine, were consistently altered during the trial. Culture-based analysis has also indicated that lactobacilli are reduced in the stomach, ileum, cecum, and colon of pigs that were fed 2500 mg Zn kg⁻¹ feed for 14 days post-weaning (Højberg et al. 2005).

High-throughput 16S rRNA gene and metagenomic sequencing

While the above methods allow for the relatively rapid visualization of large shifts in the microbiota, they may miss more subtle changes. More recently, higher-resolution high-throughput sequencing techniques have been employed to better understand the changes that take

place in the swine gut microbiota in response to dietary antimicrobial supplementation (Table 1). Depending upon the sequencing platform used, high-throughput sequencing methods can generate millions to billions of sequences in a single run (Caporaso et al. 2012).

Using 454 pyrosequencing of the metagenome and the 16S rRNA gene (V3 region), Looft and others (2012) investigated the effect of in-feed ASP250 administered to three post-weaned pigs for three weeks on the abundance and diversity of antimicrobial resistance genes. The diversity of antimicrobial resistance genes was increased as was the abundance of six different classes of resistance genes in the medicated pigs. These resistance gene classes included not only those which confer resistance to the administered antibiotics but also to antibiotics not used in the study, suggesting potential co-selection of these resistance gene classes (Looft et al. 2012). Genes associated with horizontal gene transfer (transposases), virulence (P pilus), as well as energy production and conversion were also reported to be more prevalent in the medicated swine metagenomes. In addition, ASP250 caused identifiable shifts in the swine gut microbiota, including a decrease in the relative abundance of *Bacteroidetes* along with an increase in *Proteobacteria* (from 1% in non-medicated to 11% in medicated), most of which were *E. coli* (Looft et al. 2012). The bacterial community structure was also changed following 14 days of ASP250 supplementation in the diet as measured using the Bray-Curtis index (Looft et al. 2012). However, this effect did not extend to carbadox-treated (10 to 50 mg kg⁻¹ feed) pigs in a similar study (Allen et al. 2011).

In this earlier study, Allen et al. (2011) determined that ASP250 altered both the fecal bacterial microbiota as well as the fecal bacteriophage community in pigs fed ASP250 for 14 days beginning at 77 days of age. In this work, both metagenomic and the 16S rRNA gene (V1 to V3 region) libraries were sequenced using 454 pyrosequencing. The diversity of the bacterial

gut microbiota was also diminished by ASP250 supplementation and the viromes of these pigs contained more integrase genes, indicating that antibiotics may induce prophages. A decrease in the relative abundance of the genera *Coprococcus*, *Succinivibrio*, *Streptococcus*, *Treponema*, and *Turicibacter* was also observed in the ASP250 supplemented pigs (Allen et al. 2011).

A recent study by Looft et al. (2014b) used 454 pyrosequencing of the V1-V3 regions of the 16S rRNA gene to examine the effect of carbadox (50 mg kg⁻¹ feed) on the gut microbiota of six week old pigs over a three-week period followed by a 42-day withdrawal phase. These authors reported decreased bacterial richness and diversity in carbadox-fed pigs during the first four days of treatment as well as significant changes in the bacterial community structure. In particular, the relative abundance of the *Bacteroidetes* phylum and the *Prevotella* genus were most notably increased during this time period, as were the genera *Roseburia*, *Faecalibacterium*, and *Asteroleplasma*. Looft et al. (2014b) suggest that *Prevotella*, *Roseburia*, and *Faecalibacterium* may provide growth benefits to the host through the production of short chain fatty acids such as butyrate. Carbadox, however, did not induce changes in the medicated vs. non-medicated pigs after 7, 14, or 21 days of treatment demonstrating the resilience of the swine gut microbiota (Looft et al. 2014b). Interestingly, the fecal bacterial communities of the non-medicated and medicated pigs diverged from one another again in the 21 to 42-day portion of the withdrawal phase, although diversity metrics remained unchanged (Looft et al. 2014b). A similar phenomenon has recently been reported in pigs that received a single injection of tulathromycin (a macrolide antibiotic) at four days of age at 2.5 mg kg⁻¹ body weight (Schokker et al. 2015). This study used a DNA microarray, together with 16S rRNA gene sequences, to determine that the jejunal microbiota of pigs injected with tulathromycin was altered in comparison with untreated pigs only at 176 days of age but not at 55 days (Schokker et al. 2015).

Other studies using high-throughput sequencing have reported no obvious alterations due to in-feed antibiotic supplementation. 454 pyrosequencing of the 16S rRNA gene (V1-V3 regions) was also used by Poole et al. (2013) to investigate the effect of 50 mg of chlortetracycline kg^{-1} feed on the fecal microbiota of post-weaned swine over a 28-day period. These authors found that chlortetracycline supplementation had no effect on bacterial diversity and richness or on community structure diversity. However, the lack of significant effects could have been a result of the relatively small number of pigs used in the study (two pigs per treatment). Similarly, Kim et al. (2012) documented no difference at the bacterial phylum or class-levels in 10 pigs continuously fed either tylosin (44 mg kg^{-1} feed) or a control, antibiotic-free diet, for 12 weeks starting at 10 weeks of age. These researchers sequenced the V3 region of the 16S rRNA gene using 454 pyrosequencing (Kim et al. 2012). There were also no identifiable changes in bacterial richness and diversity, or community structure metrics, although the swine gut microbiota may have already been well established and relatively stable at this stage of production (>10 weeks). However, alterations in the relative abundance at the genus and OTU-levels were observed. *Prevotella* and *Lactobacillus* were among six genera that were more abundant in tylosin-fed pigs.

Recently, we used Illumina sequencing of the V4 region of the 16S rRNA gene to investigate the effect of subtherapeutic levels of in-feed tylosin and chlortetracycline over a 19-week period (Holman and Chénier 2014). While archaeal and bacterial diversity and richness in pigs fed tylosin (11 to 44 mg kg^{-1} feed) starting at weaning was not altered compared to controls, tylosin administration did result in numerous shifts at the phylum, genus, and OTU-levels. The relative abundance of the genera *Akkermansia*, *Coprococcus*, and *Streptococcus* was enriched in tylosin-fed pigs while *Bacteroidetes* and the genera *Succinivibrio* and *Anaerovibrio* were

reduced. With the exception of the *Coprococcus* genus, however, these alterations in the major (>0.1% of sequences) phyla and genera were not sustained from one sampling time to the next, indicating that the dominant taxa are adaptable or resilient to perturbations by long-term antibiotic administration. The overall community structure was also changed by in-feed tylosin as measured using weighted UniFrac, which takes into account OTU abundance. These results were not replicated in the chlortetracycline (5.5 mg kg⁻¹ feed) pigs, although the relative abundance of specific taxa such as *Firmicutes* and *Lactobacillus* were temporally altered (Holman and Chénier 2014).

Looft et al. 2014 (2014a) combined 454 pyrosequencing of the 16S rRNA gene with metagenomic analysis to monitor the impact of ASP250 on the ileal, cecal, colonic, and fecal microbiota of three-month old pigs over a two-week period. It was found that changes in bacterial taxa due to ASP250 administration were restricted to a specific location in the pig gastrointestinal tract, while other changes occurred in multiple locations. For example, *Helicobacter* was only reduced in the cecum and *Turicibacter* in the colon of ASP250-treated pigs. *Escherichia* were increased in all gut locations in medicated pigs, most notably in the lumen and mucosa of the ileum. The proportion of the genera *Treponema* and *Streptococcus* were both diminished in the cecum and colon. Interestingly, although 12 antibiotic resistance genes were differentially more abundant in the ASP250 pigs, 8 antibiotic resistance genes were more abundant in the untreated pigs (Looft et al. 2014a).

Using 454 pyrosequencing of the V3-V5 region of the 16S rRNA gene, researchers in China (Sun et al. 2014) evaluated the effect of feeding lincomycin hydrochloride (500 mg kg⁻¹ feed), chlortetracycline (100 mg kg⁻¹ feed), and amoxicillin (500 mg kg⁻¹ feed) to sows before farrowing over a 12 day period. These authors reported a significant decrease in the relative

abundance of *Proteobacteria* after treatment, in particular *Escherichia/Shigella* which were reduced by 40%. The *Streptococcus* genus was also reduced in the medicated sows whereas *Treponema*, *Enterococcus*, and *Staphylococcus* were temporarily enriched. It should be noted that in total, although nine samples were pooled into one composite sample, only one composite sample was used per time point. Also, this treatment represents a relatively high concentration of antibiotics being added to feed, at least compared to levels typically used in North America. Sun et al. (2014) also used real-time PCR in this work and they observed greater numbers of aminoglycoside, lincomycin, macrolide, and tetracycline resistance genes following antibiotic treatment.

Two separate studies by Vahjen et al. (2010; 2011) have examined the effect of 3000 mg Zn kg⁻¹ feed on the ileal microbiota of post-weaned swine using pyrosequencing. At the genus-level, *Weissella*, *Leuconostoc*, and *Streptococcus* were all increased in pigs receiving high levels of Zn, while *Sarcina* was reduced (Vhagen et al. 2010). In the 2nd study, bacterial diversity and richness were reported to be enhanced in the high Zn dosage pigs. Species of *Weissella* and *Leuconostoc* were again found to be more relatively abundant in the pigs fed high doses of Zn and the proportion of *Lactobacillus reuteri* and *Sarcina ventriculi* was decreased in these pigs (Vhagen et al. 2011).

Effect of antimicrobial use on antimicrobial resistance determinants in the swine gut microbiota

In addition to those studies already discussed, several other researchers have also investigated the association of antimicrobial use and the abundance and detection of antimicrobial resistance genes in swine feces and manure. In China, Zhu et al. (2013) detected a total of 149 different antibiotic resistance genes in swine manure and composted manure from

farms where at least six different antimicrobials were used as in-feed growth promoters and several more were used for therapeutic reasons. The abundance of *bla*_{TEM} genes conferring resistance to ampicillin increased in the feces of pigs given ampicillin (20 mg kg⁻¹ body weight) either orally or via intramuscular injection (Bibbal et al. 2007). A recent study by Agga et al. (2015) found a higher abundance of the *tet*(A) gene in the feces of pigs fed 550 mg kg⁻¹ feed post-weaning, although the abundance of *tet*(B) was not affected.

Similar to studies that measure phenotypic resistance to antimicrobials in anaerobic bacteria, there is a wide dissemination of antimicrobial resistance determinants among pigs in modern production facilities even in the absence of antimicrobial exposure (Pakpour et al. 2012; Holman and Chénier 2013; Agga et al. 2015; Kalmokoff et al. 2011). In particular, the tetracycline resistance genes *tet*(M), *tet*(O), *tet*(Q), and *tet*(X) (Agga et al. 2015; Barkovskii and Bridges 2012; Holman and Chénier 2013) and the macrolide resistance genes *erm*(B), *erm*(F), and *erm*(G) (Holman and Chénier 2013; Kalmokoff et al. 2011; Looft et al. 2012) are frequently detected in pigs not directly exposed to antimicrobials.

The ubiquitous presence of these genes among antimicrobial-free pigs means that a reduction in antimicrobial use in swine production will likely not immediately reduce antimicrobial resistance in the gut microbiota of pigs. The complexity of this issue has been demonstrated in Denmark where antimicrobial growth promoters were first banned in weanling pigs in 1999 and in all pigs by 2000. While overall antimicrobial use has decreased in Denmark, antimicrobial resistance in swine gut bacteria has largely persisted (Jensen and Hayes 2014). This may be a result of the continued use of therapeutic levels of some antimicrobials and/or agents that may co-select for resistance or provide cross-resistance such as heavy metals and disinfectants (Hasman et al. 2006; Yazdankhah et al. 2014). At the same time, a reduction in

496 macrolide use in swine in Denmark has resulted in a lower prevalence of macrolide-resistant
497 *Campylobacter* spp. (Alban et al. 2008).

498 **Conclusions**

499 Antimicrobials are used in swine production for a variety of purposes including animal
500 welfare, disease treatment, and growth promotion. It is well established that the use of these
501 antimicrobial agents helps provide the necessary selective pressure to maintain and increase the
502 abundance and dissemination of antimicrobial-resistant bacteria and resistance determinants.
503 Antimicrobial resistance, however, can also persist in the swine gut microbiota in the absence of
504 antimicrobial exposure. For this reason, the benefits of using relatively high levels of heavy
505 metals in swine diets should also be examined.

506 It is hoped that by characterizing the response of the swine gut microbiota to
507 antimicrobials, alternative agents such as prebiotics, probiotics, or other feed additives can be
508 developed to replace or reduce the use of antimicrobials. Although a number of studies have
509 now been conducted on the swine gut microbiota following antimicrobial perturbation using
510 high-throughput sequencing methods, no clear pattern has emerged in terms of the alteration of
511 specific bacterial genera or species. For example, the relative abundance of the lactic acid
512 bacterial genera *Lactobacillus* and *Streptococcus*, as well as *Coprococcus*, has been reported to
513 be both increased and reduced following in-feed antibiotic administration (Allen et al. 2011;
514 Holman and Chénier 2014; Looft et al. 2014a; Looft et al. 2014b). The same is true for the
515 phylum *Bacteroidetes* (Holman and Chénier 2014; Looft et al. 2014b; Sun et al. 2014).

516 These differences may be due to genetic and environmental factors (e.g. commercial vs.
517 research farm setting) as well as the experimental design of each study in relation to the age of
518 the pigs and the type and dosage of antibiotic used. In addition, the analytical methods and

sequencing platforms used, as well as the choice of 16S rRNA variable region that is sequenced, can all influence study outcomes. Nonetheless, the resilience of the swine gut microbiota to long-term changes due to the administration of antimicrobials is apparent. Older pigs also appear to exhibit fewer changes in their gut microbiota compared with younger swine in response to in-feed antibiotics. This early growth stage also is also the time period where antibiotics tend to be most effective in improving feed efficiency and/or growth rate. Rare bacterial taxa, which are frequently ignored or removed during sequence analysis, may have a larger role in the swine gut microbiota following antibiotic exposure than is currently known. This also includes bacterial subspecies diversity which is undetectable with current sequencing technologies (Allen and Stanton 2014). Finally, it is also possible that antibiotics have a positive growth effect on the host that is independent of the gut microbiota (Niewold 2007).

The reporting of growth rate data in these types of studies might also help identify those cases where microbial changes should be expected in response to antimicrobial supplementation. As well, although feces are often the sample of choice because it allows for the continuous sampling from a single animal, it may not accurately represent the changes that occur in the entire swine gastrointestinal tract in response to antimicrobial administration.

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Table 1. High throughput sequencing studies on the effect of antibiotics on the swine gut microbiota.

Author	Antibiotic and dosage (mg kg ⁻¹ feed)	16S rRNA hypervariable region sequenced	Number of pigs used per treatment group	Duration of study (age of pigs in weeks)	Sequencing platform	Sample type	Notable findings
Allen et al. 2011	Carbadox: 10; carbadox: 50; ASP250: (chlortetracycline: 100,sulfamethazine: 100, penicillin:50)	Whole genome sequencing of virome; 16S rRNA V1-V3	Six (pooled for WGS)	3 to 7	454 GS-FLX	Feces	ASP250 pigs: Shift in phage and bacterial communities; ↑ <i>Escherichia</i> ; ↑ integrase genes in ASP250 + carbadox pigs
Holman and Chénier 2014	Tylosin: 11, 22, 44 Chlortetracycline: 5.5	V4	Six	3 to 19	Illumina MiSeq	Feces	Tylosin pigs: Shift in bacterial community structure; ↑ <i>Coprococcus</i> (3 to 9 wk); ↓ <i>Fibrobacteres</i> , (3 wk); ↑ <i>Streptococcus</i> (6 wk); ↓ <i>Bacteroidetes</i> (9 wk); ↓ <i>Succinivibrio</i> (9 wk) Chlortetracycline pigs: ↓ <i>Bacteria</i> (3 wk); ↓ <i>Lactobacillus</i> (6 wk); ↓ <i>Firmicutes</i> (16 wk) Tylosin pigs: ↓ <i>Prevotella</i> , <i>Megasphaera</i> , <i>Blautia</i> , <i>Sarcina</i> ; ↑ <i>Lactobacillus</i> , <i>Sporacetigenium</i> No effect on alpha or beta diversity
Kim et al. 2012	Tylosin: 40	V3	Two farms – 10 pigs per treatment at each farm (pooled)	10 to 22	454 GS-FLX	Feces	Tylosin pigs: ↓ <i>Prevotella</i> , <i>Megasphaera</i> , <i>Blautia</i> , <i>Sarcina</i> ; ↑ <i>Lactobacillus</i> , <i>Sporacetigenium</i> No effect on alpha or beta diversity
Looft et al. 2012	ASP250	Whole genome sequencing; 16S rRNA V1-V3	Three	18 to 21	454 GS-FLX	Feces	ASP250 pigs: ↓ <i>Bacteroidetes</i> , <i>Anaerobacter</i> , <i>Barnesiella</i> , <i>Papillibacter</i> , <i>Sporacetigenium</i> , <i>Sarcina</i> ; ↑ <i>Deinococcus-Thermus</i> , <i>Proteobacteria</i> , <i>Succinivibrio</i> , <i>Ruminococcus</i> , <i>E. coli</i> ; ↑ diversity and abundance of antibiotic resistance genes
Looft et al. 2014a	ASP250	Whole genome sequencing; 16S rRNA V1-V3	Six	12 to 14	454 GS-FLX	Ileum, cecum, colon: tissue and	ASP250 pigs: ↑ <i>Tenericutes</i> in cecum, ↑ <i>Escherichia</i> in cecum and colon; ↑ <i>Paraprevotella</i> , <i>Anaeroplasma</i> in the cecum; ↓

						lumen	<i>Treponema</i> in cecum and colon,
Looft et al. 2014b	Carbadox: 50	V1-V3	Six	6 to 9 (treatment) 10 to 15 (withdrawal period)	454 GS-FLX	Feces	Carbadox pigs: ↑ at days 1-4 <i>Bacteroidetes</i> , <i>Prevotella</i> , <i>Roseburia</i> , <i>Faecalibacterium</i> , <i>Asteroleplasma</i> ; ↓ at days 1-4 <i>Lactobacillus</i> ; ↓ alpha-diversity in 1-4 day samples but not 7 days+; shifts in beta-diversity in 1-4 day samples but not 7 days+
Poole et al. 2013	Chlortetracycline: 50	V1-V3	Two	4 to 11 7 to 11 (treatment)	454 GS-FLX	Feces	No differences in alpha or beta-diversity, nor at the taxa-level
Sun et al. 2014	Mixture of: lincomycin hydrochloride: 500; chlortetracycline:100; amoxicillin 500	V3-V5	24 pregnant sows (nine samples pooled at each time point)	Treatment duration: 12 days	454 GS-FLX	Feces	Following treatment: ↓ <i>Proteobacteria</i> (3 to 12 days); ↓ <i>Escherichia/Shigella</i> ; <i>Streptococcus</i> (days 3 and 6); ↑ <i>Bacteroidetes</i> , <i>Spirochaetes</i> , <i>Euryarchaeota</i> , <i>Actinobacteria</i> (days 3 and 6); ↑ aminoglycoside, lincomycin, macrolides, and tetracycline resistance genes

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Author	Antibiotic and dosage (mg kg ⁻¹ feed)	16S rRNA hypervariable region sequenced	Number of pigs used per treatment group	Duration of study (age of pigs in weeks)	Sequencing platform	Sample type	Notable findings
Allen et al. 2011	Carbadox: 10; carbadox: 50; ASP250: (chlortetracycline: 100,sulfamethazine: 100, penicillin:50)	Whole genome sequencing of virome; 16S rRNA V1-V3	Six (pooled for WGS)	3 to 7	454 GS-FLX	Feces	ASP250 pigs: Shift in phage and bacterial communities; ↑ <i>Escherichia</i> ; ↑ integrase genes in ASP250 + carbadox pigs
Holman and Chénier 2014	Tylosin: 11, 22, 44 Chlortetracycline: 5.5	V4	Six	3 to 19	Illumina MiSeq	Feces	Tylosin pigs: Shift in bacterial community structure; ↑ <i>Coprococcus</i> (3 to 9 wk); ↓ <i>Fibrobacteres</i> , (3 wk); ↑ <i>Streptococcus</i> (6 wk); ↓ <i>Bacteroidetes</i> (9 wk); ↓ <i>Succinivibrio</i> (9 wk) Chlortetracycline pigs: ↓ <i>Bacteria</i> (3 wk); ↓ <i>Lactobacillus</i> (6 wk); ↓ <i>Firmicutes</i> (16 wk) Tylosin pigs: ↓ <i>Prevotella</i> , <i>Megasphaera</i> , <i>Blautia</i> , <i>Sarcina</i> ; ↑ <i>Lactobacillus</i> , <i>Sporacetigenium</i> No effect on alpha or beta diversity
Kim et al. 2012	Tylosin: 40	V3	Two farms – 10 pigs per treatment at each farm (pooled)	10 to 22	454 GS-FLX	Feces	Tylosin pigs: ↓ <i>Prevotella</i> , <i>Megasphaera</i> , <i>Blautia</i> , <i>Sarcina</i> ; ↑ <i>Lactobacillus</i> , <i>Sporacetigenium</i> No effect on alpha or beta diversity
Looft et al. 2012	ASP250	Whole genome sequencing; 16S rRNA V1-V3	Three	18 to 21	454 GS-FLX	Feces	ASP250 pigs: ↓ <i>Bacteroidetes</i> , <i>Anaerobacter</i> , <i>Barnesiella</i> , <i>Papillibacter</i> , <i>Sporacetigenium</i> , <i>Sarcina</i> ; ↑ <i>Deinococcus-Thermus</i> , <i>Proteobacteria</i> , <i>Succinivibrio</i> , <i>Ruminococcus</i> , <i>E. coli</i> ; ↑ diversity and abundance of antibiotic resistance genes
Looft et al. 2014a	ASP250	Whole genome sequencing; 16S rRNA V1-V3	Six	12 to 14	454 GS-FLX	Ileum, cecum, colon: tissue and	ASP250 pigs: ↑ <i>Tenericutes</i> in cecum, ↑ <i>Escherichia</i> in cecum and colon; ↑ <i>Paraprevotella</i> , <i>Anaeroplasma</i> in the cecum; ↓

						lumen	<i>Treponema</i> in cecum and colon,
Looft et al. 2014b	Carbadox: 50	V1-V3	Six	6 to 9 (treatment) 10 to 15 (withdrawal period)	454 GS-FLX	Feces	Carbadox pigs: ↑ at days 1-4 <i>Bacteroidetes</i> , <i>Prevotella</i> , <i>Roseburia</i> , <i>Faecalibacterium</i> , <i>Asteroleplasma</i> ; ↓ at days 1-4 <i>Lactobacillus</i> ; ↓ alpha-diversity in 1-4 day samples but not 7 days+; shifts in beta-diversity in 1-4 day samples but not 7 days+
Poole et al. 2013	Chlortetracycline: 50	V1-V3	Two	4 to 11 7 to 11 (treatment)	454 GS-FLX	Feces	No differences in alpha or beta-diversity, nor at the taxa-level
Sun et al. 2014	Mixture of: lincomycin hydrochloride: 500; chlortetracycline:100; amoxicillin 500	V3-V5	24 pregnant sows (nine samples pooled at each time point)	Treatment duration: 12 days	454 GS-FLX	Feces	Following treatment: ↓ <i>Proteobacteria</i> (3 to 12 days); ↓ <i>Escherichia/Shigella</i> ; <i>Streptococcus</i> (days 3 and 6); ↑ <i>Bacteroidetes</i> , <i>Spirochaetes</i> , <i>Euryarchaeota</i> , <i>Actinobacteria</i> (days 3 and 6); ↑ aminoglycoside, lincomycin, macrolides, and tetracycline resistance genes