Impact of subtherapeutic administration of tylosin and chlortetracycline on antibiotic resistance and the gut microbiota in farrow-to-finish swine

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Abstract

The use of antibiotics in swine production for the purposes of growth promotion dates back to the 1950s. This practice remains controversial, however, as it may lead to the maintenance of a reservoir of antibiotic resistant bacteria and resistance determinants. The underlying mechanisms for the growth-promoting antibiotics also remain poorly characterized. We investigated the effect of two commonly employed antibiotics, chlortetracycline and tylosin, given at subtherapeutic concentrations on the swine gut microbiota, antibiotic resistome, and on the culturable anaerobic bacterial population. This study was carried out over the entire swine production cycle (19 wk). Tylosin-fed pigs had significantly higher concentrations of *erm*(B) in their feces as well as a higher frequency of detection for *erm*(A), *erm*(F), and *erm*(G). Tylosin-resistant anaerobes increased significantly at weaning (6 wk) and remained stable at this level throughout the study (21 wk). A two-week withdrawal of tylosin prior to slaughter did not significantly reduce *erm*(B) concentrations or the proportion of tylosin-resistant anaerobes. Chlortetracycline had no significant effect on the concentration of chlortetracycline-resistant anaerobes or on the frequency of detection or concentration of tetracycline-resistance genes. Pigs given either tylosin or chlortetracycline did not exhibit increases in growth rate. In-feed subtherapeutic tylosin also altered the relative abundance of several taxa and OTUs in the swine gut microbiota. In contrast, chlortetracycline caused fewer changes to the biodiversity of the gut microbiota. Samples taken from suckling piglets (3 wk) were significantly different from all subsequent sampling times in terms of the relative abundance of specific taxa and OTUs. Extruded flaxseed was fed to growing pigs as a potential antibiotic alternative. The addition of

flaxseed did not cause detectable changes in the gut microbiota as measured using DGGE and real-time PCR.

Résumé

L'utilisation d'antibiotiques en production porcine comme promoteurs de croissance remonte aux années 1950. Toutefois, cette pratique demeure controversée car elle peut favoriser le maintien d'un réservoir de bactéries résistantes et de déterminants de résistance aux antibiotiques. Les mécanismes sous-jacents des antibiotiques promoteurs de croissance demeurent mal caractérisés. Nous avons étudié l'effet de deux antibiotiques couramment utilisés en production porcine, la chlortétracycline et la tylosine, administrés à des concentrations sous-thérapeutiques sur le microbiote intestinal porcin, sur le résistome antibiotique, ainsi que sur la population bactérienne anaérobie cultivable. Cette étude a été réalisée sur l'ensemble du cycle de production porcine (19 sem). Les porcs dont la moulée contenait de la tylosine avaient des concentrations significativement plus élevées de *erm*(B) dans leurs excréments, ainsi qu'une plus grande fréquence de détection pour *erm*(A), *erm*(F) et *erm*(G). La concentration de la population d'anaérobes résistants à la tylosine a augmenté de manière significative au sevrage (6 semaines) et est demeurée stable à ce niveau tout au long de l'étude (21 semaines). Une période de retrait de deux semaines de la tylosine avant l'abattage n'a pas réduit de façon significative la concentration du gène *erm*(B) ni la proportion d'anaérobes résistants à la tylosine. La chlortétracycline n'a eu aucun effet significatif sur la concentration de la population d'anaérobes résistants à la chlortétracycline ni sur la fréquence de détection ou la concentration des gènes de résistance aux tétracyclines. Les porcs dont les moulées contenaient soit de la tylosine ou de chlortétracycline n'ont pas présenté d'augmentation de leur taux de croissance. La tylosine ajoutée aux moulées a

modifié l'abondance relative de plusieurs taxons et OTU dans le microbiote intestinal porcin. Par contre, la chlortétracycline a induit moins de changements dans la biodiversité phylogénique de la flore intestinale. Les échantillons prélevés sur les porcelets sevrés (3 sem) étaient significativement différents de tous les échantillons prélevés ultérieurement en termes de l'abondance relative d'un grand nombre de taxons ainsi que OTU. Un extrait de graine de lin ajouté à la moulée de porcs en croissance comme une alternative potentielle aux antibiotiques n'a pas causé de modification détectable dans la concentration ou la biodiversité du microbiote intestinal, tel que mesurée à l'aide de l'électrophorèse sur gel avec gradient dénaturant (Denaturing Gradient Gel Electrophoresis, DGGE) et de la réaction en chaîne de la polymérase en temps réel.

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Contribution to knowledge

Chapter 3. These experiments focused on the commensal microbiota rather than strictly pathogenic *Bacteria* as many other studies have done. Pigs were raised from farrowing (birth) to the finishing production stage and the development of antibiotic resistance was monitored over a five-month period. This is the longest monitoring period that has been used to date in the literature using molecular-based methods. We demonstrated that pigs that had been given tylosin beginning at weaning (6 wk) shed a significantly greater proportion of tylosin-resistant anaerobic bacteria in their feces than did the control group after only 16 days of treatment. Tylosin treated pigs also had significantly greater concentrations of *erm*(B) in their fecal microbiota. Other macrolide resistance genes (*erm*) were also detected more frequently in the tylosin-fed group. We also noted no improvement in growth rate was noted for either tylosin or chlortetracycline supplemented pigs, a significant finding given that this is the most common reason given for antibiotic use in swine. We also determined that a two-week withdrawal period prior to shipping had no significant effect on decreasing tylosin-resistant bacteria and macrolide-resistant genes. Our findings provide evidence-based data for scientific regulators, veterinarians, and producers.

Chapter 4. This study investigated the impact of tylosin and chlortetracycline on the swine gut microbiota using Illumina-based high throughput sequencing. This study is novel in its design as pigs were sampled over the entire swine production cycle rather than at limited time periods. To date, this is the longest monitoring period in the literature, as well as the largest number of pigs monitored at a single farm, using high throughout sequencing methods. It was

determined that tylosin alters the fecal microbiota at both the taxa and OTU-level and that these changes are temporary rather than permanent. Suckling piglets were also observed to have significantly different gut microbial communities than older pigs. Overall, this work provides evidence of changes in the swine gut microbiota that can be used to aid in the development of antibiotic alternatives.

Chapter 5. This is the first study to examine the impact of flaxseed supplementation on the entire swine gut microbiota. It was found that extruded flaxseed did not alter the abundance of specific bacterial taxa or structure of the swine gut microbiota over a 51 day period. In addition, no effect of extruded flaxseed was observed for archaeal populations. No association between weight and the *Bacteriodetes:Firmicutes* ratio was observed either, despite claims by some researchers of a correlation between decreased *Bacteriodetes:Firmicutes* ratio and weight gain in pigs and humans. This work demonstrated that flaxseed has no negative impact on the swine gut microbiota and therefore flaxseed can be safely used as a feed additive to produce value-added omega-3 fatty acid enriched pork products.

Contributions of authors

Three co-authored manuscripts are included in this thesis.

Chapter 3: D.B. Holman and M.R. Chénier.

D.B. Holman designed and carried out all the experiments, analyzed and interpreted the results, and wrote the manuscript. M.R. Chénier reviewed the manuscript. Published in FEMS Microbiology Ecology. 2013. Impact of subtherapeutic administration of tylosin and chlortetracycline on antimicrobial resistance in farrow-to-finish swine. 85(1):1-13.

Chapter 4: D.B. Holman and M.R. Chénier.

D.B. Holman designed and carried out all the experiments, analyzed and interpreted the results, and wrote the manuscript. M.R. Chénier reviewed the manuscript. Temporal changes and the effect of subtherapeutic concentrations of antibiotics in the gut microbiota of swine. Published in FEMS Microbiology Ecology, 2014. DOI: 10.1111/1574-6941.12419.

Chapter 5: D.B. Holman, Bushansingh Baurhoo, M.R. Chénier.

D.B. Holman designed and carried out all the experiments, analyzed and interpreted the results, and wrote the manuscript. B. Baurhoo designed the experiments and reviewed the manuscript. M.R. Chénier reviewed the manuscript. Temporal analysis of the effect of extruded flaxseed on the swine gut microbiota. Published in the Canadian Journal of Microbiology, 2014. DOI: 10.1139/cjm-2014-0317.

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List of abbreviations

bp	Base pair
CFIA	Canadian Food Inspection Agency
CFU	Colony forming unit
d	Day
DGGE	Denaturing gradient gel electrophoresis
erm	Erythromycin resistance methylase gene
HGT	Horizontal gene transfer
IS	Insertion sequence
MIC	Minimum inhibitory concentration
MLS_B	Macrolide, lincosamide, and streptogramin B
OTU	Operational taxonomic unit
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
tet	Tetracycline resistance gene
U	Units
UPGMA	Unweighted pair group method with arithmetic mean
wk	Week
WHO	World Health Organization

1. General introduction

Pork is the most widely consumed meat around the world with an estimated 114.3 million tonnes produced in 2013 (Food and Agriculture Organization, 2014). While the total number of pigs raised in North America has remained relatively stable over the past 50 years, modern swine operations have become increasingly larger with greater numbers of animals raised per farm (United States Department of Agriculture, 2008). This industrialization of the pork industry has coincided with the use of antimicrobials in all stages of swine production (MacDonald and McBride, 2009). Antimicrobials have now been used in swine production since the 1950s for several purposes. These include: 1) the treatment of infectious disease, 2) as a prophylactic measure to prevent infectious disease during high risk circumstances, and 3) growth promotion (Viola and DeVincent, 2006). Worldwide, the majority of antimicrobials produced are used in animals raised for human food consumption. Most of these antimicrobials are administered via feed or water (Silbergeld et al., 2008). Furthermore, swine production accounts for 60% of all antimicrobials used in animals (Bibbal et al., 2007).

The concern surrounding antimicrobial use in agricultural animals is that their use selects for resistant bacteria, which in turn can serve as a reservoir of resistance determinants (Wegener, 2003). Resistant bacteria, both pathogenic and commensal, may then be passed to humans through food, direct contact with animals, or release of animal waste into the environment (Barza, 2002; Silbergeld et al., 2008; Witte, 1998). Antimicrobial resistance in pathogenic bacteria is increasing and infections caused by resistant organisms are more difficult and expensive to treat, along with being associated with higher mortality and poorer health outcomes (Jones et al., 2004; Roberts et al., 2009). In addition, the problem of antimicrobial resistance is

exacerbated by the current lack of new antimicrobial drugs in development and available for use (Livermore et al., 2012; Piddock, 2012).

While the European Union (EU) has banned the use of all antimicrobials at subtherapeutic levels for the purpose of growth promotion, they are still used for this indication in Canada, the United States, and many other countries (Aarestrup and Jenser, 2007). In Canada, the province of Quebec is the largest producer with an inventory of just over 4 million pigs (Statistics Canada, 2013). In Quebec, a prescription from a veterinarian is required to purchase any antibiotic to be used in livestock production. In most areas of North America, however, there are few restrictions on their use in agriculture.

To date, there has been limited research on the impact of antimicrobial use on the gut microbiota of swine. Until very recently, most of the studies involving swine and antimicrobial resistance have been focused on antimicrobial resistance in pathogenic bacteria rather than commensal bacteria which are in far greater abundance (Akwar et al., 2008; Mathew et al., 2005; Thakur and Gebreyes, 2005). In addition, the majority of work has relied upon culture-dependent methods to identify and characterize resistance. This can lead to misrepresentation of the microbiota as only 20-40% of gut bacteria are estimated to be culturable (Licht et al., 2006).

The use of subtherapeutic levels of antibiotics such as tylosin and chlortetracycline in swine production for the purposes of growth promotion remains controversial due to its potential impact on public health. As discussed, the continuous delivery of antimicrobials provides the selective pressure necessary for the emergence and maintenance of reservoirs of antimicrobial resistant bacteria in animals such as swine (Teuber, 2001). Therefore, this study is designed to simulate the use of antimicrobial supplemented feed at subtherapeutic levels in swine.

1.1 Objectives

- To monitor pigs (via fecal sampling) that have been given subtherapeutic concentrations of either tylosin or chlortetracycline beginning at weaning (4 weeks of age) over the entire swine production cycle, i.e. farrow-to-finish. The proportion of cultivable tylosin- and chlortetracycline-resistant fecal anaerobes will be measured using anaerobic culture techniques. The impact of these two antibiotics on the identification, via PCR, of commonly identified macrolide and tetracycline resistance genes in swine feces, as well as on the abundance of selected macrolide and tetracycline genes via real-time PCR, will be investigated. In addition, the growth rate of the pigs in response to subtherapeutic concentrations of tylosin and chlortetracycline will be evaluated.
- Identify changes in the swine gut microbiota as a result of subtherapeutic tylosin and chlortetracycline supplementation using high throughput Illumina 16S rRNA gene sequencing. Also to analyze longitudinal changes in the swine gut microbiota from farrow-to-finish.
- To evaluate the effect of feeding co-extruded flaxseed to pigs in the growing phase on the swine gut microbiota using DGGE.

2. Literature review

2.1 Antimicrobial use in swine production

Antimicrobials are used in commercial swine operations for growth promotion, as a prophylactic measure to prevent disease or to treat active disease (Mackie et al., 2006). Prophylaxis refers to the treatment of individual or small numbers of pigs while metaphylaxis involves treating the whole herd with an antimicrobial agent for disease control (McEwen and Fedorka-Cray, 2002). It has been estimated that approximately 70-80% of starter pigs, 70-80%

of growers, 50 to 60% of finishers, and 40-50% of sows consume feed that is supplemented with antimicrobials. Most of these antimicrobials are administered via feed or water (Cromwell, 2002).

2.1.1 Growth promotion

Antimicrobials have long been added to feed for use in swine production. Cunha et al. (1950) first demonstrated in 1950 that pigs given feed supplemented with the antibiotic chlortetracycline had higher growth rates. In addition, the use of antimicrobials in swine feed at subtherapeutic levels has been shown to increase both growth rate and feed efficiency (Cromwell, 2002; Dibner and Richards, 2005). For example, Zimmerman (1986) reported a 15% increase in the average growth rate and a 6.5% decrease in the amount of feed needed per unit of weight gain during the starter period (8 to 26 kg body weight). During the growth-finisher stage (27 to 92 kg body weight), the growth rate was increased by 3.6% and the feed required per unit of weight gain was decreased by 2.4%. More recent research, however, has suggested that the effect of growth-promoting antibiotics on growth rate and feed efficiency in modern swine husbandry may not be as pronounced or may be limited to the nursery phase of production (Dritz et al., 2002; Holt et al., 2011). Pig herds with a higher disease load and those raised in unsanitary conditions also have a better response to antibiotics used for growth promotion than do healthy pigs in clean environments (Jin et al., 2008).

In Canada, there is a lack of comprehensive information available as it relates to antimicrobial use in livestock. Estimates derived from producer and veterinarian surveys have indicated, however, that 90 to 95% of all pigs are exposed to antibiotics at some point during production (Deckert et al., 2010; Rosengren et al., 2008a). In the United States, 91.8% of farms surveyed reported using antimicrobials for growth promotion in nursery-age pigs, with larger

operations more likely to use antimicrobials for this purpose. The average time for antimicrobial administration via feed in nursery-age pigs for the purpose of growth promotion is 32.4 days (United States Department of Agriculture, 2007). Similarly, 93% of farms used antimicrobials for growth promotion in grower-finisher pigs (United States Department of Agriculture, 2007).

Although the exact mechanism(s) by which antibiotics increase swine growth rate is unknown, several potential modes of action have been proposed. These include: 1) reduction of harmful metabolites produced by intestinal bacteria, 2) an increase in the availability of nutrients in the gut that would otherwise be consumed by the gut microbiota, 3) an increase in the 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). The last proposed mechanism, the control of non-specific subclinical disease through antibiotic administration, is the most popular explanation. Antibiotics also tend to be more efficacious in terms of growth promotion in younger pigs, which are more susceptible to disease, than in older pigs (Cromwell, 2002). Alternatively, it has also been proposed that antibiotics have anti-inflammatory effects that are independent of any interaction with the gut microbiota (Niewold, 2007).

The use of growth promoting antimicrobials in animals is also known to increase feed intake, energy and nitrogen retention, as well as nutrient, glucose, fatty acid, vitamin, calcium, and trace element absorption. In addition, antimicrobials may decrease gut energy loss, vitamin synthesis, gut wall thickness and diameter, fecal moisture, and mucosal cell turnover (Gaskins et al., 2002).

2.1.2 Medicinal uses – treatment of disease

Modern animal husbandry often involves a large number of animals being kept in close contact with one another and as a result it provides optimal conditions 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). Antimicrobials are important in veterinary medicine as they are not easily replaceable due to a lack of alternatives such as vaccines (Ungemach 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 and (Friendship, 2006).

2.2 Common antimicrobials used in swine production and mode of action

There are a relatively large number of antimicrobials approved for use in North America. In the United States, 17 antimicrobials are approved for use in swine production with 14 approved for growth promotion purposes (United States Department of Agriculture, 2007). In Canada, a total of 24 antimicrobials or combinations are approved for use in swine production, however, only 7 are approved for growth promotion (Canadian Food Inspection Agency, 2014). The two most commonly used antimicrobials in North American swine production are tylosin and chlortetracycline (Stone et al., 2009). In Canada, chlortetracycline is approved for use in growth promotion in swine at a dose of 5.5 mg kg^{-1} feed at all stages of production. Tylosin can be used at the following levels for growth promotion: 44 mg kg^{-1} feed in starters, 22 mg kg^{-1} feed in growers, and 11 mg kg^{-1} feed in finishers (Canadian Food Inspection Agency, 2014).

The various classes of antibiotics are found in Table 2.1. In general, antibiotics act on one of three bacterial targets: 1) cell wall biosynthesis, 2) protein synthesis, and 3) DNA

replication or repair (Walsh, 2000). Antibiotics can be further classified as either bacteriostatic or bactericidal. Bacteriostatic agents inhibit cell growth without affecting cell viability, while bactericidal antibiotics induce cell death (Kohanski et al., 2010). Bactericidal antibiotics are able to generate hydroxyl radicals which damage proteins, DNA, and membrane lipids (Kohanski et al., 2007). The cell-wall inhibitors (i.e. β-lactams, glycopeptides) as well as aminoglycosides and fluoroquinolones constitute the major classes of bactericidal antibiotics. Bacteriostatic antibiotics include macrolides, tetracyclines, chloramphenicol, and streptogramins (Kohanski et al., 2010).

Table 2.1. Major antibiotic classes, their targets, mechanism of action, and classification by Health Canada based on their importance in human medicine. Their use in swine production refers to drug classes approved for use in-feed in Canada for swine. G+ = Gram-positive and G- = Gram-negative.

Class	Drugs	Mode of inhibition	Target		Range of species	Derivation	Health Canada classification	Class used in swine (Yes/no)
Fluoroquinolones	Nalidixic acid, ciprofloxacin, levofloxacin, gemifloxacin	DNA synthesis	Topoisomerase II,topisomerase IV	Bactericidal	Aerobic G+ and G-, anaerobic	Synthetic	Very high importance	No
Trimethoprim-sulfamethoxazole	Co-trimoxazole	DNA synthesis	Tetrahydrofolic acid synthesis inhibitors	Bacteriostatic	Aerobic G+ and G-	Synthetic	High importance	No
Rifamycins	Rifamycin, rifampin, rifapentine	RNA synthesis	DNA-dependent RNA polymerase	Bactericidal	G+ and G-and *M. tuberculosis*	Natural	Very high importance	No
β-lactams	Penicillin, cephalosporin, carbapenem	Cell wall synthesis	Penicillin binding proteins	Bactericidal	Aerobic and anaerobic G+ and G-	Natural and semi-synthetic	Very high/high importance	Yes
Glycopeptides	Vancomycin, teicoplanin	Cell wall synthesis	Peptidoglycan subunits (terminal D-Ala-D-Ala)	Bactericidal	Gram-positive	Natural and semi-synthetic	Very high importance	No
Aminoglycosides	Gentamycin, tobramycin, streptomycin, kanamycin	Protein synthesis	30S ribosome	Bactericidal	Aerobic G+ and G-, *M. tuberculosis*	Natural and semi-synthetic	High importance	Yes
Tetracyclines	Tetracycline, doxycycline, chlortetracycline	Protein synthesis	30S ribosome	Bacteriostatic	Aerobic G+ and G-	Natural and semi-synthetic	Medium importance	Yes
Macrolides	Erythromycin, azithromycin, tylosin	Protein synthesis	50S ribosome	Bacteriostatic	Aerobic and anaerobic G+ and G-	Natural and semi-synthetic	High importance	Yes
Streptogramins	Dalfopristin, pristinamycin, virginiamycin	Protein synthesis	50S ribosome	Bacteriostatic	Aerobic and anaerobic G+ and G-	Natural and semi-synthetic	High importance	Yes
Amphenicols	Chloramphenicol	Protein synthesis	50S ribosome	Bacteriostatic	G+ and G-	Natural and semi-synthetic	Medium importance	No

2.2.1 Chlortetracycline

Chlortetracycline (Figure 2.1) was the first tetracycline antibiotic discovered and was isolated from *Streptomyces aureofaciens* (Duggar, 1948). It has a broad-spectrum of activity, affecting both Gram-negative and Gram-positive bacteria, as well as some protozoa. Tetracyclines function by binding to the 30S ribosomal subunit and preventing the association of aminoacyl-tRNA with the ribosome. As a result, protein synthesis is inhibited (Chopra and Roberts, 2001). Resistance to the tetracyclines was virtually unheard of prior to the mid-1950s; however, the widespread use of tetracyclines in human and veterinary medicine has led to an increase in resistance to this class of antibiotics (Chopra and Roberts, 2001).

In addition to growth promotion, chlortetracycline is approved for treatment of swine infections in Canada. It may be used at a level of 55 mg kg^{-1} feed to maintain weight during atrophic rhinitis or prevent bacterial enteritis. Higher concentrations (110 mg kg^{-1}) are permitted for the treatment of bacterial enteritis, while porcine proliferative enteropathy caused by *Lawsonia intracellularis* may be prevented using feed supplemented with 22 mg chlortetracycline kg^{-1} feed. In all cases chlortetracycline must be withdrawn seven days prior to slaughter (Canadian Food Inspection Agency, 2014).

In humans, tetracyclines are used as either a primary or alternative treatment to treat infections due to a variety of agents, such as: *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Chlamydia trachomatis*, mefloquine-resistant *Plasmodium falciparum, Borrelia burgdorferi*, *Bartonella henselae, Yersinia pestis*, and *Francisella tularensis*. The antibiotic tetracycline also has non-antibacterial properties such as immunosuppression, lipase and collagenase inhibition,

increased wound healing, and anti-inflammation. As a result, tetracyclines often get used in acne or rosacea treatment (Eliopoulos and Roberts, 2003).

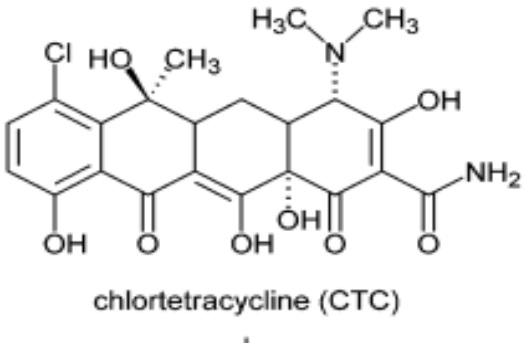

Figure 2.1. Structure of chlortetracycline (Reproduced from Eichhorn and Aga, 2004).

2.2.2 Tylosin

Tylosin (Figure 2.2) is a macrolide antibiotic which binds to the 50S ribosomal subunit and interacts with 23S RNA. More specifically, aminoacyl-tRNA is blocked from binding to the A site on the ribosome thereby inhibiting protein synthesis (Katz and Ashley, 2005). Tylosin is produced through fermentation by the bacterium *Streptomyces fradiae* (Zalacain and Cundliffe, 1989). Macrolides such as tylosin are generally more effective against Gram-positive bacteria compared to Gram-negative bacteria due to the relative impermeability of the outer membrane in the latter (Leclercq and Courvalin, 1991). Tylosin is a first-generation macrolide with a 16-member lactone ring and has been used to treat respiratory infections in animals caused by *Pasteurella multocida*, *Mannheimia haemolytica,* and *Haemophilus* spp. (Katz and Ashley, 2005). In pigs, tylosin can also be used to treat porcine proliferative enteropathy by *Lawsonia intracellularis,* an obligate intracellular Gram-negative bacterium (Lee et al., 2001).

Concentrations of tylosin used for therapeutic purposes range from 44 mg kg^{-1} to 110 mg kg^{-1} feed in Canada (Canadian Food Inspection Agency, 2014).

Tylosin, as used in agriculture, is comprised of 80-90% tylosin A with smaller amounts of tylosin B, tylosin C, and tylosin D (Loke et al., 2000). In animals receiving tylosin orally, approximately 29% of the microbiologically active component may be recovered in fecal samples (Teeter and Meyerhoff, 2003). Although tylosin is used only in animals, it is structurally related to erythromycin, another macrolide antibiotic which is used in humans and animals (Chen et al., 2008). In humans, macrolides may be used to treat infections caused by *Streptococcus pneumoniae*, *S. pyogenes*, *Staphylococcus aureus*, and some other pathogens such as *Legionella pneumophila*, *Chlamydophila pneumoniae,* and *Mycoplasma pneumoniae* (Wierzbowski et al., 2006).

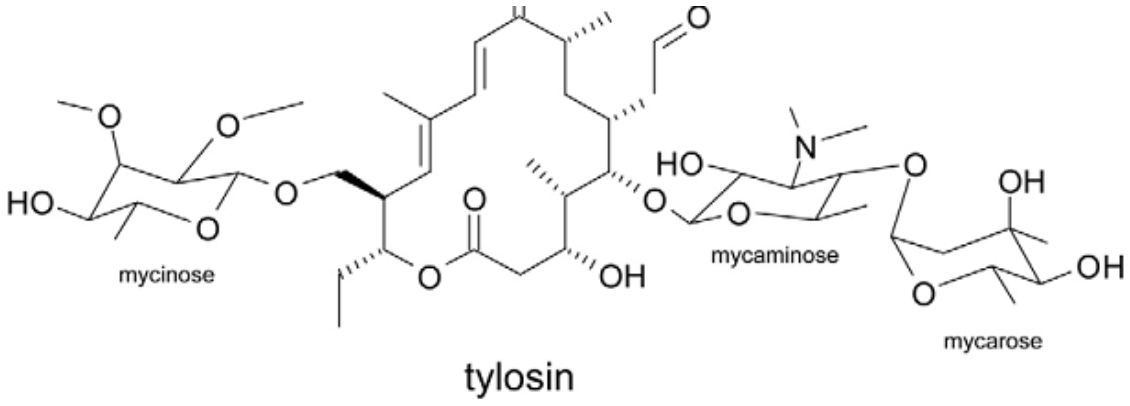

Figure 2.2. Structure of tylosin (Reproduced from Mankin, 2008).

2.3 Antimicrobial resistance

The introduction of any new antimicrobial agent has frequently been followed by the emergence of resistance to that particular agent (Aarestrup, 2004). Multidrug resistant strains were first observed in Japan in the 1950s where *Shigella dysenteriae* and *Escherichia coli* strains resistant to chloramphenicol, tetracycline, streptomycin, and sulfonamides were isolated (Akiba et al., 1960). Akiba et al. (1960) were able to demonstrate experimentally that *E. coli* resistant to these antibiotics were able to transfer the same resistance phenotype to *S. dysenteriae.* Asai and colleagues (2005) found that the volume of antimicrobials used in swine correlated significantly with resistance rates.

In Canada, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) is a national surveillance program designed to monitor antimicrobial use and resistance in humans, animals, and food products. The CIPARS monitors and characterizes samples from humans in the clinical setting, from animals and the environment at the farm level, abattoirs, and retail market. Much of the focus is on swine, poultry, and cattle, with particular attention given to resistance in foodborne pathogens such as *Salmonella* spp., *E. coli*, and *Campylobacter*. For example, 83% of *E. coli* and 71% of *Salmonella* isolates from swine farms are resistant to at least one antimicrobial agent (Public Health Agency of Canada, 2009).

The use of antimicrobial agents inhibits or kills susceptible organisms while those carrying resistance determinants survive and propagate (Levy and Marshall, 2004). Resistance to antimicrobials can either be intrinsic or acquired. Intrinsic mechanisms of resistance are most often due to a lack of required antimicrobial targets in the cell or the inaccessibility of the antimicrobial agent to these targets. Intrinsic resistance is a characteristic present in all bacterial

cells of a given species or genera, whereas acquired resistance is a strain phenomenon (Schwarz et al., 2006).

Acquired resistance includes gene mutations that alter drug targets, as well as the transfer of resistance determinants via plasmids, transposons, integrons, bacteriophages, and other mobile genetic elements (Alekshun and Levy, 2007). These resistance determinants can be transferred among bacteria of different species and evolutionary background (Levy, 2002). In bacteria, the majority of phenotypic antimicrobial resistance is a result of horizontal gene transfer (HGT) (Boerlin and Reid-Smith, 2008). Although the origin of these resistance genes is often unknown, most are generally believed to come from environmental microorganisms, such as the natural producers of the antibiotic (Lu et al., 2004).

2.3.1 Mobile genetic elements

Mobile genetic elements are DNA sequences that contain genes that mediate their movement between bacterial cells and genomes. Mobile genetic elements include plasmids, transposons, insertion sequences, and integrons and are where the majority of antimicrobial resistance genes are located (Nwosu, 2001, Frost et al., 2005).

2.3.1.1 Plasmids

Plasmids are self-replicating extra-chromosomal DNA molecules that often carry genes needed for survival under certain environmental conditions such as exposure to antibiotics, heavy metals, or sanitizers. Other genes found on plasmids may be needed to utilize a particular nutrient, or function as virulence factors (Bennett, 2008). Plasmids can be either conjugative or mobilizable. Conjugative plasmids encode all the genes necessary for cell-to-cell transfer, while mobilizable plasmids require the presence of a conjugative plasmid to transfer between cells (Smillie et al., 2010). As such, conjugative plasmids tend to be larger than mobilizable plasmids,

>30 kb vs. <10 kb respectively (Bennett, 2008). Resistance genes encoded on multi-resistance plasmids may be physically linked together and as a result the presence of one antimicrobial agent can provide sufficient selective pressure for the maintenance of all plasmid-borne resistance determinants (Schwarz et al., 2006). Virulence genes may also be physically linked with antimicrobial resistance genes and this is one of the possible explanations for the increased phenotypic display of antimicrobial resistance in pathogens compared to commensals (Boerlin and White, 2006).

2.3.1.2 Transposons and insertion sequences

Transposons are mobile DNA elements that can carry antimicrobial resistance genes, in addition to the genes required for their own transposition (e.g. transposase). They are able to move from one DNA molecule to another, such as from a plasmid to the chromosome. In general, DNA homology is not required for the insertion of the transposon into a DNA molecule, although there are a few exceptions (Bennett, 2008). Insertion sequences (ISs) are similar to transposons, differing in that they encode only those genes necessarily for transposition (Mahillon and Chandler, 1998). Composite transposons are comprised of regions of DNA, e.g. antibiotic resistance genes, flanked by ISs. These ISs can be either direct or inverted repeats. Simple transposons on the other hand, only have short inverted repeats on either end of the DNA region. There also exist so-called conjugative transposons which can be located on the chromosome or a plasmid. Unlike other transposons, however, conjugative transposons do not encode for a transposase, instead they use other mechanisms for transposition such as site-specific recombination (Roy, 2009). Conjugative transposons may also aid in the transfer of mobilizable plasmids to other cells (Alekshun and Levy, 2007).

A large number of transposable elements have been identified in swine feces. For example, a recent study in China involved screening swine manure taken from farms where antimicrobials are widely used. A total of 149 different antibiotic resistance genes were detected and these genes were highly correlated with the number of transposases in each sample (Zhu et al., 2013). Similarly, Lamendella et al. (2011) conducted a comparative metagenomic study of the swine gut microbiome and observed a large diversity of mobile genetic elements with 42% of the contigs over 500 bp in length matching putative transposases.

2.3.1.3 Integrons

Integrons are mobile genetic elements that incorporate and express exogenous open reading frames (ORFs), also known as gene cassettes (Mazel, 2006). Integrons are composed of an integrase gene (*intI*), an attachment site (*attI*), and a promoter which ensures the expression of the acquired ORF (Carattoli, 2001). Integrons can acquire resistant gene cassettes and reside on conjugative plasmids, transposons, or the chromosome where they can contribute to the dissemination of antibiotic resistance genes (Boucher et al., 2007). Mobile integrons are classified based on the sequence of the *intI* gene. Although five classes of integrons have been identified, the majority of clinically-relevant integrons with antibiotic resistance genes belong to class 1 (Gillings et al., 2008).

Antibiotic-resistant *E. coli* and *Salmonella* spp. isolated from swine fecal samples have been found to be carrying class 1 and class 2 integrons (Lapierre et al., 2010; San Martin et al., 2008). Marchant et al. (2013) reported that 80% of swine intestinal *E. coli* isolates were carrying class 1 or class 2 integrons. The *aad* (streptomycin 3'-adenyltransferase) gene and *dfrA* (dihydrofolatereductase – trimethoprim resistance) gene were found to be the most frequently detected antibiotic resistance gene cassettes in these integrons. In addition, the presence of an

integron was associated with resistance to 10 different antimicrobials (Marchant et al., 2013). A recent study by de la Torre and others (2014) also noted an increase in class 1 and class 2 integrons over time in swine production.

2.3.1.4 Bacteriophages

Viruses that infect bacterial cells are referred to as bacteriophages and they are widely disseminated across almost all environments, including the mammalian gut (Brabban et al., 2005). Depending on their life cycle within the bacterial cell, they can be classified as either lyric or temperate. After attaching and entering a bacterial cell, a lytic bacteriophage replicates using the host bacterium's cellular machinery, followed by the lysis of the host bacterial cell whereby new bacteriophage particles are released. Temperate or lysogenic bacteriophages, do not result in bacterial cell lysis, instead the bacteriophage integrates into the host bacterium's genome where the bacteriophage becomes a prophage and is replicated alongside the host bacterium's genome (Dalmasso et al., 2014). A temperate bacteriophage may be induced to enter the lytic lifecycle following exposure to environmental triggers such as UV or chemicals that affect DNA metabolism (Brabban et al., 2005).

Recently, Allen et al. (2011) reported that prophages in the swine gut microbiota could be induced following exposure to the antibiotic AS250 (100 mg chlortetracycline kg^{-1} feed, 100 mg sulfamethazine kg^{-1} feed, 50 mg penicillin kg^{-1} feed). AS250, however, had no effect on the number of resistance genes identified within the swine virome (Allen et al., 2011). Although infrequent, host cell DNA may be incorporated and assembled into the bacteriophage head during the excision of the bacteriophage from the host chromosome or plasmid. As a result, the bacteriophage is carrying foreign DNA as well as bacteriophage DNA, which can be introduced

into another bacterial cell following attachment and entry of the bacteriophage. This mechanism of genetic exchange between bacterial cells is termed transduction (Frost et al., 2005).

2.3.2 Horizontal gene transfer

Horizontal gene transfer refers to the exchange of mobile genetic elements among bacteria, and moreover, it is the lateral nonsexual transmission of genetic material from one organism to another. Horizontal gene transfer occurs mainly through three mechanisms: conjugation, transduction, and transformation (van Elsas and Bailey, 2002).

2.3.2.1 Conjugation

The self-exchange or transfer of a conjugative plasmid or transposon from a donor cell to a recipient cell is referred to as conjugation. In order for conjugation to occur, the donor and recipient cells must be in close proximity to one another, and a transfer apparatus is necessary to promote exchange (Schwarz et al., 2006). Due to the fact that conjugation can occur between unrelated species, this is the primary method of HGT of resistance genes in the environment (Jonas et al., 2001). In relation to swine production, the ability of *rmtB*-positive *Enterobacteriaceae* isolates from two swine farms to transfer *rmtB* through conjugation was demonstrated *in vitro* by Chen et al. (2007). The rmtB gene confers resistance to aminoglycoside antibiotics through the post-transcriptional methylation of 16S rRNA (Chen et al., 2007).

2.3.2.2 Transduction

Transduction is mediated by bacteriophages which attach to bacterial cells, release their DNA inside the cell, and then replicate or integrate into the host cell genome. This HGT mechanism is limited by the size of the DNA molecule that can be packaged into the phage as well as the requirement of specific receptors on host cells for transduction (Schwarz et al., 2006;

Zaneveld et al., 2008). In specialized transduction, DNA near the bacteriophage integration site in the host bacterium is exchanged for bacteriophage DNA. It is important to note, however, that certain genes must be included in the bacteriophage genome for the newly recombined bacteriophage to remain viable (Brabban et al., 2005). In generalized transduction, any DNA from the host bacterium may be included in the bacteriophage capsid. While both temperate and lytic bacteriophages can be involved in generalized transduction the contribution by lytic bacteriophages is thought to be relatively small (Brabban et al., 2005).

2.3.2.3 Transformation

Transformation is a HGT mechanism which involves the uptake of naked DNA from the environment followed by recombination and expression in the host cell. Natural transformation as a means of HGT is limited by the fact that most exogenous DNA is degraded and that host cells must be competent for DNA uptake (Oyarzabal et al., 2007, Thomas and Nielsen, 2005). Among known bacterial species, around 1% are thought to be naturally competent for transformation (Thomas and Nielsen, 2005). *In vitro*, erythromycin resistance via natural transformation has been observed in *Campylobacter coli* isolates from swine (Kim et al., 2006). Similarly, a chromosomally located *erm*(B) in *C. coli* isolates taken from swine exhibiting a high level of resistance to macrolides could be transferred into *Campylobacter jeuni* via natural transformation (Qin et al., 2014).

2.3.3 Mechanisms of antibiotic resistance

There are three main mechanisms of antibiotic resistance: 1) enzymatic inactivation, 2) target site modification, and 3) efflux or permeability changes in the membrane (Silva, 1996). Enzymatic inactivation as a means of resistance can result in either the irreversible destruction or modification of the antimicrobial agent. The production of β-lactamase, which inactivates and

hydrolyzes β-lactam antibiotics such as penicillin, is an example of the former. The latter is represented by enzymes that acylate, phosphorylate, glycosylate, or adenylate the antimicrobial agent (D'Costa and Wright, 2009; Nikaido, 2009). The antimicrobial target in the cell can also be altered or modified to prevent the agent from binding. Ribosomal protection proteins and rRNA methylases, are two examples of resistance mediated by target modification (Connell et al., 2003; Vester and Douthwaite, 2001).

Efflux systems provide resistance by pumping antimicrobials out of the cell. Efflux systems can be either specific for a particular antimicrobial agent or class, or they remove a number of unrelated antimicrobials. Antimicrobial-specific efflux systems tend to be located on the aforementioned mobile genetic elements, i.e. plasmids, transposons, or integrons, while the multidrug efflux systems are usually found on the chromosome (Poole, 2007). Mutations in efflux systems may result in an increase in the expression of the efflux pump protein or increase the export efficiency of the protein (Piddock, 2006). In Gram-negative bacteria, porin molecules in the outer membrane may be altered such that the ability of the antibiotic to cross the membrane and enter the cell is greatly diminished (Delcour, 2009).

2.3.4 Resistance to chlortetracycline

Resistance to the tetracyclines is usually a result of the acquisition of new genes rather than a chromosomal mutation, with these genes often located on mobile genetic elements (Roberts and Schwarz, 2009). To date, 45 tetracycline resistance genes have been identified (Table 2.2). The *tet* genes are considered to be distinct genes if they contain ≤ 79% amino acid sequence identity. These genes fall into three categories: efflux proteins, ribosomal protection proteins, and enzymatic inactivation. There is also one gene whose product function is unknown, *tet*(U) (Roberts, 2005). There are currently 29 known genes that encode efflux

proteins, 12 that encode for ribosomal resistance proteins, and three that encode for inactivating enzymes.

Table 2.2 Known tetracycline resistance genes as of October 2014 (Roberts, 2014a).

Efflux	Ribosomal protection	Enzymatic	Unknown
tet(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(V), *tet*(Y), *tet*(Z), *tet(*30), *tet*(31), *tet*(33), *tet*(35), *tet*(39), *tet*(41), *tet*(K), *tet*(L), *tet*(38), *tet*(45), *tetA*(P), *tet*(40) *otr*(B), *otr*(C), *tcr* *tet*(42), *tet*(43), *tet*AB(46)	*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(32) *tet*(Q), *tet*(T), *tet*(36) *otr*(A), *tetB*(P), *tet, tet*(44)	*tet*(X),*tet*(37), *tet*(34)	*tet*(U)

All of the *tet* efflux genes encode for a 46 kDa membrane-bound efflux protein (Roberts and Schwarz, 2009). These efflux proteins are membrane proteins that function in an energy-dependant manner to export tetracycline out of the cell by exchanging a proton for a tetracycline-cation complex against a concentration gradient (Roberts, 2005). In Gram-negative bacteria, the tetracycline efflux genes have a repressor that is upstream of the structural gene and transcribed in the opposite direction. In the presence of tetracycline, a tetracycline-Mg^{2+} complex forms which is able to bind to the *tet* repressor protein and induce transcription of the *tet* resistance gene. In the absence of tetracycline, the *tet* repressor protein binds to the operator site and prevents transcription by blocking access to the promoter site (Zakeri and Wright, 2008). In Gram-negative bacteria isolated from humans and animals, *tet*(A), *tet*(B), *tet*(C), *tet*(D), and *tet*(H) are most commonly identified (Schwarz et al. 2006). The *tet*(K) and *tet*(L) genes are more

frequently associated with Gram-positive bacteria and are not regulated by a repressor but rather their expression is controlled by translational attenuation which requires the presence of tetracycline for induction (Poole, 2007).

Ribosomal protection proteins (RPP) are cytoplasmic proteins that are able to release tetracycline from its binding site through conformational change of the ribosome (Connell et al., 2003). They exhibit a high degree of similarity to elongation factors EF-Gu and EF-Tu, and have similar GTPase activity to these two proteins (Zakeri and Wright, 2008). Tet(O) and Tet(M) are the best studied of the RPPs. Tet(O) for example, binds near the A-site on the ribosome causing a conformational change which prevents the binding of tetracycline (Spahn et al. 2001). The RPPs are Gram-positive in origin, but have also been found in a variety of Gram-negative genera (Schwarz et al., 2006).

Tetracyclines may also be inactivated with the enzymatic products of *tet*(X), *tet*(34), and *tet*(37). The *tet*(X) protein product is an oxygen-dependent flavoprotein that hydroxylates tetracycline in a regioselective manner, yielding a product that is unstable and decomposes non-enzymatically. Although oxygen-dependent, this gene has only been identified on transposons isolated from the anaerobic *Bacteriodes* genus (Yang et al., 2004). The *tet*(34) and *tet*(37) genes are also classified as encoding tetracycline inactivation proteins, however, both remain poorly characterized (Thaker et al., 2010). Mutants that downregulate the expression of porin channels on the outer membranes may also exhibit greater impermeability to tetracyclines. A change at base 1058 (*E. coli* numbering) in the 16S rRNA from guanosine to cytosine has been demonstrated to confer resistance to tetracycline in *Propionibacterium* (Ross et al., 1998).

2.3.4.1 Chlortetracycline resistance in swine

Blake et al. (2003) reported that 80% of the tetracycline-resistant *E. coli* isolated from a group of pigs raised under conventional conditions carried either *tet*(B) or *tet*(G). The *tet*(C) gene was found in 20% of the isolates, while none had *tet*(A), *tet*(D), *tet*(E), or *tet*(H). In samples taken from a farm where antimicrobials hadn't been used for 3 years, 62% of *E. coli* isolates were resistant to 16 μg ml^{-1} of tetracycline. The gene resistance pattern for these isolates was different, however, with tetracycline-resistant *E. coli* harbouring only *tet*(A) or *tet*(C). Isolates with *tet*(B) or *tet*(G) had significantly higher MICs than those carrying *tet*(A) or *tet*(C) indicating that efflux proteins do not all have the same efficacy (Blake et al., 2003). These researchers also demonstrated that the MICs of tetracycline-resistant *E. coli* in samples taken from antimicrobial use farms were higher than from the antimicrobial-free operation. In addition, the presence of *tet*(B) or *tet*(G) conferred greater resistance to tetracycline than the other *tet* genes identified (Blake et al., 2003). In *E. coli* isolated from healthy swine, the genes *tet*(A) and *tet*(B) have been found to reside on large conjugative plasmids, with both genes located within transposons (Sunde and Sorum, 2001). In fact, almost all *tet* genes are found on mobile genetic elements (Poole, 2007).

Chuanchuen and Padungtod (2009) isolated a number of *Salmonella enterica* serovars from swine and screened them for resistance to several antimicrobials, including tetracycline. Using PCR, they further screened the tetracycline-resistant isolates for *tet*(A) and *tet*(B). It was found that 45% of isolates carried *tet*(A), 4% had *tet*(B) only, and 8% had both genes (Chuanchuen and Padungtod ,2009). Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen in humans and can also cause infection in swine. One study found that all MRSA isolates taken from diseased swine in Germany were also resistant to tetracycline.

Resistance to tetracycline in these isolates was shown to be conferred through *tet*(M), *tet*(K) and/or *tet*(L). The majority (74%) had both *tet*(M) and *tet*(K), while 20% carried all three genes. In addition, 44% of the MRSA isolates also carried the macrolide resistance genes *erm*(A), *erm*(B), or *erm*(C) (Kadlec et al., 2009). A recent survey of Chinese swine farms reported the detection of 22 out of 28 targeted *tet* genes in manure samples (Zhu et al., 2013). Soil samples that are taken from agricultural fields where swine manure is applied as a fertilizer have also been observed to harbour a variety of different *tet* genes (Ghosh and LaPara, 2007).

Several studies have reported a relatively high level of tetracycline resistance even in isolates from swine that received no antibiotics (Gebreyes et al., 2005; Wagner et al., 2008). A study by Gellin et al. (1989) found a significantly lower prevalence of tetracycline resistance (MIC 64 μg ml^{-1}) in Gram-negative enteric bacteria isolated from swine who had not been exposed to any antibiotics in 13 years (4.8 to 20.3%) compared to swine that had received subtherapeutic doses (86.1 to 96.8%) or therapeutic concentrations (53.5 to 67%) of antimicrobials, including chlortetracycline. Tetracycline resistance was also significantly higher in swine given subtherapeutic concentrations of antimicrobials when compared to swine given antimicrobials only to treat disease (Gellin et al., 1989). It is again interesting to note the relatively high prevalence (up to 20.3%) of tetracycline resistance in isolates from swine reared in antimicrobial-free conditions (Gellin et al., 1989). In Alberta and Saskatchewan (Canada), 47%, 27.4%, and 22.9% of fecal *Salmonella* isolates from nursery, grow-finish pigs, and sows respectively, were determined to be resistant to tetracycline (Rosengren et al., 2008b). Also of note, wild small animals such as shrews, voles, and mice, found on or near swine farms, have been observed to carry a significantly greater number of tetracycline-resistant *E. coli* than those found in natural areas (Kozak et al., 2009).

Looft et al. (2012) used a metagenomic approach together with real-time PCR to investigate the effect of feeding ASP250 (100 mg chlortetracycline kg^{-1} feed, 100 mg sulfamethazine kg^{-1} feed, 50 mg penicillin kg^{-1} feed) to post-weaned swine for a 21 d period. These authors found a higher abundance of *tet*(B), *tet*B(P), *tet*(M), *tet*(O), and *tet*(Q) in the ASP250 treated pigs, along with several other antibiotic-resistance genes (Looft et al., 2012). The diversity of antibiotic resistance genes detected was also increased as a result of ASP250 treatment. In addition, non-medicated swine carried a large variety of antibiotic resistance genes, with a total of 50 different antibiotic resistance genes detected in these untreated pigs (Looft et al., 2012)

2.3.5 Resistance to tylosin

Resistance to tylosin and other macrolides, as well as the structurally unrelated lincosamide and streptogramin B (MLS_B) antibiotics, is mediated by rRNA methylases, efflux proteins, or inactivation enzymes. To date, there are 67 genes that are known to confer resistance to macrolides (Table 2.3). The most widespread and clinically important of these resistance mechanisms are the rRNA methylases (Eliopoulos and Roberts, 2003). The erythromycin ribosome methylation (*erm*) genes reduce the binding of MLS_B antibiotics to 23S rRNA by adding one or two methyl groups to an adenine residue at position 2058 (*E. coli* numbering) in domain V of 23S rRNA. As a result, macrolides can no longer bind to this target. This methylation confers resistance to all MLS_B antibiotics as a result of the overlap in binding sites (Chen et al., 2008; Leclercq, 2002).

Table 2.3. Known macrolide resistance genes as of October 2014 (Roberts, 2014b).

rRNA methylase	Efflux	Inactivating enzymes			
		esterases	lyases	transferases	phosphorylases
erm(A), *erm*(B), *erm*(C), *erm*(D), *erm*(E), *erm*(F), *erm*(G), *erm*(H), *erm*(I), *erm*(N), *erm*(O), *erm*(Q), *erm*(R), *erm*(S), *erm*(T), *erm*(U), *erm*(V), *erm*(W), *erm*(X), *erm*(Y), *erm*(Z), *erm*(30), *erm*(31), *erm*(33), *erm*(32), *erm*(33), *erm*(34), *erm*(35) *erm*(36), *erm*(37), *erm*(38), *erm*(39), *erm*(40), *erm*(41), *erm*(42), *erm*(43)	*mef*(A),*mef*(B), *msr*(A),*msr*(C), *msr*(D),*msr*(E), *car*(A), *lmr*(A), *ole*(B), *ole*(C) *srm*(B), *tlc*(C) *lsa*(A), *lsa*(B), *lsa*(C), *lsa*(E), *vga*(A)*, *vga*(B), *vga*(C), *vga*(D), *vga*(E), *eat*(A)$_v$, *sal*(A)	*ere* (A), *ere*(B)	*vgb*(A), *vgb*(B)	*lnu*(A), *lnu*(B), *lnu*(C), *lnu*(D), *lnu*(E), *lnu*(F), *vat*(A), *vat*(B), *vat*(C), *vat*(D), *vat*(E), *vat*(F), *vat*(H)	*mph*(A), *mph*(B), *mph*(C), *mph*(D), *mph*(E), *mph*(F)

Similar to the tetracycline resistance determinants, the *erm* genes are most often located on mobile genetic elements (Leclercq, 2002). These resistance genes have been isolated from a number of Gram-positive and Gram-negative bacteria, including anaerobes (Roberts et al., 1999). The *erm* genes are regulated by translational attenuation and can be either constitutive or inducible. Inducible resistance is a posttranscriptional regulatory system that requires the presence of a macrolide (inducer) for translation of the mRNA to occur. For example, in *erm*(C), the inducer-ribosome complex causes a change in the conformation of the upstream leader peptide permitting translation of the *erm*(C) mRNA (Roberts et al., 1999; Weisblum, 1995). Constitutively resistant bacteria translate *erm* mRNA even in the absence of the macrolide inducer. Most often this form of *erm* expression is due to a mutation/deletion event in the attenuator region. Constitutively resistant *erm* bacteria have become more prevalent than inducible ones in recent years (Depardieu et al., 2007; Roberts et al., 1999).

The efflux proteins confer resistance to a macrolides by pumping the antibiotic out of the cell, thereby reducing intracellular antibiotic concentration. Most of these belong to either the ABC transporter superfamily or major facilitator superfamily of efflux proteins. The drug-resistance pattern varies depending on the gene (Leclercq, 2002; Roberts et al., 1999). The inactivating enzymes are encoded by 23 different genes are comprised of 2 esterases, 2 lyases, 13 transferases, and 6 phosphotransferases (Roberts, 2014).

2.3.5.1 Tylosin resistance in swine and other livestock

In a study by Berrang et al. (2007), broiler chicken were inoculated with *Campylobacter jejuni* and fed 22 mg tylosin phosphate kg^{-1} feed. All *Campylobacter* spp. subsequently isolated were resistant to tylosin phosphate (MIC 16 μg ml^{-1}) and erythromycin (MIC 128 μg ml^{-1}), while none of the control broiler chickens carried resistant *Campylobacter* spp. The addition of tylosin to feed has also been shown to increase the incidence of both erythromycin and tetracycline resistant genes in cattle (Chen et al., 2008). The *erm*(ABCF) genes have been isolated from swine waste lagoons as well as in wells impacted by these lagoons (Koike et al., 2010).

Jackson et al. (2004) investigated the effect of tylosin at concentrations used for growth promotion or disease treatment in swine production as it relates to erythromycin resistance in *Enterococci*. Use for growth promotion was defined as finishing swine being given feed supplemented with 10 mg tylosin kg^{-1} feed at the finishing stage while disease treatment involved giving tylosin to pigs for 5 days or less as they entered the nursery to control scours. These researchers found that the incidence of erythromycin-resistant *Enterococci* spp. (MIC ≥ 8 μg ml^{-1}) was 59%, 28%, and 2% for the growth promotion, disease treatment, and no tylosin groups respectively. Of the resistant isolates, 96% were positive for *erm*(B) while none were positive for *erm*(A) or *erm*(C). In addition, these authors reported that *erm*(B) was found on the

chromosome in 86% of the *erm*(B)-positive isolates and located on a plasmid in the remaining 14% (Jackson et al., 2004).

Tylosin fed to swine as a growth promoter has been demonstrated to increase resistance not only in the intestinal bacteria of swine but in the microbiota of the skin as well. Aarestrup and Carstensen (1998) recorded an increase in erythromycin resistance in *Staphylococcus hyicus* isolated from swine skin swabs, as well as enterococci recovered from swine feces. In Denmark during the mid-to-late 1990s, legislation and voluntary bans reduced the amount of antimicrobials (including tylosin) that could be used in food animals as a growth promoter. The result, according to Aarestrup et al. (2001), was a subsequent significant decrease in the number of *Enterococci faecalis* strains that were resistant to erythromycin in swine. Similar to tetracycline resistance, relatively high levels of resistance to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics has been reported in swine from organic farms that claimed to be antimicrobial free (Zhou et al., 2009).

Juntunen et al. (2010) fed pigs 140 mg tylosin kg^{-1} feed to treat *Lawsonia intracellularis* proliferative enteropathy. *Campylobacter coli* isolates from treated pigs had significantly higher erythromycin MICs than those obtained from untreated pigs after only four days of treatment. Whereas all *C. coli* isolates were susceptible to erythromycin prior to treatment (MIC $\leq$ 16 μg ml^{-1}) at the end of their study 13 days later, 75% of the *C. coli* isolates had MICs $\geq$ 512 μg ml^{-1}.

2.3.6 Resistance involving other antimicrobials in swine

Several other antimicrobials may also be used in swine production. Matthew and colleagues (2005) fed oxytetracycline (100 mg kg^{-1} feed) to sows 14 days prior to farrowing. The sows had had no previous antibiotic exposure at either therapeutic or subtherapeutic levels. After farrowing, piglets were left to nurse for 21 days, weaned, and then given feed containing

the aminoglycoside apramycin at a dose of 150 mg kg^{-1} feed on day 7 post-weaning and continued for 14 days. Following this period, oxytetracycline was then added at 50 mg kg^{-1} feed through the growing-finish phase. These researchers observed that *E. coli* isolated from pigs farrowed from sows treated with oxytetracycline, were consistently more resistant to tetracycline than control groups (for the first 28 d: 100% vs. 67 to 88% for control pigs). Resistance to apramycin was also higher in *E. coli* isolated from pigs treated with apramycin (for the first 28 d: 8.5 to 53.9% vs. 0 to 23.1% for control pigs). The apramycin resistance was determined to be plasmid-mediated (Mathew et al., 2005).

Similarly, Akwar (2008) reported a significantly higher prevalence of antimicrobial resistance in *E. coli* isolated from farms in Ontario and British Columbia (Canada) that used antimicrobials in feed compared to those farms that did not. On farms using antimicrobials, 68.2% of *E. coli* isolates were resistant to at least one antimicrobial while only 21.8% of isolates from antimicrobial-free farms were. Resistance to two or more antimicrobial agents was also significantly higher in *E. coli* isolates from farms that used antimicrobials in feed (79.9% vs. 52.3%). In addition, weanling pigs had significantly higher numbers of antimicrobial-resistant *E. coli* than did finisher pigs (46.7% vs. 43.3%). Resistance to tetracycline was most frequently observed (Akwar et al., 2008).

Campylobacter spp., particularly *C. coli*, are frequently isolated from swine. Thakur and Gebreyes (2005) found that all *C. coli* isolates tested from both conventional and antimicrobial-free swine operations were positive for *tet*(O) and resistant to tetracycline at the highest MIC (32 mg L^{-1}). The authors also recorded a high level of erythromycin resistance in 16 of 21 *C. coli* isolates. A single base change of adenine to guanine at position A2075G in the peptidyl transferase region of 23S rRNA was identified in all erythromycin-resistant isolates (Thakur and

Gebreyes, 2005). A study by Rollo et al. (2010) recorded significantly higher numbers of azithromycin (69.0% vs. 20.1%), erythromycin (68.3% vs. 21.3%), or tetracycline (74.5% vs. 48.8%) resistant *Campylobacter* spp. isolated from conventional swine farms than from antimicrobial-free operations. These workers also observed a significant decrease in azithromycin- or erythromycin-resistant *Campylobacter* spp. related to the length of time a farm had been antimicrobial-free. The longer a farm was antimicrobial-free, the fewer the number of azithromycin- or erythromycin-resistant bacteria isolated (Rollo et al., 2010).

In *Salmonella* spp., Varga et al. (2009) found no association between reported tetracycline use in swine and tetracycline resistance. They did, however, find a significant association between tylosin use and resistance to ampicillin and streptomycin (Varga et al., 2009). In nursery pigs, 16.9% to 41% of fecal *Salmonella* isolates were found to be resistant to sulfamethoxazole, streptomycin, ampicillin, and/or kanamycin (Rosengren et al., 2008b).

As mentioned previously, antimicrobial resistance can also persist in swine herds even in the absence of antimicrobial use (Kalmokoff et al., 2011; Pakpour et al., 2012). This likely reflects the long period of time that antimicrobials have been used in swine production. In addition, the fitness costs of carrying antimicrobial resistance determinants may be variable in nature (Andersson, 2006). This may also be a result of other dietary additives in swine feed that provide selective pressure for the maintenance of antimicrobial resistance determinants. Zinc and copper, for example, are frequently added to swine feed at levels that exceed normal dietary requirements, usually to promote growth in swine (Jondreville et al., 2003). Due to the fact that genes conferring resistance to these metals are often physically linked together on the same MGE as antimicrobial resistance genes, the continued use of these dietary additives may result in the

persistence of antimicrobial-resistance genes even in the absence of antimicrobial use (Frye et al., 2011).

2.4 Impact of antimicrobial resistance in food animals on public health

Antimicrobial resistance has been called one of the greatest threats to public health (Marshall and Levy, 2011; World Health Organization, 2014). Antimicrobial-resistant infections not only increase morbidity and mortality, but they are more costly to treat (Roberts et al., 2009; Mauldin et al. 2010). This is a situation that has been made worse by the lack of new antimicrobial drugs in the discovery pipeline (Livermore et al., 2012; Piddock, 2012). The magnitude of the role that agriculture plays in this issue is unknown, however, antimicrobial use in agricultural animals selects for resistant bacteria, which in turn may serve as a reservoir of resistant bacteria and resistance determinants (Wegener, 2003). Resistant bacteria, both pathogenic and commensal, and/or resistance determinants may then be passed to humans through food, direct contact with animals, or release of animal waste into the environment (Barza, 2002; Silbergeld et al., 2008; Witte, 1998). Antimicrobial residues may also persist in animal food products (Kantiani et al., 2010). An increase in antimicrobial resistance may also reduce the efficacy of antimicrobial drugs used to treat patients empirically before microbiological analysis can be performed (Molbak, 2006).

The threat to human health posed by the agricultural use of antimicrobials has been a source of concern dating back over 40 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, 1969). It was also recommended that drugs such as tylosin and tetracycline not be used in feed without a prescription. Despite these recommendations, antimicrobials continued to be used in animal agriculture around the world. Since 2006, the European Union

has banned the use of antibiotics in food-producing animals for the purpose of growth promotion (European Union, 2005). Recently, however, in both Canada and the United States, the government has 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 (United States Food and Drug Administration, 2013; Health Canada, 2014).

2.4.2 Direct impact on human health

Macrolides are categorized by Health Canada (2009) as antimicrobial drugs of high importance in human medicine, while tetracyclines are considered to be of medium importance. People with occupations that put them in close contact with swine have been shown to be more likely to carry antimicrobial-resistant *E. coli* than non-swine workers (Alali et al., 2010). Levy et al. (1976) found that after feeding tetracycline-supplemented feed to chickens, 5 to 6 months later 31.3% of fecal samples from farm residents had significantly higher levels of tetracycline-resistant bacteria (>80% resistant) compared to their neighbours (6.8% of fecal samples).

One of the earliest documented foodborne outbreaks involving a multi-drug resistant pathogen of animal origin occurred in the midwestern United States in 1983. A *Salmonella* Newport strain carrying a plasmid conferring resistance to ampicillin, carbenicillin, and tetracycline was isolated from beef cattle and salmonellosis patients. Patients were thought to have consumed hamburger originating from one specific farm. Cattle on the suspect farm had been fed chlortetracycline in their feed at subtherapeutic levels (110 mg kg^{-1} feed; Holmberg et al., 1984). An outbreak of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 in Denmark was directly linked to pork from a slaughterhouse. Two swine herds were found to be carrying this particular strain (Molbak et al., 1999). In fact, living on a livestock farm has been identified as one of the risk factors associated with acquiring this strain (Dore et

al., 2004). Similarly, an outbreak of multidrug resistant *Salmonella enterica* serotype Typhimurium DT193 in England was linked to a single pig farm (Maguire et al., 1993). MRSA carriage and infections in farm workers has also been demonstrated to be linked to contact with swine (Denis et al., 2009; Hartmeyer et al., 2010; Lewis et al., 2008). It is also possible that antimicrobial resistance can lead to more virulent, resistant pathogens as virulence genes and resistance genes can often be linked together when they are transferred (Barza, 2002). In porcine enterotoxigenic *Escherichia coli* isolates for example, the tetracycline resistance gene *tet*(A) has been observed to be linked with several virulence genes (Boerlin et al., 2005). Also, in 1985, an outbreak of chloramphenicol-resistant *Salmonella enterica* serovar Newport infections was linked to the consumption of ground beef that came from a dairy operation that had used chloramphenicol (Spika et al., 1987).

There is, however, some disagreement about whether or not antimicrobial use in livestock poses a significant risk to human health (Casewell et al., 2003; Phillips et al., 2004; Wassenaar, 2005). Phillips (2007) for example, argues that the ban on antimicrobial growth promoters in Denmark lead to a decline in animal health and an increase in therapeutic use of antimicrobials. Singer et al. (2007) note that diseased animals can constitute a greater threat to human health since they may have higher carriage rates of foodborne pathogens and tend to be handled more often than healthy animals, which may lead to cross-contamination between healthy and diseased animals. These authors created a model to estimate the impact of increased *Campylobacter* shedding in chickens on the rate of human campylobacteriosis infections as a result of the removal of subtherapeutic antibiotics. They concluded that a small increase in *Campylobacter*-shedding animals could lead to a large increase in human illness. Singer et al. (2007) also hypothesized that the benefits of banning the use of subtherapeutic antibiotics in

terms of a reduction of antibiotic resistance in foodborne pathogens, is outweighed by the increased risk of infection from animal sources due to presumed higher bacterial loads in these animals.

In contrast, a recent study by Aarestrup et al. (2010) reported that swine productivity in Denmark has actually improved following a ban on antimicrobials for growth promotion. In addition, these researchers found no increase in mortality rate among weanling pigs following the ban, and overall antimicrobial use declined by greater than 50%. Grave and others (2006) also concluded that an increase in therapeutic antimicrobial use was only temporary following a ban on antimicrobials for growth promotion in Sweden and Denmark. In Norway, there was no increase in therapeutic antimicrobial use following the ban (Grave et al., 2006). Likewise, veterinary prescriptions for antimicrobials did not increase following the ban of antimicrobial growth promoters in Switzerland (Arnold et al., 2004). It should be noted that a significant increase in antibiotic use for treating diarrhea in swine was observed in Denmark in the year following the ban, although there was no difference in the treatment of other infectious diseases such as pneumonia or for non-thriving pigs (Vigre et al., 2008).

The horizontal transfer of *tet*(Q) has been demonstrated among different species of *Prevotella* and *Bacteroides* colonizing either the human oral cavity, colon, or animal intestinal tract based on the nearly identical *tet*(Q) sequences found in these bacteria, regardless of host or geographic location. Furthermore, these exchanges of *tet*(Q) were postulated to have occurred very recently (Nikolich et al., 1994). In Norway, the same multi-antibiotic resistant plasmid was isolated from fecal *E. coli* recovered from cattle as well as humans that lived on the farm and from the local veterinarian. The serotypes of the *E. coli* strains differed between cattle and

humans, indicating that horizontal transfer was responsible for the presence of the plasmid in other strains (Oppegaard et al., 2001).

Relatively high levels of antibiotic-resistant *E. coli* have been reported in turkey farmers and slaughterers (79% and 61% respectively for oxytetracycline). These groups had not used any antibiotics in the three months prior to sampling (van den Bogaard et al., 2001). A study by Marshall et al. (1990), demonstrated that swine and cattle inoculated with an *E. coli* strain carrying a plasmid conferring resistance to multiple antibiotics were able to spread the organism to farm workers, adjacent livestock, and flies, in the absence of antibiotic use. Shedding of the bacterium was observed in these farm workers for several weeks. Nijsten et al. (1994) recorded a significantly greater incidence of antibiotic resistance in fecal *E. coli* isolates from pig farmers than from suburban and urban residents who lived in the same area in the Netherlands. Higher incidences of resistance were reported for amoxicillin (62% vs. 47%), neomycin (66% vs. 25%), oxytetracycline (79% vs. 36%), sulfamethoxazole (84% vs. 40%), and trimethoprim (53% vs. 15%) in pig farmers compared with suburban and urban residents.

2.4.2 Dissemination of antimicrobial resistance via groundwater and soil amendment

The amendment of soil with swine manure has been demonstrated to increase the number and diversity of antimicrobial resistance determinants in the soil. Recent work by Marti et al. (2014) showed that soil amended with raw swine manure had an increased abundance of *sul1* (sulphonamide resistance), *str*(B) (streptomycin-resistance), *erm*(B), *int1* (integron integrase), and IncW *repA* (plasmid) genes, although the abundance of each gene decreased in the soil over a one-year period. The manure in this study had been taken from a farm where pigs were medicated with Aureo SP-250 (220 mg chlortetracycline, 220 mg sulfamethazine, and 110 mg penicillin kg^{-1} feed) (Marti et al., 2014). The storage or treatment of swine manure for a period

of time before application to soils may mitigate the impact in terms of the dissemination of antimicrobial resistance to a degree (Ghosh and LaPara, 2007; Joy et al.; 2014; Zhou et al.; 2014). However, some studies have only found a limited or variable decrease in the abundance of certain resistance genes following storage and treatment of swine manure (Chen et al.; 2010).

Groundwater and surface water may also be effected by the runoff and leaching of swine manure and waste. Koike and others (2007) consistently detected seven different tetracycline resistance genes [*tet*(C), *tet*(H), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), and *tet*(Z)] over a three-year period in groundwater sampled from swine farms that used lagoons for holding waste. Antibiotics, including chlortetracycline, had been used for growth promotion and treatment of disease on these farms. These same authors also detected several macrolide resistance genes [erm(A), *erm*(B), *erm*(C), and *erm*(F)] in groundwater at these same swine operations (Koike et al., 2007). Similarly, a study by Sapkota et al. (2007) observed that enterococci isolates from groundwater and surface water down-gradient of a swine farm had higher minimum inhibitory concentrations for erythromycin, tetracycline, clindamycin, and virginiamycin, than isolates from up-gradient water samples.

2.4.3 Retail pork products

Significantly greater numbers of antibiotic-resistant *E. coli* have been isolated from retail pork produced from conventional farms than from organic farms (Miranda et al., 2008). Similarly, Schroeder and others (2003) identified a high prevalence (>70%) of tetracycline-resistant *E. coli* isolates in pork samples taken from retail supermarkets. *Staphylococcus* spp. resistant to several antibiotics have also been isolated from the various stages of swine production including: feces, feedstuffs, and processed pork products. Simeoni et al. (2008)

reported that the majority of these antibiotic-resistant staphylococci were found to carry *tet*(M), *tet*(K), *erm*(B), and *erm*(C).

In a study by Garofalo et al. (2007), all raw pork samples from a meat processing plant in Italy was found to harbour *tet*(M), *tet*(K), and *erm*(B). The authors also noted a relatively high (30% to 75%) incidence of *erm*(A), *erm*(C), and *tet*(O) in these samples. In addition, all of these resistant determinants were detected in the swine fecal samples collected from the slaughterhouses of these plants. *Salmonella* isolates from fresh pork sausages purchased from supermarkets and butcher shops have also been identified as having a high incidence (70.7%) of resistance to tetracycline (Mürmann et al., 2009).

An examination of LAB (lactic acid bacteria) from pork abattoirs in Ireland revealed that 41% of the LAB isolates were resistant to erythromycin. All erythromycin-resistant LAB carried the *erm*(B) gene. Interestingly, only one LAB isolate (6% of isolates) was resistant to tetracycline. This *Lactobacillus plantarum* isolate, however, was able to conjugally transfer *tet*(M) to a strain of *Lactococcus lactis* and *Enterococcus faecalis* through filter mating (Toomey et al., 2010).

2.5 Swine gut microbiota

The gastrointestinal tract of the pig contains a large and diverse number of microorganisms which are collectively known as the gut microbiota (Lamendella et al., 2011). The 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 mammalian gut is home to approximately 10^{14} bacterial cells and 500-1000 species of bacteria, the majority of which are members of only a few different phyla (Gill et al., 2006; Lamendella et al.; 2011; Sommer and Bäckhed, 2013). The gut microbiota is also dominated by anaerobes, with most

belonging to the *Bacteroidetes* and *Firmicutes* phyla (Kim et al., 2011; Sommer and Bäckhed, 2013).

While fewer *Bacteria* are found in the stomach and proximal small intestine (10^3 to 10^5 CFU g^{-1} digesta) due to the rapid rate of digesta movement and the relatively low pH, colonization increases in the ileum (10^8 to 10^9 CFU g^{-1} digesta). With the slower movement of digesta in the colon, 10^{10} to 10^{12} culturable bacteria per g digesta are found in this location (Dibner and Richards, 2005; Leser et al., 2000). The feces of swine is composed of nearly 40% microbial cells by weight (Savage, 1977). The gastrointestinal tract of swine is sterile prior to birth, but quickly becomes colonized with bacteria from the environment, diet, and mother (Dibner and Richards, 2005). The gut microbiota is strongly influenced by diet and anatomy, and not only tends to vary between species but also between animals of the same species. Once established, the microbiota tends to remain stable over time in terms of richness and diversity. Diet serves as both a source of microorganisms as well as substrates for the gut microbiota (Gaskins et al., 2002; Leser and Mølbak, 2009; Pedersen et al., 2013; Pieper et al. 2008). Feed texture and granulometry can also influence the swine gut microbiota (Molbak et al., 2008). The sanitary conditions of the housing environment can alter the gut microbiota, with pigs raised under poor sanitation having been shown to shed more *Lactobacillus* (increase of 0.9 $\log_{10}$ CFU g^{-1} feces) and *Enterobacteria* (increase of 1.0 $\log_{10}$ CFU g^{-1} feces) in the their feces (Montagne et al., 2010).

The gut microbiota is a vital component of a healthy animal. Colonic bacteria metabolize undigested carbohydrates into short-chain fatty acids which are then absorbed by the host. In this way, intestinal bacteria act to recover nutrients from energy sources which would otherwise be lost through excretion (Cummings and Macfarlane, 1997; Laparra and Sanz; 2010). These

commensal 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 (Gaskins, 2001; Lalles et al., 2007). Commensal gut bacteria are largely tolerated by the host immune system and at the same time help stimulate the normal immunological development and homeostasis (Kelly et al., 2005; Sommer and Bäckhed, 2013; Zoetendal et al. 2004).

Traditional culture-based techniques have revealed that the microbiota of the swine gut is comprised largely of Gram-positive, obligate anaerobic bacteria. Bacteria belonging to the genera *Streptococcus*, *Lactobacillus*, *Fusobacterium*, *Eubacterium*, and *Peptostreptococcus* predominate. Only about 10-35% of the culturable bacteria are Gram-negative bacterial species, the majority of which are *Bacteroides* spp. and *Prevotella* spp. (Konstantinov et al., 2003; Moore et al., 1987; Salanitro et al., 1977).

These culture-depended methods are strongly limited, however, by the fact that most microorganisms will not grow under laboratory conditions and therefore cannot be cultured and characterized. In fact, it has been estimated that only 20-40% of gut bacteria are culturable (Suau et al,. 1999). The growth requirements of bacteria in the gut are often unknown and it can be difficult to replicate the interactions of these bacteria with other microorganisms and host cells in the gut (Zoetendal et al., 2004). In addition, classification based on phenotypic characteristics doesn't always correspond accurately to phylogenetic identification. Culture-based methods also take more time to complete than culture-independent techniques (Gaskins, 2001).

In response to these challenges associated with culture-based studies, culture-independent techniques have been developed that eliminate the need for culturing. Most of these approaches make use of the 16S small subunit ribosomal RNA gene (16S rRNA). The archaeal and bacterial 16S rRNA gene (≈ 1550 bp; Figure 2.1) is used as a phylogenetic marker due to its ubiquitous presence in all organisms and the fact that it is functionally highly conserved (Clarridge, 2004; Woese, 1987). Importantly, there are also nine "hypervariable regions" (V1–V9, Figure 2.1) found in the 16S rRNA gene that display a high degree of sequence diversity (Chakravorty et al., 2007). A number of PCR primers have been designed which bind to the conserved regions and amplify one or more of the hypervariable regions (Baker et al., 2003). These 16S rRNA gene amplicons can then be used to determine the prokaryotic diversity of a particular environment or to identify individual isolates through techniques such as denaturing gradient gel electrophoresis (DGGE), cloning, and high throughput sequencing (Dowd et al., 2008; Leser et al., 2002; Simpson et al., 1999).

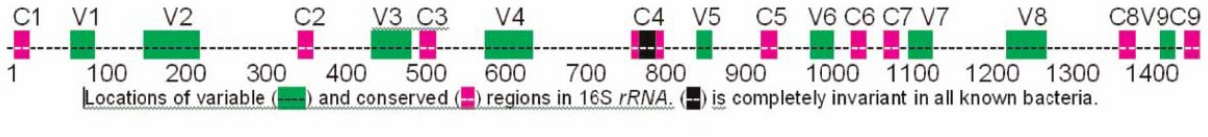

Figure 2.3. Conserved and hypervariable regions in the 16S rRNA gene. Conserved regions are indicated as C1to C9 and hypervariable regions are V1 to V9. Numbers below dotted line refer to base pair position in *E. coli* (Reproduced from Ram et al., 2011).

The intestinal tract of the piglet is sterile prior to birth and undergoes colonization first with facultative aerobic microorganisms which are later succeeded by obligate anaerobes

(Konstantinov et al., 2004; Lalles et al., 2007). Members of the *Clostridiaceae* and *Enterobacteriaceae* families have been identified as the very early colonizers in <1 day old piglets and are displaced by species in *Streptococcaceae* and then by *Lactobacillaceae* just prior to weaning (Petri et al., 2010). The source of these early colonizers is mainly the sow and the immediate environment with the diversity of the gut microbiota of the piglet generally increasing over time (Inoue et al., 2005; Thompson et al., 2008). The transition from sow's milk to less digestible solid feed at weaning has a significant impact on the gut microbiota (Richards et al., 2005). It is also a stressful period for piglets due to the separation from their sow and the loss of protective maternal IgA antibodies (Heo et al., 2013). Piglets are more susceptible to colonization by pathogenic bacteria such as *Salmonella* and *Escherichia coli*, and they exhibit reduced nutrient digestion and absorption due to the changes in the gut microbiota (Lalles et al., 2007; Pluske, 2013; Richards et al., 2005). This is also the time when obligate anaerobes become dominant in the swine gut and the abundance of facultative anaerobes decreases (Konstantinov et al., 2004). Beyond this period, the structure of the gut microbiota becomes successively more stable making it more difficult to manipulate through dietary factors (Thompson et al., 2008).

Similar to humans, high throughput sequencing studies have revealed that the swine gut microbiota is dominated by two phyla, *Firmicutes* and *Bacteroidetes* (Kim et al., 2011; Lamendella et al., 2011; Pedersen et al., 2013). At the genus level, *Prevotella*, a member of the *Bacteroidetes* phylum, has frequently been found to be the most abundant genus of bacteria (Kim et al., 2011; Looft et al., 2012; Pedersen et al., 2013). Although the actual composition of the swine gut microbiota varies from study-to-study, other dominant genera include: *Anaerobacter*, *Treponema*, *Oscillibacter*, *Streptococcus*, *Lactobacillus*, *Clostridium*, *Megasphaera*, *Blautia*,

Succinivibrio, and *Ruminococcus* (Kim et al., 2011; Looft et al., 2012; Looft et al., 2014; Upadrasta et al., 2013).

Although *Archaea* only comprise about 1% of the swine gut microbiota, they are notable for the production methane gas in the gut (Luo et al., 2012). The large majority of *Archaea* found in the swine intestinal tract are methanogens and belong to only one of two genera: *Methanobrevibacter* or *Methanosphaera* (Luo et al., 2012; Mao et al., 2011; Ufnar et al., 2007). Methanogens are obligate anaerobes and are difficult to culture in the lab due to their fastidious growth requirements (Cavicchioli, 2011). They produce methane via the reduction of carbon dioxide, acidic acid, or one of several one-carbon molecules (Saengkerdsub and Ricke, 2014).

2.5.1 Impact of antimicrobials on the swine gut microbiota

Studies using culture-dependent methods have examined the changes in the microbiota of swine treated with antibiotics, growth supplements, and effect of disease (Fuller et al., 1960; Robinson et al., 1984). However, to date, only a handful of studies have used molecular methods to characterize the changes that take place in the gut microbiota of swine in response to antimicrobials. Antimicrobial growth promoters tend not to be well absorbed in the gut and can act directly on the microbiota, mainly Gram-positive bacteria (Erik and Knudsen, 2001). In humans, antibiotic use is associated with lower taxonomic richness, diversity, and abundance as measured using 16S rRNA sequencing (Bartosch et al., 2004; Dethlefsen et al., 2008). Similarly, using massive parallel 16S rRNA gene pyrosequencing, the small intestine of canine treated with therapeutic levels of tylosin was observed to undergo a significant change in microbial diversity (Suchodolski et al., 2009).

Collier et al. (2003) investigated the effect of tylosin in-feed at 44 mg kg^{-1} feed on the ileal microbiota of swine and reported a decrease in DGGE band numbers after 14 days of

treatment, however, no differences were reported at day 21, 28, or 35. The authors also observed a decrease in total bacterial 16S rRNA gene abundance at days 14 and 21, although not after 28 or 35 days. Interestingly, the relative proportion of *Lactobacillus* 16S rRNA gene sequences was found to be more prevalent after tylosin treatment (Collier et al., 2003). In another study, Thyman et al. (2007) treated weaned pigs with 20 mg amoxicillin kg^{-1} feed and 2500 mg ZnO kg^{-1} feed and found that the treated pigs had a significantly lower incidence (13% vs. 56%) of haemolytic *E. coli* than the control group. Microbial diversity as measured by terminal restriction fragment length polymorphism was also reported to be lower in pigs treated with amoxicillin and ZnO (Thymann et al., 2007).

Using DGGE, a study by Janczyk et al. (2007) found a decrease in both diversity and richness (Shannon index: 0.79 vs. 1.03) in the gut microbiota of 39-day old piglets that had been administered a single dose of amoxicillin (15 mg kg^{-1} body weight) intramuscularly at birth. In piglets fed chlortetracycline for two weeks at 50 mg kg^{-1} feed following weaning, a significant change in ileal microbiota was recorded by Rettedal et al. (2009) using 16S rRNA gene library analysis. The authors also noted that *Lactobacillus*, the family *Clostridiaceae*, and the genus *Turicibacter* were most often identified. Furthermore there was a large variation in the microbiota of individual pigs (Rettedal et al., 2009). A study by Kalmokoff and colleagues (2011) used DGGE and a 16S rRNA gene clone library analysis to investigate the effect that either tylosin or virginiamycin has on the gut microbiota of growing-finishing pigs. These authors did not detect any changes attributable to either antibiotic in terms of the gut microbiota or in the level of phenotypic resistance using culture-based methods. Of note was the fact that fecal anaerobes from pigs had high levels of both tylosin and virginamycin resistance at the beginning of this study and prior to treatment (Kalmokoff et al., 2011).

Recent studies investigating the impact of antibiotics on the swine gut microbiota using high-throughput technologies have also revealed conflicting results. Poole et al. (2013) did not detect a difference in fecal communities between pigs fed 50 mg chlortetracycline kg^{-1} feed and a control group while using 16S rRNA gene 454 pyrosequencing. It should be noted that only two pigs per treatment were used and that pigs were only treated for a period of 28 days. Similarly, Kim et al. (2012) documented no difference at the phylum or class-levels of bacteria in pigs fed either tylosin at 44 mg kg^{-1} feed or a control, antibiotic-free diet. They did, however, observe shifts at the genus level and in OTU abundance between the two groups.

Meanwhile, Looft et al. (2012) recorded a significant shift in the fecal microbiota of pigs that had been fed ASP250 (100 mg chlortetracycline kg^{-1} feed, 100 mg sulfamethazine kg^{-1} feed, 50 mg penicillin kg^{-1} feed) for 14 days. Most notably, these authors reported an increase in the abundance of *Proteobacteria* in the antibiotic-treated pigs. This study was also conducted with 454 pyrosequencing but with only six pigs in total and two sampling times. Previous work by these researchers using pigs fed ASP250 had observed alterations in the gut microbiota as well as the gut viriome, with reduced bacterial diversity described in ASP250-treated pigs. However, this effect did not extend to carbadox-treated (10 to 50 mg kg^{-1} feed) pigs (Allen et al., 2011). A more recent study by Looft et al. (2014) found significant differences in the bacterial community structure in the ileum, cecum, colon, and feces of pigs fed ASP250 for 14 days.

2.6 Antibiotic alternatives in swine

The issues associated with antibiotic use in agriculture have increased the urgency to develop and identify alternatives (Allen et al.; 2013; Thacker; 2013). Ideally, any successful antibiotic alternative will mimic the effects of antibiotics in the gut without contributing to the emergence and dissemination of antibiotic resistance (Verstegen and Williams; 2002). To date,

numerous alternatives have been proposed. Some alternatives such as prebiotics and probiotics, attempt to modify specific populations in the gut but have so far produced varying degrees of success (Stein and Kil, 2006; Vondruskova et al., 2010). The same holds true for most other antibiotic alternatives that have been studied. These include: bacteriophages, cereal grains, vaccines, clay minerals, egg yolk antibodies, organic acids, rare earth metals, and essential oils (Allen et al. 2013, Pieper et al.. 2008; Seal et al.. 2013; Thacker. 2013).

Flaxseed represents one of these potential antibiotic alternatives in swine production as components of flaxseed may possess antimicrobial activity (Kiarie et al., 2007). Flaxseed is one of the richest plant sources of α-linolenic acid (18:3 n-3), an essential fatty acid that has been associated with several health benefits in humans such a reduced risk of cardiovascular and inflammatory diseases (Barceló-Coblijn and Murphy, 2009; Stark et al., 2008). The oil fraction of flaxseed ranges from 35 to 46%, of which 45 to 58% is α-linolenic acid (Puvirajah, 2012; Singh et al., 2011). Numerous studies have successfully increased the α-linolenic acid content of pork by adding flaxseed to the diet of swine (Juarez et al., 2010; Matthews et al., 2000; Romans et al., 1995; Turner et al., 2014).

Flaxseed is also a significant source of lignan, namely secoisolariciresinol diglucoside, a phytoestrogen which is metabolized by colonic bacteria (Zhang et al., 2008). α-Linolenic acid and oil extracted from flaxseed have bactericidal and anti-adhesion properties *in vitro* (Kankaanpaa et al., 2001). In addition, fibre, both soluble (e.g. cellulose and lignin) and insoluble (e.g. mucilage), is also an important component of flaxseed which may influence the intestinal microbiota (Singh et al., 2011). Flaxseed supplementation has been demonstrated to reduce microbial metabolic activity and anaerobic spore formers in the ileum of nursery-aged piglets (Kiarie et al., 2007). Flaxseed has also been shown to decrease the concentration of

methanogens in the rumen of dairy cattle (Li et al., 2012). Furthermore, a reduction in methane production in dairy cattle supplemented with flaxseed has been reported (Hook et al., 2010).

2.7 References

Aarestrup F, Jenser L (2007). Use of antimicrobials in food animal production. *Foodborne Disease.* Humana Pres,: Totowa, NJ. pp 405–417.

Aarestrup FM, Carstensen B (1998). Effect of tylosin used as a growth promoter on the occurrence of macrolide-resistant enterococci and staphylococci in pigs. *Microb Drug Resist* **4:** 307-312.

Aarestrup FM, Seyfarth AM, Emborg H-D, Pedersen K, Hendriksen RS, Bager F (2001). Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob Agents Chemother* **45:** 2054-2059.

Aarestrup FM (2004). Monitoring of antimicrobial resistance among food animals: principles and limitations. *J Vet Med B* **51:** 380-388.

Aarestrup FM, Jensen VF, Emborg H-D, Jacobsen E, Wegener HC (2010). Changes in the use of antimicrobials and the effects on productivity of swine farms in Denmark. *Am J Vet Res* **71:** 726-733.

Akiba T, Koyama K, Ishiki Y, Kimura S, Fukushima T (1960). On the mechanism of the development of multiple-drug-resistant clones of Shigella. *Jpn J Microbiol* **4:** 219-227.

Akwar H, Poppe C, Wilson J, Reid-Smith R, Dyck M, Waddington J et al. (2008). Prevalence and patterns of antimicrobial resistance of fecal *Escherichia coli* among pigs on 47

farrow-to-finish farms with different in-feed medication policies in Ontario and British Columbia. *Can J Vet Res* **72:** 195.

Alali W, Scott H, Norby B (2010). Assessing the similarity of antimicrobial resistance phenotypes among fecal *Escherichia coli* isolates from two aggregated occupational cohorts of humans versus swine using cluster analysis and multivariate statistics. *Prev Vet Med* **94:** 77-83.

Alekshun MN, Levy SB (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell* **128:** 1037-1050.

Allen HK, Looft T, Bayles DO, Humphrey S, Levine UY, Alt D et al. (2011). Antibiotics in feed induce prophages in swine fecal microbiomes. *MBio* **2:** e00260-00211.

Allen HK, Levine UY, Looft T, Bandrick M, Casey TA (2013). Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends Microbiol* **21:** 114-119.

Andersson, DI (2006). The biological cost of mutational antibiotic resistance: any practical conclusions? *Curr Opin Microbiol* **9**:461-5.

Arnold S, Gassner B, Giger T, Zwahlen R (2004). Banning antimicrobial growth promoters in feedstuffs does not result in increased therapeutic use of antibiotics in medicated feed in pig farming. *Pharmacoepidemiol Dr S* **13:** 323-331.

Asai T, Kojima A, Harada K, Ishihara K, Takahashi T, Tamura Y (2005). Correlation between the usage volume of veterinary therapeutic antimicrobials and resistance in *Escherichia coli* isolated from the feces of food-producing animals in Japan. *Jpn J Infect Dis* **58:** 369.

Baker GC, Smith JJ, Cowan DA (2003). Review and re-analysis of domain-specific 16S primers. *J Microbiol Methods* **55:** 541-555.

Barceló-Coblijn G, Murphy EJ (2009). Alpha-linolenic acid and its conversion to longer chain *n*-3 fatty acids: Benefits for human health and a role in maintaining tissue *n*-3 fatty acid levels. *Progress in lipid research* **48:** 355-374.

Barlow M (2009). What antimicrobial resistance has taught us about horizontal gene transfer. *Horizontal Gene Transfer*. Springer. pp 397-411.

Bartosch S, Fite A, Macfarlane GT, McMurdo ME (2004). Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl Environ Microbiol* **70:** 3575-3581.

Barza M (2002). Potential mechanisms of increased disease in humans from antimicrobial resistance in food animals. *Clin Infect Dis* **34 Suppl 3:** S123-125.

Bennett PM (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br J Pharmacol* **153 Suppl 1:** S347-357.

Berrang ME, Ladely SR, Meinersmann RJ, Fedorka-Cray PJ (2007). Subtherapeutic tylosin phosphate in broiler feed affects *Campylobacter* on carcasses during processing. *Poult Sci* **86:** 1229-1233.

Bibbal D, Dupouy V, Ferre JP, Toutain PL, Fayet O, Prere MF et al. (2007). Impact of three ampicillin dosage regimens on selection of ampicillin resistance in *Enterobacteriaceae* and excretion of bla_{TEM} genes in swine feces. *Appl Environ Microbiol* **73:** 4785-4790.

Blake D, Humphry R, Scott K, Hillman K, Fenlon D, Low J (2003). Influence of tetracycline exposure on tetracycline resistance and the carriage of tetracycline resistance genes within commensal Escherichia coli populations. *J Appl Microbiol* **94:** 1087-1097.

Boerlin P, Reid-Smith RJ (2008). Antimicrobial resistance: its emergence and transmission. *Anim Health Res Rev* **9:** 115-126.

Boerlin P, Travis R, Gyles CL, Reid-Smith R, Janecko N, Lim H et al. (2005). Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. *Appl Environ Microbiol* **71**:6753-61.

Boerlin P, White DG (2006). Antimicrobial resistance and its epidemiology. *Antimicrobial therapy in veterinary medicine, fourth edition Blackwell Publishing, Oxford, UK***:** 27-43.

Boucher Y, Labbate M, Koenig JE, Stokes H (2007). Integrons: mobilizable platforms that promote genetic diversity in bacteria. *Trends Microbiol* **15:** 301-309.

Brabban AD, Hite E, Callaway TR (2005). Evolution of foodborne pathogens via temperate bacteriophage-mediated gene transfer. *Foodborne Pathog Dis* **2**:287-303.

Butaye P, Devriese LA, Haesebrouck F (2003). Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. *Clin Microbiol Rev* **16:** 175-188.

Canadian Food Inspection Agency (2014). Compendium of medicating ingredients. http://www.inspection.gc.ca/animals/feeds/medicating-ingredients/mib/eng/1330705207970/1330714849837. Accessed on April 16, 2014.

Carattoli A (2001). Importance of integrons in the diffusion of resistance. *Vet Res* **32:** 243-259.

Casewell M, Friis C, Marco E, McMullin P, Phillips I (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J Antimicrob Chemother* **52:** 159-161.

Cavicchioli R (2011). *Archaea*—timeline of the third domain. *Nat Rev Microbiol* **9:** 51-61.

Chakravorty S, Helb D, Burday M, Connell N, Alland D (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J Microbiol Methods* **69:** 330-339.

Chen L, Chen ZL, Liu JH, Zeng ZL, Ma JY, Jiang HX (2007). Emergence of RmtB methylase-producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. *J Antimicrob Chemother* **59**:880-5.

Chen J, Fluharty F, St-Pierre N, Morrison M, Yu Z (2008). Technical note: Occurrence in fecal microbiota of genes conferring resistance to both macrolide-lincosamide-streptogramin B and tetracyclines concomitant with feeding of beef cattle with tylosin. *J Anim Sci* **86:** 2385-2391.

Chen J, Michel FC, Sreevatsan S, Morrison M, Yu Z (2010). Occurrence and persistence of erythromycin resistance genes (erm) and tetracycline resistance genes (tet) in waste treatment systems on swine farms. *Microbial Ecol* **60**: 479-486.

Chopra I, Roberts M (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* **65:** 232-260.

Chuanchuen R, Padungtod P (2009). Antimicrobial resistance genes in *Salmonella enterica* isolates from poultry and swine in Thailand. *J Vet Med Sci* **71:** 1349-1355.

Clarridge JE, III (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev* **17:** 840-862.

Collier CT, Smiricky-Tjardes MR, Albin DM, Wubben JE, Gabert VM, Deplancke B et al. (2003). Molecular ecological analysis of porcine ileal microbiota responses to antimicrobial growth promoters. *J Anim Sci* **81:** 3035-3045.

Connell SR, Tracz DM, Nierhaus KH, Taylor DE (2003). Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrob Agents Chemother* **47:** 3675-3681.

Cromwell GL (2002). Why and how antibiotics are used in swine production. *Anim Biotechnol* **13:** 7-27.

Cummings JH, Macfarlane GT (1997). Colonic microflora: nutrition and health. *Nutrition* **13:** 476-478.

Cunha TJ, Burnside JE, Meadows GB, Edwards HM, Benson RH, Pearson AM et al. (1950). Effect of APF supplement on efficiency of feed utilization for the pig. *J Anim Sci* **9:** 615-618.

Dalmasso M, Hill C, Ross RP (2014). Exploiting gut bacteriophages for human health. *Trends Microbiol* **22**:399-405.

D'Costa V, Wright GD (2009). Biochemical logic of antibiotic inactivation and modification. . In Mayers, D., Lerner, S., Sobel, J. and Ouellette, M. (ed.). Antimicrobial drug resistance: mechanisms of drug resistance. Humana Press: New York, NY, pp 81–96.

Deckert A, Gow S, Rosengren L, Leger D, Avery B, Daignault D et al. (2010). Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) Farm Program: results from finisher pig surveillance. *Zoonoses Public Health* **57 Suppl 1:** 71-84.

de la Torre E, Colello R, Padola NL, Etcheverría A Rodríguez E, Amanto F et al. (2014). Detection of integrase gene *in E. coli* isolated from pigs at different stages of production system. *Int J Microbiol* 2014:489569.

Delcour AH (2009). Outer membrane permeability and antibiotic resistance. *Biochim Biophys Acta* **1794:** 808-816.

Denis O, Suetens C, Hallin M, Catry B, Ramboer I, Dispas M et al. (2009). Methicillin-resistant *Staphylococcus aureus* ST398 in swine farm personnel, Belgium. *Emerg Infect Dis* **15:** 1098-1101.

Depardieu F, Podglajen I, Leclercq R, Collatz E, Courvalin P (2007). Modes and modulations of antibiotic resistance gene expression. *Clin Microbiol Rev* **20:** 79-114.

Dethlefsen L, Huse S, Sogin ML, Relman DA (2008). The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol* **6:** e280.

Dibner JJ, Richards JD (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poult Sci* **84:** 634-643.

Dore K, Buxton J, Henry B, Pollari F, Middleton D, Fyfe M et al. (2004). Risk factors for *Salmonella* Typhimurium DT104 and non-DT104 infection: a Canadian multi-provincial case-control study. *Epidemiol Infect* **132:** 485-493.

Dowd S, Callaway T, Wolcott R, Sun Y, McKeehan T, Hagevoort R et al. (2008). Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiol* **8:** 125.

Dritz SS, Tokach MD, Goodband RD, Nelssen JL (2002). Effects of administration of antimicrobials in feed on growth rate and feed efficiency of pigs in multisite production systems. *J Am Vet Med Assoc* **220:** 1690-1695.

Duggar BM (1948). Aureomycin; a product of the continuing search for new antibiotics. *Ann NY Acad Sci* **51:** 177-181.

Eichhorn P, Aga DS (2004). Identification of a photooxygenation product of chlortetracycline in hog lagoons using LC/ESI-ion trap-MS and LC/ESI-time-of-flight-MS. *Anal Chem* **76:** 6002-6011.

Eliopoulos GM, Roberts MC (2003). Tetracycline therapy: update. *Clin Infect Dis* **36:** 462-467.

Erik K, Knudsen B (2001). Development of antibiotic resistance and options to replace antimicrobials in animal diets. *P Nutr Soc* **60:** 291-299.

European Union (2005). Ban on antibiotics as growth promoters in animal feed enters into effect (1831/2003/EC). http://europa.eu/rapid/press-release_IP-05-1687_en.htm. Accessed July 17, 2014.

Fluit AC, van der Bruggen JT, Aarestrup FM, Verhoef J, Jansen WT (2006). Priorities for antibiotic resistance surveillance in Europe. *Clin Microbiol Infect* **12:** 410-417.

Food and Agriculture Organization. (2014). Food Outlook. http://www.fao.org/docrep/019/I3751E/I3751E.pdf. Accessed October 29, 2014.

Friendship R, Prescott, J. F. (2006). Drug therapy and prophylaxis. In: Straw BE (ed). Diseases of Swine, 9th ed. Blackwell Publishing: Ames, Iowa. pp 1131-1143.

Frost LS, Leplae R, Summers AO, Toussaint A (2005). Mobile genetic elements: the agents of open source evolution. *Nat Rev Microbiol* **3:** 722-732.

Frye JG, Lindsey RL, Meinersmann RJ, Berrang ME, Jackson CR, Englen MD, Turpin JB, Fedorka-Cray PJ (2011). Related antimicrobial resistance genes detected in different bacterial species co-isolated from swine fecal samples. Foodborne Pathog Dis **8**:663-79.

Fuller R, Newland L, Briggs C, Braude R, Mitchell K (1960). The normal intestinal flora of the pig. IV. The effect of dietary supplements of penicillin, chlortetracycline or copper sulphate on the faecal flora. *J Appl Microbiol* **23:** 195-205.

Garofalo C, Vignaroli C, Zandri G, Aquilanti L, Bordoni D, Osimani A et al. (2007). Direct detection of antibiotic resistance genes in specimens of chicken and pork meat. *Int J Food Microbiol* **113:** 75-83.

Gaskins H (2001). Intestinal bacteria and their influence on swine growth. *In*: Southern, AJ (ed). Swine Nutrition. CRC Press LLC: Florida. pp 585-608.

Gaskins H, Collier C, Anderson D (2002). Antibiotics as growth promotants: mode of action. *Anim Biotechnol* **13:** 29-42.

Gebreyes WA, Thakur S, Morrow WE (2005). *Campylobacter coli*: prevalence and antimicrobial resistance in antimicrobial-free (ABF) swine production systems. *J Antimicrob Chemother* **56:** 765-768.

Gellin G, Langlois BE, Dawson KA, Aaron DK (1989). Antibiotic resistance of Gram-negative enteric bacteria from pigs in three herds with different histories of antibiotic exposure. *Appl Environ Microbiol* **55:** 2287-2292.

Ghosh S, LaPara TM (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J* **1:** 191-203.

Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS et al. (2006). Metagenomic analysis of the human distal gut microbiome. *Science* **312:** 1355-1359.

Gillings M, Boucher Y, Labbate M, Holmes A, Krishnan S, Holley M et al. (2008). The evolution of class 1 integrons and the rise of antibiotic resistance. *Bacteriol.* **190**:5095-100.

Grave K, Jensen VF, Odensvik K, Wierup M, Bangen M (2006). Usage of veterinary therapeutic antimicrobials in Denmark, Norway, and Sweden following termination of antimicrobial growth promoter use. *Prev Vet Med* **75:** 123-132.

Hartmeyer GN, Gahrn-Hansen B, Skov RL, Kolmos HJ (2010). Pig-associated methicillin-resistant *Staphylococcus aureus*: family transmission and severe pneumonia in a newborn. *Scand J Infect Dis* **42:** 318-320.

Health Canada (2009). Categorization of antimicrobial drugs based on importance in human medicine. http://www.hc-sc.gc.ca/dhp-mps/vet/antimicrob/amr_ram_hum-med-rev-eng.php. Accessed on April 16, 2014.

Health Canada (2014). Notice to stakeholders: Collaborative efforts to promote the judicious use of medically-important antimicrobial drugs in food animal production. http://www.hc-sc.gc.ca/dhp-mps/vet/antimicrob/amr-notice-ram-avis-20140410-eng.php. Accessed July 21, 2014.

Heo J, Opapeju F, Pluske J, Kim J, Hampson D, Nyachoti C (2013). Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *J Anim Physiol Anim Nutr* **97:** 207-237.

Holmberg SD, Osterholm MT, Senger KA, Cohen ML (1984). Drug-resistant *Salmonella* from animals fed antimicrobials. *N Engl J Med* **311**:617-22.

Holt J, Van Heugten E, Graves A, See M, Morrow W (2011). Growth performance and antibiotic tolerance patterns of nursery and finishing pigs fed growth-promoting levels of antibiotics. *Livest Sci* **136:** 184-191.

Hook SE, Wright AD, McBride BW (2010). Methanogens: methane producers of the rumen and mitigation strategies. *Archaea* **2010:** 945785.

Inoue R, Tsukahara T, Nakanishi N, Ushida K (2005). Development of the intestinal microbiota in the piglet. *The Journal of general and applied microbiology* **51:** 257-265.

Jackson CR, Fedorka-Cray PJ, Barrett JB, Ladely SR (2004). Effects of tylosin use on erythromycin resistance in enterococci isolated from swine. *Appl Environ Microbiol* **70:** 4205-4210.

Janczyk P, Pieper R, Souffrant WB, Bimczok D, Rothkotter HJ, Smidt H (2007). Parenteral long-acting amoxicillin reduces intestinal bacterial community diversity in piglets even 5 weeks after the administration. *ISME J* **1:** 180-183.

Jin Z, Yang Y, Choi J, Shinde P, Yoon S, Hahn T-W et al. (2008). Effects of potato (Solanum tuberosum L. cv. Golden valley) protein having antimicrobial activity on the growth performance, and intestinal microflora and morphology in weanling pigs. *Anim Feed Sci Technol* **140:** 139-154.

Jonas DA, Elmadfa I, Engel KH, Heller KJ, Kozianowski G, Konig A et al. (2001). Safety considerations of DNA in food. *Ann Nutr Metab* **45:** 235-254.

Jondreville C, Revy PS, Dourmad, JY (2003). Dietary means to better control the environmental impact of copper and zinc by pigs from weaning to slaughter. *Livest Prod Sci* **84**:147–156.

Jones ME, Draghi DC, Thornsberry C, Karlowsky JA, Sahm DF, Wenzel RP (2004). Emerging resistance among bacterial pathogens in the intensive care unit–a European and North American Surveillance study (2000–2002). *Ann Clin Microbiol Antimicrob* **3:** 14.

Joy SR, Li X, Snow DD, Gilley JE, Woodbury B, Bartelt-Hunt SL (2014). Fate of antimicrobials and antimicrobial resistance genes in simulated swine manure storage. *Sci Total Environ* **481**:69-74.

Juarez M, Dugan ME, Aldai N, Aalhus JL, Patience JF, Zijlstra RT et al. (2010). Feeding co-extruded flaxseed to pigs: effects of duration and feeding level on growth performance and backfat fatty acid composition of grower-finisher pigs. *Meat Sci* **84:** 578-584.

Juntunen P, Heiska H, Olkkola S, Myllyniemi AL, Hänninen ML (2010). Antimicrobial resistance in *Campylobacter coli* selected by tylosin treatment at a pig farm. *Vet Microbiol* **146**:90-7.

Kadlec K, Ehricht R, Monecke S, Steinacker U, Kaspar H, Mankertz J *et al.* (2009). Diversity of antimicrobial resistance pheno- and genotypes of methicillin-resistant Staphylococcus aureus ST398 from diseased swine. *J Antimicrob Chemother* **64:** 1156-1164.

Kalmokoff M, Waddington L, Thomas M, Liang KL, Ma C, Topp E *et al.* (2011). Continuous feeding of antimicrobial growth promoters to commercial swine during the growing/finishing phase does not modify faecal community erythromycin resistance or community structure. *J Appl Microbiol* **110:** 1414-1425.

Kankaanpaa PE, Salminen SJ, Isolauri E, Lee YK (2001). The influence of polyunsaturated fatty acids on probiotic growth and adhesion. *FEMS Microbiol Lett* **194:** 149-153.

Kantiani L, Farre M, Grases IFJM, Barcelo D (2010). Determination of antibacterials in animal feed by pressurized liquid extraction followed by online purification and liquid chromatography-electrospray tandem mass spectrometry. *Anal Bioanal Chem* **398:** 1195-1205.

Katz L, Ashley GW (2005). Translation and protein synthesis: macrolides. *Chem Rev* **105:** 499-528.

Kelly D, Conway S, Aminov R (2005). Commensal gut bacteria: mechanisms of immune modulation. *Trends Immunol* **26:** 326-333.

Kiarie E, Nyachoti CM, Slominski BA, Blank G (2007). Growth performance, gastrointestinal microbial activity, and nutrient digestibility in early-weaned pigs fed diets containing flaxseed and carbohydrase enzyme. *J Anim Sci* **85:** 2982-2993.

Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ et al. (2011). Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Vet Microbiol* **153:** 124-133.

Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ et al. (2012). Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. *Proc Natl Acad Sci USA* **109:** 15485-15490.

Kim JS, Carver DK, Kathariou S (2006). Natural transformation-mediated transfer of erythromycin resistance in *Campylobacter coli* strains from turkeys and swine. *Appl Environ Microbiol* **72**:1316-21.

Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130:** 797-810.

Kohanski MA, Dwyer DJ, Collins JJ (2010). How antibiotics kill bacteria: from targets to networks. *Nat Rev Microbiol* **8:** 423-435.

Koike S, Aminov RI, Yannarell AC, Gans HD, Krapac IG, Chee-Sanford JC et al. (2010). Molecular ecology of macrolide-lincosamide-streptogramin B methylases in waste lagoons and subsurface waters associated with swine production. *Microb Ecol* **59:** 487-498.

Koike S, Krapac IG, Oliver HD, Yannarell AC, Chee-Sanford JC, Aminov RI, Mackie RI (2007). Monitoring and source tracking of tetracycline resistance genes in lagoons and

groundwater adjacent to swine production facilities over a 3-year period. *Appl Environ Microbiol* **73**:4813-23.

Konstantinov SR, Zhu WY, Williams BA, Tamminga S, Vos WM, Akkermans AD (2003). Effect of fermentable carbohydrates on piglet faecal bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. *FEMS Microbiol Ecol* **43:** 225-235.

Konstantinov SR, Favier CF, Zhu WY, Williams BA, Klüß J, Souffrant W-B *et al.* (2004). Microbial diversity studies of the porcine gastrointestinal ecosystem during weaning transition. *Anim Res* **53:** 317-324.

Kozak GK, Boerlin P, Janecko N, Reid-Smith RJ, Jardine C (2009). Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. *Appl Environ Microbiol* **75:** 559-566.

Lalles J-P, Bosi P, Smidt H, Stokes CR (2007). Nutritional management of gut health in pigs around weaning. *Proceedings of the Nutrition Society* **66:** 260-268.

Lamendella R, Domingo JW, Ghosh S, Martinson J, Oerther DB (2011). Comparative fecal metagenomics unveils unique functional capacity of the swine gut. *BMC Microbiol* **11:** 103.

Laparra JM, Sanz Y (2010). Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacol Res* **61:** 219-225.

Lapierre L, San Martin B, Araya-Jordan C, Borie C (2010). Comparison of integron-linked antibiotic resistance genes in strains of *Salmonella* spp. isolated from swine in Chile in 2005 and 2008. *Can J Microbiol* **56:** 515-521.

Leclercq R, Courvalin P (1991). Intrinsic and unusual resistance to macrolide, lincosamide, and streptogramin antibiotics in bacteria. *Antimicrob Agents Chemother* **35:** 1273-1276.

Leclercq R (2002). Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clin Infect Dis* **34:** 482-492.

Lee SW, Kim TJ, Park SY, Song CS, Chang HK, Yeh JK *et al.* (2001). Prevalence of porcine proliferative enteropathy and its control with tylosin in Korea. *J Vet Sci* **2:** 209-212.

Leser TD, Lindecrona RH, Jensen TK, Jensen BB, Moller K (2000). Changes in bacterial community structure in the colon of pigs fed different experimental diets and after infection with *Brachyspira hyodysenteriae*. *Appl Environ Microbiol* **66:** 3290-3296.

Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M, Moller K (2002). Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Appl Environ Microbiol* **68:** 673-690.

Leser TD, Mølbak L (2009). Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environ Microbiol* **11:** 2194-2206.

Levy SB, FitzGerald GB, Macone AB (1976). Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. *N Engl J Med* **295:** 583-588.

Levy SB (2002). The 2000 Garrod lecture. Factors impacting on the problem of antibiotic resistance. *J Antimicrob Chemother* **49:** 25-30.

Levy SB, Marshall B (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med* **10:** S122-129.

Lewis HC, Mølbak K, Reese C, Aarestrup FM, Selchau M, Sørum M *et al.* (2008). Pigs as source of methicillin-resistant *Staphylococcus aureus* CC398 infections in humans, Denmark. *Emerg Infect Dis* **14:** 1383.

Li L, Schoenhals KE, Brady PA, Estill CT, Perumbakkam S, Craig AM (2012). Flaxseed supplementation decreases methanogenic gene abundance in the rumen of dairy cows. *Animal* **6:** 1784-1787.

Licht TR, Hansen M, Poulsen M, Dragsted LO (2006). Dietary carbohydrate source influences molecular fingerprints of the rat faecal microbiota. *BMC Microbiol* **6:** 98.

Livermore DM, Blaser M, Carrs O, Cassell G, Fishman N, Guidos R et al. (2011). Discovery research: the scientific challenge of finding new antibiotics. *J Antimicrob Chemother* 66: 1941-4.

Loke ML, Ingerslev F, Halling-Sorensen B, Tjornelund J (2000). Stability of Tylosin A in manure containing test systems determined by high performance liquid chromatography. *Chemosphere* **40:** 759-765.

Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD et al. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proc Natl Acad Sci USA* **109:** 1691-1696.

Looft T, Allen HK, Cantarel BL, Levine UY, Bayles DO, Alt DP et al. (2014). Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. *ISME J*. e-pub ahead of print 13 Feb 2014, doi: 10.1038/ismej.2014.12.

Lu K, Asano R, Davies J (2004). Antimicrobial resistance gene delivery in animal feeds. *Emerg Infect Dis* **10:** 679-683.

Luo Y-h, Su Y, Wright A-DG, Zhang L-l, Smidt H, Zhu W-y (2012). Lean breed Landrace pigs harbor fecal methanogens at higher diversity and density than obese breed Erhualian pigs. *Archaea* **2012**:605289.

MacDonald JM, McBridge WD (2009). The Transformation of U.S. Livestock Agriculture: Scale, Efficiency, and Risks. http://www.ers.usda.gov/media/184977/eib43.pdf. Accessed October 29, 2014.

Mackie RI, Koike S, Krapac I, Chee-Sanford J, Maxwell S, Aminov RI (2006). Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. *Anim Biotechnol* **17:** 157-176.

Maguire HC, Codd AA, Mackay VE, Rowe B, Mitchell E (1993). A large outbreak of human salmonellosis traced to a local pig farm. *Epidemiol Infect* **110:** 239-246.

Mahillon J, Chandler M (1998). Insertion sequences. *Microbiol Mol Biol Rev* **62:** 725-774.

Mankin AS (2008). Macrolide myths. *Curr Opin Microbiol* **11:** 414-421.

Mao SY, Yang CF, Zhu WY (2011). Phylogenetic analysis of methanogens in the pig feces. *Curr Microbiol* **62:** 1386-1389.

Marchant M, Vinué L, Torres C, Moreno MA (2013). Change of integrons over time in *Escherichia coli* isolates recovered from healthy pigs and chickens. *Vet Microbiol* **163**(1-2)

Marshall BM, Levy SB (2011). Food animals and antimicrobials: impacts on human health. *Cli*n *Microbiol Rev* **24**:718-33.

Marshall B, Petrowski D, Levy SB (1990). Inter- and intraspecies spread of Escherichia coli in a farm environment in the absence of antibiotic usage. *Proc Natl Acad Sci USA* **87:** 6609-6613.

Mathew A, Garner K, Ebner P, Saxton A, Clift R, Liamthong S (2005). Effects of antibiotic use in sows on resistance of *E. coli* and *Salmonella enterica* Typhimurium in their offspring. *Foodborne Pathog Dis* **2:** 212-220.

Matthews K, Homer D, Thies F, Calder P (2000). Effect of whole linseed (*Linum usitatissimum*) in the diet of finishing pigs on growth performance and on the qualityand fatty acid composition of various tissues. *Br J Nutr* **83:** 637-643.

Mauldin PD, Salgado CD, Hansen IS, Durup DT, Bosso JA (2010). Attributable hospital cost and length of stay associated with health care-associated infections caused by antibiotic-resistant Gram-negative bacteria. *Antimicrob Agents Chemother* **54**:109-15.

Mazel D (2006). Integrons: agents of bacterial evolution. *Nat Rev Microbiol* **4:** 608-620.

McEwen SA, Fedorka-Cray PJ (2002). Antimicrobial use and resistance in animals. *Clin Infect Dis* **34:** S93.

Miranda J, Vazquez B, Fente C, Barros-Velazquez J, Cepeda A, Abuín CF (2008). Antimicrobial resistance in *Escherichia coli* strains isolated from organic and conventional pork meat: a comparative survey. *Eur Food Res Technol* **226:** 371-375.

Molbak K, Baggesen DL, Aarestrup FM, Ebbesen JM, Engberg J, Frydendahl K et al. (1999). An outbreak of multidrug-resistant, quinolone-resistant *Salmonella enterica* serotype Typhimurium DT104. *N Engl J Med* **341:** 1420-1425.

Molbak K (2006). The clinical importance of animal-related resistance. In: Aarestrup FM (ed). Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press: Washington, DC, pp 329-337.

Molbak L, Johnsen K, Boye M, Jensen TK, Johansen M, Møller K, et al. (2008). The microbiota of pigs influenced by diet texture and severity of *Lawsonia intracellularis* infection. *Vet Microbiol* **128**:96-107.

Montagne L, Arturo-Schaan M, Le Floc'h N, Guerra L, Le Gall M (2010). Effect of sanitary conditions and dietary fibre on the adaptation of gut microbiota after weaning. *Livest Sci* **133:** 113-116.

Moore WE, Moore LV, Cato EP, Wilkins TD, Kornegay ET (1987). Effect of high-fiber and high-oil diets on the fecal flora of swine. *Appl Environ Microbiol* **53:** 1638-1644.

Mürmann L, dos Santos MC, Cardoso M (2009). Prevalence, genetic characterization and antimicrobial resistance of *Salmonella* isolated from fresh pork sausages in Porto Alegre, Brazil. *Food Control* **20:** 191-195.

Niewold TA (2007). The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poult Sci* **86:** 605-609.

Nijsten R, London N, van den Bogaard A, Stobberingh E (1994). Resistance in faecal *Escherichia coli* isolated from pig farmers and abattoir workers. *Epidemiol Infect* **113:** 45-52.

Nikaido H (2009). Multidrug resistance in bacteria. *Annu Rev Biochem* **78:** 119-146.

Nikolich MP, Hong G, Shoemaker NB, Salyers AA (1994). Evidence for natural horizontal transfer of *tet*Q between bacteria that normally colonize humans and bacteria that normally colonize livestock. *Appl Environ Microbiol* **60:** 3255-3260.

Nwosu VC (2001). Antibiotic resistance with particular reference to soil microorganisms. *Res Microbiol* **152**:421-30.

Oppegaard H, Steinum TM, Wasteson Y (2001). Horizontal transfer of a multi-drug resistance plasmid between coliform bacteria of human and bovine origin in a farm environment. *Appl Environ Microbiol* **67:** 3732-3734.

Oyarzabal OA, Rad R, Backert S (2007). Conjugative transfer of chromosomally encoded antibiotic resistance from *Helicobacter pylori* to *Campylobacter jejuni*. *J Clin Microbiol* **45:** 402-408.

Pakpour S, Jabaji S, Chénier MR (2012). Frequency of antibiotic resistance in a swine facility 2.5 years after a ban on antibiotics. *Microb Ecol* **63**:41-50.

Pedersen R, Andersen AD, Molbak L, Stagsted J, Boye M (2013). Changes in the gut microbiota of cloned and non-cloned control pigs during development of obesity: gut microbiota during development of obesity in cloned pigs. *BMC Microbiol* **13:** 30.

Petri D, Hill J, Van Kessel A (2010). Microbial succession in the gastrointestinal tract (GIT) of the preweaned pig. *Livest Sci* **133:** 107-109.

Public Health Agency of Canada (2009). Canadian integrated program for antimicrobial resistance surveillance (CIPARS). http://www.phac-aspc.gc.ca/cipars-picra/2009/summary-sommaire-eng.php. Accessed April 16, 2014.

Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R et al. (2004). Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother* **53:** 28-52.

Phillips I (2007). Withdrawal of growth-promoting antibiotics in Europe and its effects in relation to human health. *Int J Antimicrob Agents* **30:** 101-107.

Piddock LJ (2006). Multidrug-resistance efflux pumps? not just for resistance. *Nat Rev Microbiol* **4:** 629-636.

Piddock LJ (2012). The crisis of no new antibiotics--what is the way forward? *Lancet Infect Dis* **12**:249-53.

Pieper R, Jha R, Rossnagel B, Van Kessel AG, Souffrant WB, Leterme P (2008). Effect of barley and oat cultivars with different carbohydrate compositions on the intestinal bacterial communities in weaned piglets. *FEMS Microbiol Ecol* **66:** 556-566.

Pluske JR (2013). Feed-and feed additives-related aspects of gut health and development in weanling pigs. *J Anim Sci Biotechnol* **4**.

Poole K (2007). Efflux pumps as antimicrobial resistance mechanisms. *Ann Med* **39:** 162-176.

Poole T, Suchodolski J, Callaway T, Farrow R, Loneragan G, Nisbet D (2013). The effect of chlortetracycline on faecal microbial populations in growing swine. *J Glob Antimicrob Resis* **1:** 171-174.

Puvirajah AS (2012). Quality of western Canadian flaxseed. http://www.grainscanada.gc.ca/flax-lin/harvest-recolte/2012/hqf12-qrl12-eng.pdf. Accessed April 17, 2014.

Qin S, Wang Y, Zhang Q, Zhang M, Deng F, Shen Z, Wu C, Wang S, Zhang J, Shen J (2014). Report of ribosomal RNA methylase gene *erm*(B) in multidrug-resistant *Campylobacter coli*. J Antimicrob Chemother **69**:964-8.

Ram JL, Karim AS, Sendler ED, Kato I (2011). Strategy for microbiome analysis using 16S rRNA gene sequence analysis on the Illumina sequencing platform. *Syst Biol Reprod Med* **57:** 162-170.

Rettedal E, Vilain S, Lindblom S, Lehnert K, Scofield C, George S et al. (2009). Alteration of the ileal microbiota of weanling piglets by the growth-promoting antibiotic chlortetracycline. *Appl Environ Microbiol* **75:** 5489-5495.

Richards J, Gong J, De Lange C (2005). The gastrointestinal microbiota and its role in monogastric nutrition and health with an emphasis on pigs: Current understanding,

possible modulations, and new technologies for ecological studies. *Can J Anim Sci* **85:** 421-435.

Roberts M (2014a). Mechanism of resistance for characterized *tet* and *otr* genes. http://faculty.washington.edu/marilynr/tetweb1.pdf. Accessed April 17, 2014.

Roberts M (2014b). Mechanisms of MLS resistance. http://faculty.washington.edu/ marilynr/ermwebA.pdf. Accessed April 17, 2014.

Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J, Seppala H (1999). Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob Agents Chemother* **43:** 2823-2830.

Roberts MC (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiol Lett* **245:** 195-203.

Roberts MC, Schwarz S (2009). Tetracycline and chloramphenicol resistance mechanisms. *Antimicrobial Drug Resistance*. Springer. pp 183-193.

Roberts RR, Hota B, Ahmad I, Scott RD, 2nd, Foster SD, Abbasi F *et al.* (2009). Hospital and societal costs of antimicrobial-resistant infections in a Chicago teaching hospital: implications for antibiotic stewardship. *Clin Infect Dis* **49:** 1175-1184.

Robinson IM, Whipp SC, Bucklin JA, Allison MJ (1984). Characterization of predominant bacteria from the colons of normal and dysenteric pigs. *Appl Environ Microbiol* **48:** 964-969.

Rollo SN, Norby B, Bartlett PC, Scott HM, Wilson DL, Fajt VR et al. (2010). Prevalence and patterns of antimicrobial resistance in *Campylobacter* spp isolated from pigs reared under antimicrobial-free and conventional production methods in eight states in the Midwestern United States. *J Am Vet Med Assoc* **236:** 201-210.

Romans JR, Wulf DM, Johnson RC, Libal GW, Costello WJ (1995). Effects of ground flaxseed in swine diets on pig performance and on physical and sensory characteristics and omega-3 fatty acid content of pork: II. Duration of 15% dietary flaxseed. *J Anim Sci* **73:** 1987-1999.

Rosengren LB, Waldner CL, Reid-Smith RJ, Harding JC, Gow SP, Wilkins WL (2008a). Antimicrobial use through feed, water, and injection in 20 swine farms in Alberta and Saskatchewan. *Can J Vet Res* **72:** 143.

Rosengren LB, Waldner CL, Reid-Smith RJ, Checkley SL, McFall ME, Rajic A (2008b). Antimicrobial resistance of fecal *Salmonella* spp. isolated from all phases of pig production in 20 herds in Alberta and Saskatchewan. *Can J Vet Res* **72:** 151.

Ross JI, Eady EA, Cove JH, Cunliffe WJ (1998). 16S rRNA mutation associated with tetracycline resistance in a Gram-positive bacterium. *Antimicrob Agents Chemother* **42:** 1702-1705.

Roy PH (2009). Genetic Mechanisms of Transfer of Drug Resistance. In: Mayers, DL (ed). Antimicrobial Drug Resistance. Humana Press: New York, NY, pp 53-64.

Saengkerdsub S, Ricke SC (2014). Ecology and characteristics of methanogenic archaea in animals and humans. *Crit Rev Microbiol* **40:** 97-116.

Salanitro JP, Blake IG, Muirhead PA (1977). Isolation and identification of fecal bacteria from adult swine. *Appl Environ Microbiol* **33:** 79-84.

San Martin B, Lapierre L, Cornejo J, Bucarey S (2008). Characterization of antibiotic resistance genes linked to class 1 and 2 integrons in strains of *Salmonella* spp. isolated from swine. *Can J Microbiol* **54:** 569-576.

Sapkota AR, Curriero FC, Gibson KE, Schwab KJ (2007). Antibiotic-resistant enterococci and fecal indicators in surface water and groundwater impacted by a concentrated Swine feeding operation. *Environ Health Perspect* 115:1040-5.

Savage DC (1977). Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol* **31:** 107-133.

Schroeder CM, White DG, Ge B, Zhang Y, McDermott PF, Ayers S *et al.* (2003). Isolation of antimicrobial-resistant *Escherichia coli* from retail meats purchased in Greater Washington, DC, USA. *Int J Food Microbiol* **85:** 197-202.

Schwarz S, Cloeckaert A, Roberts MC (2006). Mechanisms and spread of bacterial resistance to antimicrobial agents. In: Aarestrup FM, (ed) Antimicrobial Resistance in Bacteria of Animal Origin. Washington, DC:ASM Press, pp 73-98.

Seal BS, Lillehoj HS, Donovan DM, Gay CG (2013). Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. *Anim Health Res Rev* **14:** 78-87.

Silbergeld EK, Graham J, Price LB (2008). Industrial food animal production, antimicrobial resistance, and human health. *Annu Rev Public Health* **29:** 151-169.

Simeoni D, Rizzotti L, Cocconcelli P, Gazzola S, Dellaglio F, Torriani S (2008). Antibiotic resistance genes and identification of staphylococci collected from the production chain of swine meat commodities. *Food Microbiol* **25:** 196-201.

Simpson JM, McCracken VJ, White BA, Gaskins HR, Mackie RI (1999). Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. *J Microbiol Methods* **36:** 167-179.

Singer RS, Cox Jr LA, Dickson JS, Hurd HS, Phillips I, Miller GY (2007). Modeling the relationship between food animal health and human foodborne illness. *Prev Vet Med*

79: 186-203.

Singh KK, Mridula D, Rehal J, Barnwal P (2011). Flaxseed: a potential source of food, feed and fiber. *Crit Rev Food Sci Nutr* **51:** 210-222.

Smillie C, Garcillan-Barcia MP, Francia MV, Rocha EP, de la Cruz F (2010). Mobility of plasmids. *Microbiol Mol Biol Rev* **74:** 434-452.

Sommer F, Bäckhed F (2013). The gut microbiota—masters of host development and physiology. *Nat Rev Microbiol* **11:** 227-238.

Spahn CM, Blaha G, Agrawal RK, Penczek P, Grassucci RA, Trieber CA et al. (2001). Localization of the ribosomal protection protein Tet(O) on the ribosome and the mechanism of tetracycline resistance. *Mol Cell* **7:** 1037-1045.

Spika JS, Waterman SH, Hoo GWS, St. Louis ME, Pacer RE, James SM et al. (1987). Chloramphenicol-resistant *Salmonella* Newport traced through hamburger to dairy farms. *N Engl J Med* **316:** 565-570.

Stark AH, Crawford MA, Reifen R (2008). Update on alpha-linolenic acid. *Nutr Rev* **66:** 326-332.

Statistics Canada (2013). Hog inventories by province. http://www.statcan.gc.ca/tables-tableaux/sum-som/l01/cst01/prim51a-eng.htm. Accessed April 16, 2014.

Stein HH, Kil DY (2006). Reduced use of antibiotic growth promoters in diets fed to weanling pigs: dietary tools, part 2. *Anim Biotechnol* **17:** 217-231.

Stone JJ, Clay SA, Zhu Z, Wong KL, Porath LR, Spellman GM (2009). Effect of antimicrobial compounds tylosin and chlortetracycline during batch anaerobic swine manure digestion. *Water Res* **43:** 4740-4750.

Suau A, Bonnet R, Sutren M, Godon J-J, Gibson GR, Collins MD et al. (1999). Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol* **65:** 4799-4807.

Suchodolski J, Dowd S, Westermarck E, Steiner J, Wolcott R, Spillmann T et al. (2009). The effect of the macrolide antibiotic tylosin on microbial diversity in the canine small intestine as demonstrated by massive parallel 16S rRNA gene sequencing. *BMC Microbiol* **9:** 210.

Sunde M, Sorum H (2001). Self-transmissible multidrug resistance plasmids in *Escherichia coli* of the normal intestinal flora of healthy swine. *Microb Drug Resist* **7:** 191-196.

Swann M (1969). Report of the joint committee on the use of antibiotics in animal husbandry and veterinary medicine. London: Her Majesty's Stationary Office.

Teeter JS, Meyerhoff RD (2003). Aerobic degradation of tylosin in cattle, chicken, and swine excreta. *Environ Res* **93:** 45-51.

Teuber M (2001). Veterinary use and antibiotic resistance. *Curr Opin Microbiol* **4:** 493-499.

Thacker PA (2013). Alternatives to antibiotics as growth promoters for use in swine production: a review. *J Anim Sci Biotechnol* **4:** 35.

Thaker M, Spanogiannopoulos P, Wright GD (2010). The tetracycline resistome. *Cell Mol Life Sci* **67:** 419-431.

Thakur S, Gebreyes WA (2005). Prevalence and antimicrobial resistance of *Campylobacter* in antimicrobial-free and conventional pig production systems. *J Food Prot* **68:** 2402-2410.

Thomas CM, Nielsen KM (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Microbiol* **3:** 711-721.

Thompson CL, Wang B, Holmes AJ (2008). The immediate environment during postnatal development has long-term impact on gut community structure in pigs. *ISME J* **2:** 739-748.

Thymann T, Sørensen KU, Hedemann MS, Elnif J, Jensen BB, Banga-Mboko H et al. (2007). Antimicrobial treatment reduces intestinal microflora and improves protein digestive capacity without changes in villous structure in weanling pigs. *Br J Nutr* **97:** 1128-1137.

Toomey N, Bolton D, Fanning S (2010). Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. *Res Microbiol* **161:** 127-135.

Turner TD, Mapiye C, Aalhus JL, Beaulieu AD, Patience JF, Zijlstra RT et al. (2014). Flaxseed fed pork: n-3 fatty acid enrichment and contribution to dietary recommendations. *Meat Sci* **96:** 541-547.

Ufnar JA, Ufnar DF, Wang SY, Ellender RD (2007). Development of a swine-specific fecal pollution marker based on host differences in methanogen *mcr*A genes. *Appl Environ Microbiol* **73:** 5209-5217.

Ungemach FR, Müller-Bahrdt D, Abraham G (2006). Guidelines for prudent use of antimicrobials and their implications on antibiotic usage in veterinary medicine. *Int J Med Microbiol* **296:** 33-38.

Upadrasta A, O'Sullivan L, O'Sullivan O, Sexton N, Lawlor PG, Hill C et al. (2013). The effect of dietary supplementation with spent cider yeast on the Swine distal gut microbiome. *PLOS ONE* **8:** e75714.

United States Department of Agriculture (2007). Part II: Reference of swine health and health management practices in the United States. http://www.aphis.usda.gov/animal_health/nahms/swine/downloads/swine2006/Swine2006_dr_PartII.pdf. Accessed April 16, 2014.

United States Department of Agriculture (2008). Part IV: Changes in the U.S. Pork Industry, 1990-2006 http://www.aphis.usda.gov/animal_health/nahms/swine/downloads/swine2006/Swine2006_dr_PartIV.pdf. Accessed October 29, 2014.

United States Food and Drug Administration (2013). Guidance for Industry. New Animal Drugs and New Animal Drug Combination Products Administered in or on Medicated Feed or Drinking Water of Food-Producing Animals: Recommendations for Drug Sponsors for Voluntarily Aligning Product Use Conditions with GFI #209. http://www.fda.gov/downloads/animalveterinary/guidancecomplianceenforcement/guidanceforindustry/ucm299624.pdf. Accessed July 21, 2014.

van den Bogaard AE, London N, Driessen C, Stobberingh EE (2001). Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *J Antimicrob Chemother* **47:** 763-771.

van Elsas JD, Bailey MJ (2002). The ecology of transfer of mobile genetic elements. *FEMS Microbiol Ecol* **42**:187-97.

Varga C, Rajić A, McFall ME, Reid-Smith RJ, McEwen SA (2009). Associations among antimicrobial use and antimicrobial resistance of *Salmonella* spp. isolates from 60 Alberta finishing swine farms. *Foodborne Pathog Dis* **6:** 23-31.

Verstegen MW, Williams BA (2002). Alternatives to the use of antibiotics as growth promoters for monogastric animals. *Anim Biotechnol* **13:** 113-127.

Vester B, Douthwaite S (2001). Macrolide resistance conferred by base substitutions in 23S rRNA. *Antimicrob Agents Chemother* **45:** 1-12.

Vigre H, Larsen P, Andreasen M, Christensen J, Jorsal S (2008). The effect of discontinued use of antimicrobial growth promoters on the risk of therapeutic antibiotic treatment in Danish farrow-to-finish pig farms. *Epidemiol Infect* **136:** 92-107.

Viola C, DeVincent S (2006). Overview of issues pertaining to the manufacture, distribution, and use of antimicrobials in animals and other information relevant to animal antimicrobial use data collection in the United States. *Prev Vet Med* **73:** 111-131.

Vondruskova H, Slamova R, Trckova M, Zraly Z, Pavlik I (2010). Alternatives to antibiotic growth promoters in prevention of diarrhoea in weaned piglets: a review. *Vet Med-Czech* **55:** 199-224.

Wagner BA, Straw BE, Fedorka-Cray PJ, Dargatz DA (2008). Effect of antimicrobial dosage regimen on *Salmonella* and *Escherichia coli* isolates from feeder swine. *Appl Environ Microbiol* **74:** 1731-1739.

Walsh C (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature* **406:** 775-781.

Wassenaar TM (2005). Use of antimicrobial agents in veterinary medicine and implications for human health. *Crit Rev Microbiol* **31:** 155-169.

Wegener H (2003). Antibiotics in animal feed and their role in resistance development. *Curr Opin Microbiol* **6:** 439-445.

Weisblum B (1995). Insights into erythromycin action from studies of its activity as inducer of resistance. *Antimicrob Agents Chemother* **39:** 797.

Wierzbowski AK, Hoban DJ, Hisanaga T, DeCorby M, Zhanel GG (2006). The use of macrolides in treatment of upper respiratory tract infections. *Curr Allergy Asthma* **6:** 171-181.

Witte W (1998). Medical consequences of antibiotic use in agriculture. *Science* **279:** 996.

Woese CR (1987). Bacterial evolution. *Microbiol Rev* **51:** 221-271.

World Health Organization (2014). Antimicrobial resistance: global report on surveillance. http://www.who.int/drugresistance/documents/surveillancereport/en/. Accessed July 21, 2014.

Yang W, Moore IF, Koteva KP, Bareich DC, Hughes DW, Wright GD (2004). TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. *J Biol Chem* **279:** 52346-52352.

Zakeri B, Wright GD (2008). Chemical biology of tetracycline antibiotics. *Biochem Cell Biol* **86:** 124-136.

Zalacain M, Cundliffe E (1989). Methylation of 23S rRNA caused by *tlr*A (*erm*SF), a tylosin resistance determinant from *Streptomyces fradiae*. *J Bacteriol* **171:** 4254-4260.

Zaneveld JR, Nemergut DR, Knight R (2008). Are all horizontal gene transfers created equal? Prospects for mechanism-based studies of HGT patterns. *Microbiology* **154:** 1-15.

Zhang W, Wang X, Liu Y, Tian H, Flickinger B, Empie MW et al. (2008). Dietary flaxseed lignan extract lowers plasma cholesterol and glucose concentrations in hypercholesterolaemic subjects. *Br J Nutr* **99:** 1301-1309.

Zhou Z, Raskin L, Zilles JL (2009). Macrolide resistance in microorganisms at antimicrobial-free swine farms. *Appl Environ Microbiol* **75:** 5814-5820.

Zhou Z, Raskin L, Zilles JL (2010). Effects of Swine manure on macrolide, lincosamide, and streptogramin B antimicrobial resistance in soils. *Appl Environ Microbiol* **76**:2218-24.

Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD et al. (2013). Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc Natl Acad Sci USA* **110:** 3435-3440.

Zimmerman DR (1986). Role of subtherapeutic levels of antimicrobials in pig production. *J Anim Sci* **62:** 6-16.

Zoetendal EG, Collier CT, Koike S, Mackie RI, Gaskins HR (2004). Molecular ecological analysis of the gastrointestinal microbiota: a review. *J Nutr* **134:** 465-472.

Chapter 3. Impact of Subtherapeutic Administration of Tylosin and Chlortetracycline on Antimicrobial Resistance in Farrow-to-Finish Swine

Running title: Antibiotic Resistance in Swine Production.

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3.1 Abstract

The use of antimicrobial agents in swine production at subtherapeutic concentrations for the purpose of growth promotion remains controversial due to the potential impact on public health. Beginning at weaning (3 wk), pigs received either non-medicated feed or feed supplemented with subtherapeutic levels of either tylosin (11 to 44 mg kg^{-1} feed) or chlortetracycline (5.5 mg kg^{-1} feed). After only 3 weeks, pigs given feed supplemented with tylosin had significantly higher levels of tylosin-resistant anaerobes ($p < 0.0001$) compared to the control group, increasing from 11.8% to 89.6%, a level which was stable for the duration of the study, even after a two-week withdrawal prior to slaughter. Tylosin-fed pigs had a higher incidence of detection for *erm*(A), *erm*(F), and *erm*(G), as well as significantly ($p < 0.001$) higher concentrations of *erm*(B) in their feces. The continuous administration of chlortetracycline-supplemented feed, however, had no significant effect on the population of chlortetracycline-resistant anaerobes in comparison with non-treated pigs ($p > 0.05$). The resistance genes *tet*(O), *tet*(Q), and *erm*(B) were detected in all pigs at each sampling time, while *tet*(G), *tet*(L), and *tet*(M) were also frequently detected. Neither chlortetracycline nor tylosin increased the growth rate of pigs.

3.2 Introduction

Antimicrobials have been used in livestock at subtherapeutic levels for the purpose of growth promotion since the 1950s (Viola and DeVincent, 2006). While the exact mechanism(s) that is responsible for the growth-promoting effects of antibiotics is unknown, it is believed to be related to a direct impact on the intestinal microbiota (Dibner and Richards, 2005). Worldwide, the majority of antimicrobials produced are used in animals raised for human food consumption. Most of these antimicrobials are administered via feed or water (Silbergeld et al., 2008). Furthermore, swine production accounts for 60% of all antimicrobials used in animals (Bibbal et al., 2007). The use of antimicrobials in agricultural animals selects for resistant bacteria, which in turn can serve as a reservoir of resistant bacteria and resistance genes (Wegener 2003). Resistant bacteria, both pathogenic and commensal, may then be passed to humans through food, direct contact with animals, or release of animal waste into the environment (Barza, 2002; Witte, 1998).

In Canada and in the United States, the tetracycline antibiotic chlortetracycline and the macrolide antibiotic tylosin represent the two most commonly used antibiotics in swine production (Apley et al., 2012, Deckert et al., 2010). Macrolides antibiotics have been declared by the World Health Organization to be critically important for human medicine while tetracyclines are deemed highly important (WHO, 2012). In the province of Quebec, a veterinary prescription is required to purchase antibiotic-supplemented feeds; however, in the rest of Canada and in the United States they can usually be purchased without a prescription. Outbreaks of antibiotic-resistant pathogens such as *Salmonella enterica* serotype Typhimurium DT104 have been epidemiologically linked to swine operations (Molbak et al., 1999) and swine and farm workers have been colonized with the same strain of methicillin-resistant

Staphylococcus aureus (Smith et al.. 2009). Individuals working on swine farms also frequently carry a higher prevalence of antibiotic-resistant bacteria in their gut (Aubry-Damon et al., 2004). The problem of antibiotic resistance has been further exacerbated by the fact that there are fewer antibiotics in development, which underlines the need to preserve the efficacy of currently available antibiotics for both animal and human therapy (Spellberg et al., 2008). It is against this backdrop that there have been calls in North America for a reduction in the use of antibiotics for growth promotion purposes or even an outright ban similar to the one enacted by the European Union in 2006 (Mathew et al., 2007).

While the antibiotic resistance profiles of pathogens and opportunistic bacteria isolated from swine such as *Salmonella*, *Campylobacter*, *Enterococcus* spp., and *Escherichia coli*, have been well characterized, the whole gut microbiota has, until recently, received far less attention (Lamendella et al., 2011; Looft et al., 2012,; Marshall and Levy, 2011). Most of the antibiotics fed to animals, however, have a broad-spectrum of activity, and therefore nearly all bacteria in the host animal are affected. Resistance determinants in commensals can be passed to pathogenic bacteria via horizontal gene transfer, as many of them are located on mobile genetic elements such as plasmids, transposons, and integrons (Alekshun and Levy, 2007; Marshall and Levy, 2011).

Studies that rely solely on traditional culture-dependent methods may bias or underestimate the full extent of antibiotic resistance. For example, only 20-40% of the gut microbiota is believed to be culturable by traditional means (Suau et al., 1999). The use of molecular-based methods helps overcome some of these limitations by providing a more comprehensive view of the gut microbiota, although these methods are also be subject to bias.

Therefore, culture-dependent and independent methods can be used together to complement one another.

Given the contentious nature of using growth-promoting antibiotics in swine there is still a need for a better understanding of how antibiotic resistance develops in pigs. The objectives of this work are to evaluate what impact the continuous feeding of subtherapeutic concentrations of either tylosin or chlortetracycline has on (1) resistance in the cultivable anaerobic gut microbiota, (2) the prevalence and abundance of several tetracycline and macrolide-lincosamide-streptogramin B resistance genes, and (3) and the growth rate of pigs. This was investigated over the entire length of the swine production cycle, that is, from farrow-to-finish.

3.3 Materials and methods

3.3.1 Animals and housing

Two Landrace x Yorkshire sows born in late-March early-April 2010, were received from a local swine farm (Ferme Rouslay, Ste-Perpétue, QC, Canada) on October 6th, 2010. The sows were housed at the McGill Swine Complex and fed antibiotic-free, pelleted, equilibrated, cereal-based feed specific for gestation and lactation, as well as water (via a self-controlled nipple waterer), ad libitum for the entirety of the study (Agribrands-Purina, St-Hubert, QC, Canada). Antibiotics have not been used at the McGill Swine Complex since January 2007 (Pakpour et al., 2012a).

All piglets were born on April 8th, 2011 and were housed together with their respective sow in the farrowing-suckling room in a farrowing crate (1.5 x 2.1 m) with a metal slatted floor. At 10 days of age, antibiotic-free creep feed (~ 500 g day^{-1} per feeder) was introduced into each

pen using a circular feeder placed in the back of the pen. Piglets also had access to water ad libitum via a self-controlled nipple waterer.

The farrowing-suckling, weanling-starter, and growing-finishing rooms had all been vacant for at least one year and had been cleaned with high-pressure water and painted prior to the introduction of the pigs in this study. All pens had either metal or concrete slated floors. Where possible, this study was designed to duplicate commercial swine husbandry practices. All animal handling procedures were carried out in accordance with McGill University Animal Care and Use Committee guidelines.

3.3.2 Experimental design

Piglets were weaned at 24 days of age and 24 piglets (12 males and 12 females) of approximately the same weight from the two sows were randomly assigned to either a control group or one of two treatment groups (tylosin and chlortetracycline). Each treatment group was comprised of one pen of four males and one pen of four females. Each pen housing pigs was separated from other pigs by two empty pens in order to minimize physical contact between groups and reduce the transmission of microorganisms and antibiotic resistance determinants between pigs of different experimental groups. Pigs were given a pelleted, equilibrated, cereal-based diet specific for each production stage, as well as water, ad libitum (Agribrands-Purina, St-Hubert, QC, Canada). The tylosin-supplemented group received feed with 44 mg tylosin kg^{-1} feed for 21 d beginning at the weanling phase, then 22 mg kg^{-1} feed for 21 d at the starter phase, and finally 11 mg kg^{-1} feed for the remaining 70 d (growing-finishing) prior to withdrawal of antibiotics. Pigs in the chlortetracycline cohort were given feed supplemented with 5.5 mg chlortetracycline kg^{-1} feed for the entirety of the study beginning at weaning. These dosages

were based on the Canadian Food Inspection Agency (CFIA) allowance for growth promotion in swine (CFIA, 2012). All feeds were otherwise identical in nutritional content.

3.3.3 Sample collection

Fresh fecal samples were obtained from pigs at suckling (3 weeks), weanling (6 weeks), starting (9 weeks), growing (12 weeks), finishing (19 weeks) and after a two-week withdrawal of antibiotics (21 weeks). Fecal samples were also obtained from the sows before and after farrowing. Sampling was carried out as previously described (Pakpour et al., 2012a) with the modification of using liquid nitrogen at the farm to preserve fecal samples destined for molecular analysis. Each treatment group was sampled on a different day of the week to minimize any potential cross-contamination associated with handling. Protective clothing and boots were also washed in between sampling days as a further precaution. Access to the pigs was limited to researchers.

3.3.4 Growth rate of pigs

Pigs were weighed individually using an electronic scale immediately prior to starting antibiotic supplemented feeds (4 wk) and at sampling times for weanling (6 wk), growing (12 wk), and finishing (19 wk). The growth rate was measured as kg gained per day.

3.3.5 Enumeration of anaerobic fecal bacteria

A spread-plate method was used for the enumeration of total and antibiotic-resistant anaerobic bacteria in swine feces as per Pakpour et al. (2012a). The Forma 1025 anaerobic chamber (Thermo Fisher Scientific, Nepean, ON, Canada) had a gas mixture of N_2-H_2-CO_2 (90:5:5). For enumerations, each piglet was randomly paired with another piglet from the same pen and therefore the same sex and treatment group. A 5 g composite sample was produced using 2.5 g of feces from each pig. Total, tylosin-resistant, and chlortetracycline-resistant

anaerobic bacteria were enumerated in the anaerobic chamber according to Pakpour et al. (2012a), with the exceptions that plates were incubated at 39°C and enumerated after 7 days. The pH of BHIA (Brain Heart Infusion Agar) plates was also adjusted with 1 N HCl to either 6.7 (the rectal pH of adult swine) or 6.4 for suckling pigs (rectal pH of suckling piglets) (Snoeck et al., 2004).

3.3.6 DNA extraction

Total DNA was extracted from individual swine fecal samples (approximately 100-150 mg) using the ZR Fecal DNA Miniprep kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. This kit provides optimal extracted DNA in terms of yield and purity, based on previous work in our laboratory (Pakpour et al. 2012b). DNA to be used as a positive control in PCR experiments (Table 3.1) was prepared using either an EZNA plasmid mini kit (Omega Biotek Inc., Norcross, GA, USA) for plasmid-mediated genes or a GenElute bacterial genomic DNA kit (Sigma) for chromosomal resistance genes. The total DNA concentration in each extract was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Extracted DNA was stored at -20°C until use.

3.3.7 Detection of antibiotic resistance determinants

Conventional PCR was employed to detect MLS_B and tetracycline resistance genes in swine feces. Amplification of DNA was performed using a Veriti thermocycler (Applied Biosystems, Foster City, CA, USA). A total of 13 tetracycline resistance genes (*tet*) and 5 MLS_B resistance genes (*erm*) were targeted for screening (Table 3.1). Each PCR reaction consisted of 1X PCR buffer (20 mM Tris-HCl pH 8.4, 20 mM KCl) (Bioshop Canada Inc., Burlington, ON, Canada), 1.5 mM $MgCl_2$, 0.5 μM of each primer (AlphaDNA, Montreal, QC, Canada, Table 3.2), 200 μM of each deoxyribonucleoside triphosphate (dNTP) (Bioshop), 1.25 U *Taq* DNA

polymerase (Bioshop), 50 ng of template DNA extracted from the fecal sample of each pig, and autoclaved distilled deionized water in a total volume of 25 µl. The $MgCl_2$ concentration was adjusted to 2 mM for *erm*(B) and *erm*(C), and to 4 mM for *erm*(A). Each DNA extract was evaluated in duplicate for each gene. Positive controls used are described in Table 3.1. An inhibition control consisting of DNA extract and positive control template was used to ensure that there were no inhibitory compounds in the extracted fecal sample DNA. A no-template negative control was also included in each run.

Table 3.1. Oligonucleotide primers, annealing temperatures, and positive control strains or plasmids used for the detection of resistance genes by conventional PCR.

Gene	Sequence (5′ → 3′)	Amplicon size (bp)	Annealing temperature (°C)	Strain or plasmid	Reference
tet(A)	F-GCGCGATCTGGTTCACTCG R-AGTCGACAGYRGCGCCGGC	164	61	*S. aureus* SAS1393	(Aminov et al. 2002)
tet(B)	F-TACGTGAATTTATTGCTTCGG R-ATACAGCATCCAAAGCGCAC	206	59	*E. coli* Ct4afooB (Tn10)	(Aminov et al. 2002)
tet(C)	F-GCGGGATATCGTCCATTCCG R-GCGTAGAGGATCCACAGGACG	207	63	pBR322	(Aminov et al. 2002)
tet(D)	F-GGAATATCTCCCGGAAGCGG R-CACATTGGACAGTGCCAGCAG	187	62	*E. coli* D7-5	(Aminov et al. 2002)
tet(E)	F-GTTATTACGGGAGTTTGTTGG R-AATACAACACCCACACTACGC	199	61	pSL1504	(Aminov et al. 2002)
tet(G)	F-CAGCTTTCGGATTCTTACGG R-GATTGGTGAGGCTCGTTAGC	844	55	pJA8122	(Ng et al. 2001)
tet(K)	F-TTATGGTGGTTGTAGCTAGAAA R-AAAGGGTTAGAAACTCTTGAAA	348	55	pSL1504	(Gevers et al. 2003)
tet(L)	F-TCGTTAGCGTGCTGTCATTC R-EGTATCCCACCAATGTAGCCG	267	55	pVB.A15	(Ng et al. 2001)
tet(M)	F-ACAGAAAGCTTATTATATAAC R-TGGCGTGTCTATGATGTTCAC	171	53	pJ13	(Aminov et al. 2001)
tet(O)	F-AACTTAGGCATTCTGGCTCAC R-TCCCACTGTTCCATATCGTCA	515	55	pUOA1	(Ng et al. 2001)
tet(Q)	F-AGAATCTGCTGTTTGCCAGTG R-CGGAGTGTCAATGATATTGCA	169	63	pNFD13-2	(Aminov et al. 2001)
tet(Y)	F-ATTTGTACCGGCAGAGCAAAC R-GGCGCTGCCGCCATTATGC	181	60	AF070999	(Aminov et al. 2002)
erm(A)	F-TCTAAAAAGCATGTAAAAGAA R-CTTCGATAGTTTATTAATATTAGT	645	52	*S. epidermidis* CCRI-9930	(Sutcliffe et al. 1996)
erm(B)	F-GAAAAGGTACTCAACCAAATA R-AGTAACGGTACTTAAATTGTTTAC	639	52	*S. aureus* CCRI-1317	(Sutcliffe et al. 1996)
erm(C)	F-TCAAAACATAATATAGATAAA R-GCTAATATTGTTTAAATCGTCAAT	642	51	*S. aureus* CCRI-1317	(Sutcliffe et al. 1996)
erm(F)	F-CGACACAGCTTTGGTTGAAC R-GGACCTACCTCATAGACAAG	309	50	pBS2	(Chen et al. 2007)
erm(G)	F-ACATTTCCTAGCCACAATC R-CGCTATGTTTAACAAGC	442	50	pGERM	(Shoemaker et al. 2001)

The PCR programs for tetracycline resistance genes were as described by Pakpour et al. (2012a), while *tet*(Q) was amplified according to Koike et al. (2007). The PCR conditions for the MLS_B resistance genes *erm*(A), *erm*(B) and *erm*(C) were as detailed in Chénier and Juteau (2009). The PCR program for *erm*(F) started with an initial DNA denaturation (94°C for 5 min), followed by 30 cycles of 30 sec at 94°C (denaturing), 1 min at 50°C (annealing), and 2 min at 72°C (extension), followed by a final extension of 5 min at 72°C. The PCR program for *erm*(G) started with an initial DNA denaturation (95°C for 5 min), followed by 30 cycles of 1 min at 95°C (denaturing), 1 min at 50°C (annealing), and 2 min at 72°C (extension), followed by a final extension of 10 min at 72°C. PCR products were detected and analyzed by electrophoresis as per Chénier and Juteau (2009).

3.3.8 Standard curve for real-time PCR

The resistance genes *tet*(O), *tet*(Q) and *erm*(B) were detected in all pigs at all production stages and *tet*(M) was detected in at least 50% of the samples. As a result, these genes were selected for quantification by real-time PCR. The genes *erm*(A) and *erm*(F) were also chosen as they were detected more frequently in the tylosin-treated pigs. The strains and plasmids described in Table 3.2 were used to generate standards in order to determine the number of copies of the corresponding resistance gene in the samples. Each target gene was amplified by conventional PCR using the reaction mixture, programs and thermocycler described above but with the primers described in Table 3.2. These amplicons (standards) were gel purified using the QIAEX II gel extraction kit (Qiagen Inc, Toronto, ON, Canada). The concentration of each standard (ng μl^{-1}) was determined using a NanoDrop 2000 Spectrophotometer. The number of copies μl^{-1} of each standard was calculated using the formula found in Malorny et al. (2003).

Subsequently, serial tenfold dilutions of each standard were prepared in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) in order to generate a 5-point standard curve from 10^2 to 10^6 copies.

3.3.9 Real-time PCR

All real-time PCR experiments were carried out in a Stratagene Mx3005P system with MxPro software version 4.10 (Agilent Technologies, Santa Clara, CA, USA). Primers used for real-time PCR are listed in Table 3.2. Each real-time PCR reaction mixture consisted of 50 ng of swine fecal DNA as a template, 0.75 µM of each forward and reverse primer for *tet*(M), *tet*(O), and *tet*(Q), 0.3 µM of each forward and reverse primer for *erm*(A), *erm*(B), and *erm*(F), 1X Brilliant III Ultra-Fast SYBR Green QPCR master mix (Agilent Technologies), and autoclaved distilled deionized water in a total reaction volume of 20 µl. All standards and samples were run in parallel and in triplicate. Each run also included a no-template negative control. The cycling conditions for all six genes were as follows: an initial denaturation of 95°C for 3 min, followed by 40 cycles of 95°C for 20 sec (denaturing) and 60°C for 20 sec (annealing and extension). A melt curve analysis from 55°C to 95°C was performed at the end of each run to ensure the amplification of only one product.

The number of copies of each target gene in swine fecal DNA extracts was determined by comparing the C_t (cycle threshold) of the samples with the standard curve using the MxPro software. The concentration of each target gene in the swine fecal DNA extract was calculated using the following formula:

$$\text{Concentration (copies ng}^{-1}\text{ total DNA)} = \frac{\text{number of copies of target gene in extract}}{\text{concentration (ng total DNA µl}^{-1}\text{) x volume (µl)}}$$

Concentration of total DNA was measured by the NanoDrop after DNA extraction; volume is the volume of DNA extract used in the real-time PCR reaction.

Table 3.2. Oligonucleotide primers and positive control strains or plasmids used for the quantification of resistance genes by real time PCR.

Gene	Primer sequence 5′ → 3′	Amplicon size (bp)	Strain or plasmid	Reference
tet(M)	F-GTGGACAAAGGTACAACGAG R-CGGTAAAGTTCGTCACACAC	406	pJ13	(Ng et al. 2001)
tet(O)	F-AACTTAGGCATTCTGGCTCAC R-TCCCACTGTTCCATATCGTCA	515	pUOA1	(Ng et al. 2001)
tet(Q)	F-AGAATCTGCTGTTTGCCAGTG R-CGGAGTGTCAATGATATTGCA	169	pNFD13-2	(Aminov et al. 2001)
erm(A)	F-GAAATYGGRTCAGGAAAAGG R-AAYAGYAAACCYAAAGCTC	332	*S. aureus* CCRI-1317	(Chen et al. 2007)
erm(B)	F-GATACCGTTTACGAAATTGG R-GAATCGAGACTTGAGTGTGC	364	*S. epidermidis* CCRI-9930	(Chen et al. 2007)
erm(F)	F-CGACACAGCTTTGGTTGAAC R-GGACCTACCTCATAGACAAG	309	pBS2	(Chen et al. 2007)

3.3.10 Statistical analysis

Analysis of variance for bacterial enumerations and real-time PCR results was performed using PROC GLM with repeated measures in SAS (SAS Inst., Inc., Cary, NC, USA) followed by Tukey's honestly significant difference post hoc test for multiple comparisons. Pearson correlation coefficients for the correlation between the presence of resistance genes and percent resistance was determined using PROC CORR. An individual pig or a pig-pair was considered the experimental unit (n). Treatment, sex, and sampling time were included in the model for the analysis of total anaerobes, while treatment, sex, sow, and sampling time were included in the

model for real-time PCR data, as well as their interactions. Results were considered significant at the $\alpha = 0.05$ level.

3.4 Results

3.4.1 Antibiotic resistance in the cultivable anaerobic fecal bacterial populations

There were no significant differences between treatment groups in terms of abundance of total anaerobic bacteria at any sampling time (data not shown). Overall, combining the total number of anaerobes from all groups, there was a significant decrease from 5.4×10^9 CFU g^{-1} wet feces at suckling (3 wk) to 1.9×10^8 CFU g^{-1} wet feces at weanling (6 wk) ($p < 0.0001$). From weaning to the two-week withdrawal period (21 wk), the total number of anaerobic bacteria remained stable, varying from 1.8×10^8 to 2.0×10^8 CFU g^{-1} wet feces.

Pigs that were given tylosin-supplemented feed had a significantly higher rate of tylosin-resistant anaerobes than either the control or chlortetracycline treated pigs at weanling ($p < 0.0001$) (6 wk), starting ($p < 0.0001$) (9 wk), growing ($p < 0.001$) (12 wk), finishing ($p < 0.0001$) (19 wk), and even two weeks after tylosin had been removed from their diet (21 wk) ($p < 0.0001$) (Figure 3.1A). After only 3 weeks, the percentage of tylosin-resistant anaerobes in tylosin-fed pigs increased from 11.8% (suckling, 3 wk) to 89.6% (weanling, 6 wk) and remained relatively stable throughout the study. The withdrawal of tylosin for a two week period prior to slaughter decreased the percentage of tylosin-resistant anaerobes from 75.8% to 57.7%, but this decrease was not significant ($p > 0.05$). The percentage of tylosin-resistant anaerobes did not increase significantly for the control and chlortetracycline groups from suckling (3 wk) to weanling (6 wk) ($p > 0.05$) (Figure 3.1A). Anaerobic culturing of sow fecal samples yielded 8.0% and 6.0% tylosin-resistant anaerobes 16 days prior to and 32 days after farrowing, respectively (data not shown).

The administration of chlortetracycline at 5.5 mg kg^{-1} feed had no significant effect on the percentage of chlortetracycline-resistant anaerobes in comparison with the control and tylosin-treated pigs ($p > 0.05$) (Figure 3.1B). From suckling (3 wk) to weanling (6 wk), the percentage of chlortetracycline-resistant anaerobes increased significantly across all three treatment groups ($p < 0.01$), decreased below suckling levels at growing (12 wk) ($p = 0.015$) and remained stable through finishing (19 wk) and after the withdrawal of antibiotics (21 wk).

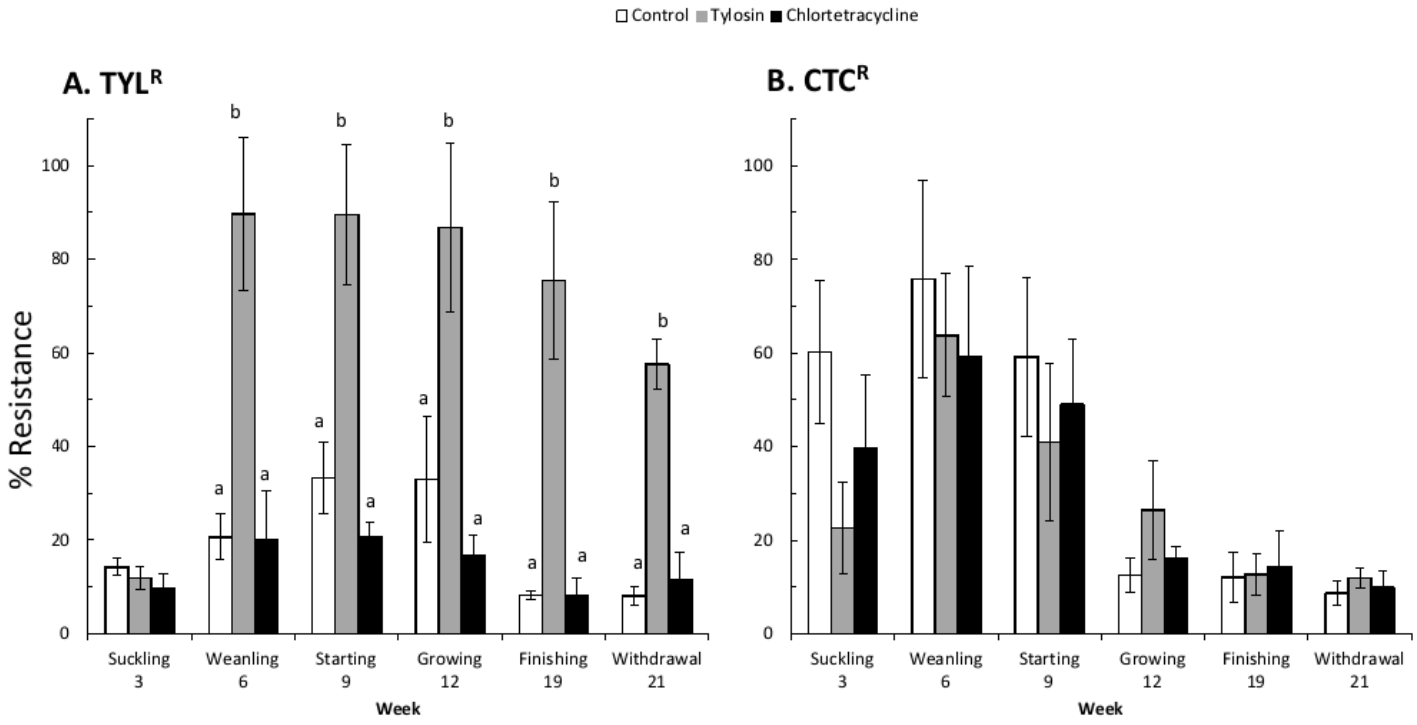

Figure 3.1. Percentage of anaerobes that are A) tylosin-resistant (TYL^R) and B) chlortetracycline-resistant (CTC^R) in the control, tylosin, and chlortetracycline treatment groups at suckling (wk 3), weanling (wk 6), starting (wk 9), growing (wk 12), finishing (wk 19) and two weeks after the withdrawal (wk 21) of each antibiotic. Error bars represent standard deviation of the mean (n = 4) and different letters indicate means that differ significantly ($p < 0.05$) at each production phase. Tylosin-treated pigs received 44 mg tylosin kg^{-1} feed during weanling (wk 4 to 6), then 22 mg tylosin kg^{-1} feed during starting (wk 7 to 9), and finally 11 mg tylosin kg^{-1} feed during growing (wk 10 to 12) and finishing (wk 13 to 19).

3.4.2 Detection of resistance determinants

Of the 18 tetracycline and MLS_B resistance genes screened by PCR, only *erm*(C) was not detected at least once over the course of the study (i.e. suckling to withdrawal of antibiotics)

(Table 3.3). The genes *tet*(O), *tet*(Q), and *erm*(B) were detected in all pigs in each treatment group at each sampling time while *tet*(G), *tet*(L), and *tet*(M) were detected in at least 50% of the samples. The MLS_B resistance genes *erm*(A), *erm*(F), *erm*(G) were detected with greater frequency in the tylosin-treated pigs than either the control or chlortetracycline group (Table 3.3). In the feces of sows, however, only 8 of the 18 genes were detected either before or after farrowing (Table 3.3). A Pearson correlation analysis revealed a weak but significant positive correlation between the percentage of tylosin-resistant anaerobes and the frequency of detection of *erm*(A) ($r = 0.485$ $p < 0.0001$), *erm*(F) ($r = 0.390$ $p = 0.0007$), and *erm*(G) ($r = 0.314$ $p = 0.0072$). The percentage of chlortetracycline-resistant anaerobes was also weakly correlated with the presence of *tet*(G) ($r = 0.253$ $p = 0.032$) and *tet*(M) ($r = 0.420$ $p = 0.0002$).

Correlation analysis between the detection of two genes was performed by substituting binary presence-absence values (1 or 0) for the screening of each gene in each pig at each time point. The genes with the strongest positive correlations were: *tet*(B) and *tet*(M) ($r = 0.301$ $p = 0.0003$), *tet*(B) and *erm*(G) ($r= 0.302$, $p = 0.0003$), *tet*(C) and *tet*(k) ($r = 0.351$, $p < 0.0001$), *tet*(G) and *tet*(L) ($r = 0.377$ $p < 0.0001$), *erm*(A) and *erm*(G) ($r = 0.503$, $p < 0.0001$), *erm*(F) and *tet*(L) ($r = 0.406$, $p < 0.0001$).

Table 3.3 Detection of tetracycline and MLS_B resistance genes using conventional PCR. Results are presented as the number of pigs in which the resistance gene was detected, with n = 8 for each treatment at each production phase. ND: not detected. *erm*(C) was not detected at any time.

Production phase	Treatment	*tet*(A)	*tet*(B)	*tet*(C)	tet(D)	*tet*(E)	*tet*(G)	*tet*(K)	*tet*(L)	*tet*(M)	*tet*(O)	*tet*(Q)	*tet*(S)	*tet*(Y)	*erm*(A)	*erm*(B)	*erm*(F)	*erm*(G)
Suckling	Control	1/8	ND	ND	1/8	ND	4/8	ND	ND	8/8	8/8	8/8	1/8	ND	ND	8/8	ND	ND
	Tylosin	1/8	1/8	ND	ND	2/8	2/8	ND	ND	8/8	8/8	8/8	ND	ND	ND	8/8	ND	ND
	Chlortetracycline	ND	2/8	ND	ND	1/8	1/8	ND	1/8	8/8	8/8	8/8	2/8	ND	ND	8/8	ND	ND
Weanling	Control	ND	2/8	ND	ND	ND	6/8	ND	8/8	7/8	8/8	8/8	2/8	ND	ND	8/8	7/8	ND
	Tylosin	ND	2/8	ND	ND	ND	6/8	ND	8/8	3/8	8/8	8/8	3/8	ND	ND	8/8	8/8	ND
	Chlortetracycline	ND	ND	ND	ND	ND	7/8	ND	8/8	5/8	8/8	8/8	ND	ND	ND	8/8	5/8	ND
Starter	Control	1/8	6/8	ND	ND	ND	8/8	ND	8/8	8/8	8/8	8/8	1/8	ND	ND	8/8	3/8	3/8
	Tylosin	1/8	6/8	ND	ND	ND	7/8	ND	7/8	6/8	8/8	8/8	ND	ND	8/8	8/8	8/8	8/8
	Chlortetracycline	ND	5/8	ND	ND	ND	8/8	ND	6/8	6/8	8/8	8/8	1/8	ND	ND	8/8	3/8	3/8
Growing	Control	1/8	ND	ND	ND	ND	6/8	1/8	5/8	ND	8/8	8/8	ND	4/8	1/8	8/8	1/8	ND
	Tylosin	ND	1/8	ND	ND	ND	1/8	2/8	4/8	ND	8/8	8/8	ND	ND	4/8	8/8	5/8	ND
	Chlortetracycline	ND	4/8	ND	ND	ND	6/8	1/8	5/8	ND	8/8	8/8	7/8	ND	1/8	8/8	ND	ND
Finishing	Control	ND	ND	2/8	ND	ND	3/8	8/8	8/8	ND	8/8	8/8	ND	ND	ND	8/8	2/8	ND
	Tylosin	ND	ND	4/8	ND	ND	3/8	8/8	5/8	ND	8/8	8/8	ND	ND	1/8	8/8	3/8	2/8
	Chlortetracycline	ND	ND	4/8	ND	ND	4/8	6/8	6/8	ND	8/8	8/8	1/8	ND	ND	8/8	1/8	ND
Withdrawal	Control	ND	8/8	ND	ND	ND	6/8	8/8	5/8	8/8	8/8	8/8	2/8	ND	ND	8/8	2/8	ND
	Tylosin	ND	4/8	ND	ND	ND	5/8	7/8	8/8	8/8	8/8	8/8	1/8	ND	ND	8/8	7/8	ND
	Chlortetracycline	ND	5/8	ND	ND	ND	8/8	5/8	8/8	8/8	8/8	8/8	ND	ND	ND	8/8	5/8	ND
Sows	Pre-farrowing	ND	2/2	ND	ND	ND	2/2	ND	2/2	2/2	2/2	2/2	ND	ND	ND	2/2	2/2	ND
	Post-farrowing	ND	2/2	ND	ND	ND	2/2	ND	2/2	2/2	2/2	2/2	ND	ND	ND	2/2	2/2	ND

3.4.3 Real-time PCR

The genes *tet*(M), *tet*(O), *tet*(Q), and *erm*(B) were chosen for absolute quantification using real-time PCR based on their frequency of detection by conventional PCR (Table 3.3). As a result of their potential relevance to the differences observed in tylosin-treated pigs, *erm*(A) and *erm*(F) were also selected for absolute quantification. A linear standard curve ($R^2 > 0.98$) was generated for each gene using purified PCR amplicons as described above and plotting C_t values vs. total copy number. The efficiency of all standard curves fell between 90 and 105%. Melting curve analysis of all samples revealed only one product for each sample.

The concentration of *tet*(M) did not differ significantly between the chlortetracycline cohort and the control pigs at any sampling period ($p > 0.05$; Figure 3.2A), nor was there any significant effect of chlortetracycline on the level of *tet*(O) at any sampling time ($p > 0.05$; Figure 3.2B). At the weanling stage (6 wk) and at all subsequent samplings, however, all groups had significantly greater concentrations of *tet*(O) than at suckling (3 wk) ($p < 0.0001$). The concentration of *tet*(Q) also increased significantly from suckling (3 wk) to weanling (6 wk) ($p < 0.05$) in all groups although the withdrawal period (21 wk) was the only time when chlortetracycline-treated pigs differed significantly from the control group ($p = 0.05$). (Figure 3.2C).

The concentration of *erm*(B) was significantly higher in the tylosin-treated pigs at all sampling times except suckling, i.e. weanling (6 wk, $p < 0.05$), starting (9 wk, $p < 0.05$), growing (12 wk, $p < 0.005$), finishing (19 wk, $p < 0.0001$), and antibiotic withdrawal (21 wk, $p < 0.0001$) (Figure 3.2D). At the starting phase (9 wk), there was a significantly higher concentration of *erm*(A) in the feces of tylosin-treated pigs (2.38×10^1 copies ng^{-1} total DNA) than in the control pigs (below detection limit) and the chlortetracycline-treated pigs (below detection limit) ($p <$

0.01). At all other sampling times, however, nearly all samples were below the detection limit of 2.75 copies ng^{-1} total DNA (data not shown). Similarly, there were a large number of samples that had concentrations of *erm*(F) below the detection limit (2.93 copies ng^{-1} total DNA), accounting for 39.5%, 25.0%, and 27.1% of all control, tylosin- and chlortetracycline-treated pigs respectively (data not shown). Statistical analysis for *erm*(F) was done by substituting the detection limit value (2.93 copies ng^{-1} DNA) for those samples that fell below the detection limit. At starting (9 wk, $p < 0.0028$), growing (12 wk, $p < 0.0224$), and finishing (19 wk, $p < 0.0135$), there was a significantly higher concentration of *erm*(F) in the tylosin-treated pigs than in pigs from the other treatment groups (data not shown).

Overall, across all time points and treatment groups, there was a moderate and significant correlation between the concentration of *erm*(B) and the proportion of tylosin-resistant anaerobes ($r = 0.619$, $p < 0.0001$). Concentrations of *tet*(O) and *tet*(Q) were moderately and significantly correlated with each other as well ($r = 0.663$, $p < 0.001$). There was no significant correlation between the concentrations of *tet*(M), *tet*(O), *tet*(Q) and the percentage of chlortetracycline-resistant anaerobes, or between the concentrations of *erm*(A), *erm*(F) and the percentage of tylosin-resistant anaerobes. The two-week withdrawal period had no significant impact on the concentrations of *tet*(O), *erm*(B), or *erm*(F) ($p > 0.05$), while the chlortetracycline-supplemented pigs actually had significantly higher levels of *tet*(Q) ($p < 0.05$) compared to the control group after chlortetracycline was removed from the diet. The feces of sows contained concentrations of *tet*(M), *tet*(O), *tet*(Q), *erm*(B), and *erm*(F) that were similar to those found in the piglets at suckling. Similarly, *erm*(A) levels were below the detection limit both before and after farrowing (data not shown).

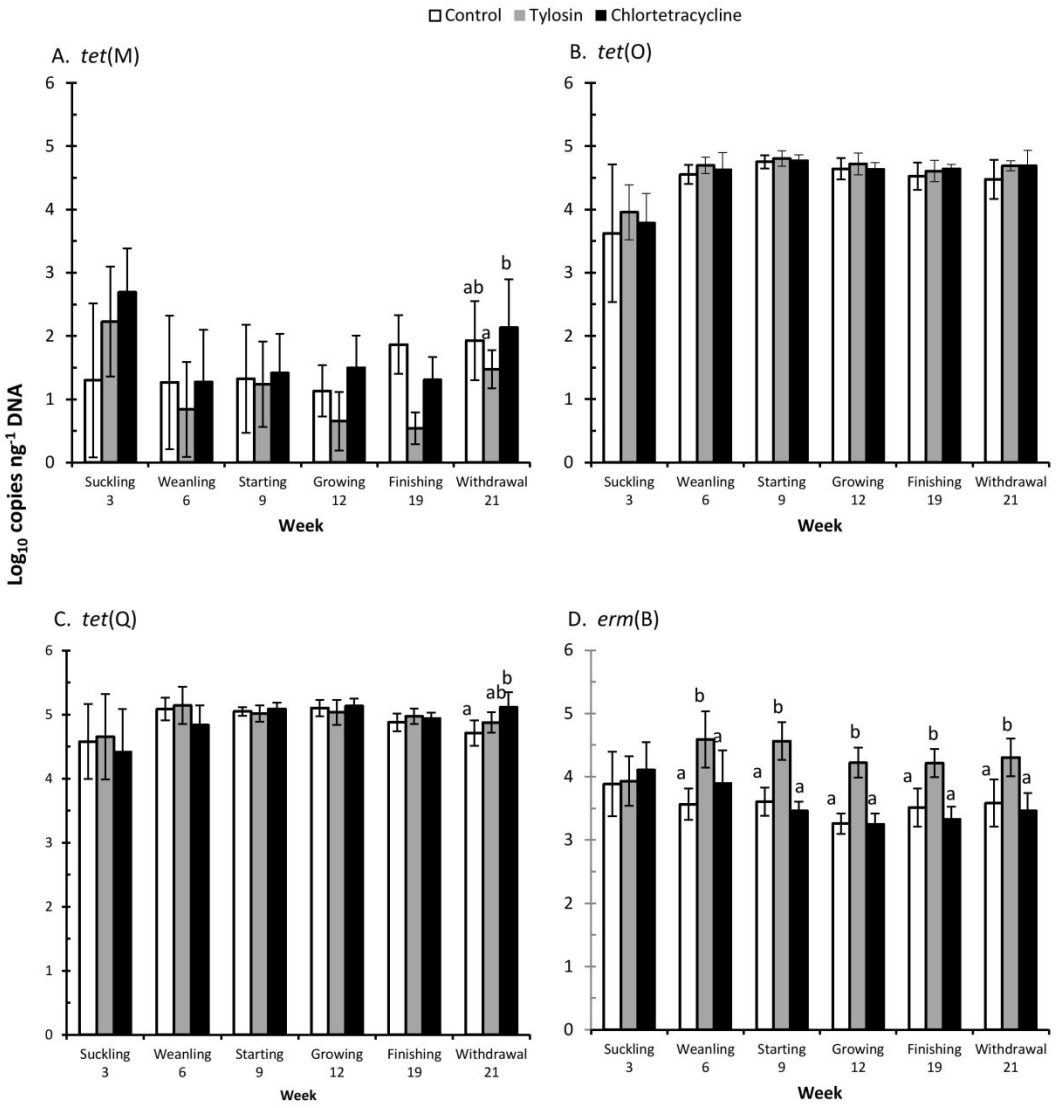

Figure 3.2. Concentration of A) *tet*(M), B) *tet*(O), C) *tet*(Q), D) *erm*(B) in the fecal microbiota of pigs in the control, tylosin, and chlortetracycline groups at suckling (wk 3), weanling (wk 6), starting (wk 9), growing (wk 12), finishing (wk 19), and antibiotic withdrawal (wk 21). Error bars represent standard deviation of the mean (n = 8) and different letters indicate means that differ significantly ($p < 0.05$) at each production phase. Tylosin-treated pigs received 44 mg tylosin kg^{-1} feed during weanling (weeks 4 to 6), then 22 mg tylosin kg^{-1} feed during starting (wk 7 to 9), and finally 11 mg tylosin kg^{-1} feed during growing (wk 10 to 12) and finishing (wk 13 to 19).

3.4.4 Growth rate of pigs in response to antibiotics

Pigs were weighed individually on the first day of weaning (4 wk), and at the weanling (6 wk), growing (12 wk), and finishing (19 wk) sampling times. In terms of growth rate (kg gained day^{-1}), there was no significant difference between any of the groups at any time ($p > 0.05$; Figure 3.3). In the weanling phase (4-6 wk), pigs in the control cohort actually had 4.0% and 2.6% higher growth rates than tylosin- and chlortetracycline-treated pigs, respectively. In the starter and growing phases (7-12 wk), tylosin and chlortetracycline pigs grew 3.0% and 1.3% faster than the control pigs, respectively. Tylosin-treated pigs also had a 2.5% higher growth rate than the control pigs during finishing (13-19 wk) but the control pigs grew 6.7% faster than the chlortetracycline-treated pigs.

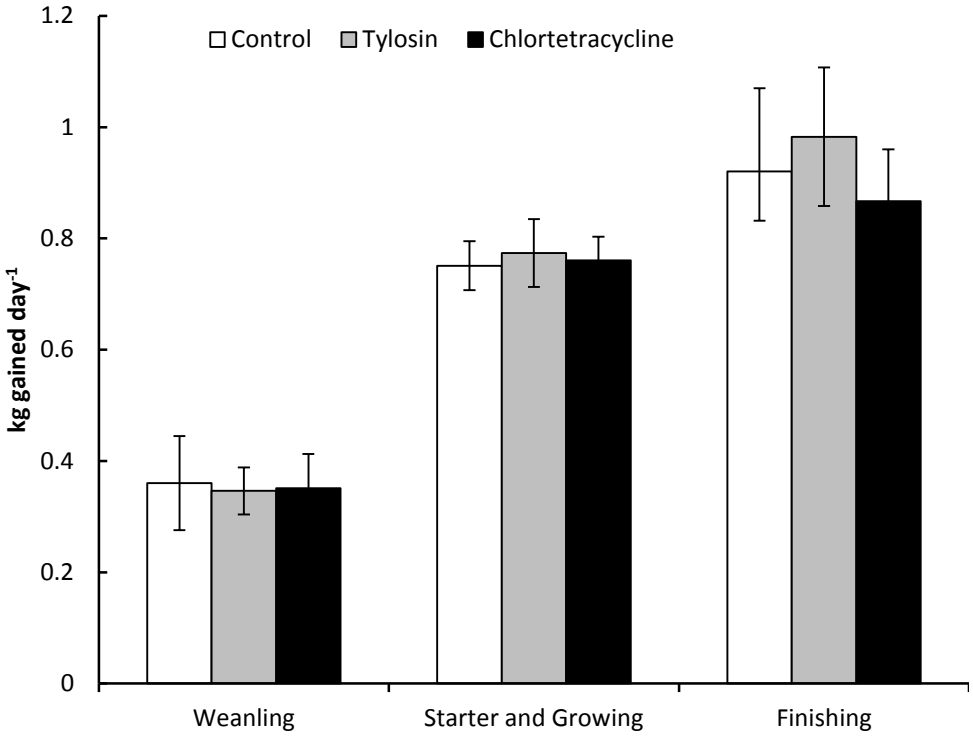

Figure 3.3. Growth rate of pigs during weanling (weeks 4 to 6), starting and growing (weeks 7 to 12), and finishing (weeks 13 to 19). Error bars represent standard deviation of the mean (n = 8).

3.5 Discussion

Previous studies of antibiotic resistance in swine have used either a relatively narrow period in the production cycle for monitoring (Kim et al., 2012; Looft et al, 2012) or focused mainly on pathogenic or opportunistic bacterial species, rather than the entire gut microbiota (Aarestrup and Carstensen, 1998; Jackson et al., 2004; Mathew et al., 1998). The commensal bacteria, however, are in far greater abundance and may serve as a reservoir of antibiotic-resistant bacteria and resistance determinants (Marshall and Levy, 2011). These resistant

bacteria and determinants may then end up in the human food chain during the slaughter process, manure spread on crops as fertilizer, or through water contaminated with animal waste (Wegener, 2003). Transfer may also occur directly between swine and farm or slaughterhouse workers (Aubry-Damon et al., 2004).

In the current study, we investigated the effect that the continuous feeding of subtherapeutic (growth promoting) concentrations of antibiotics has on the tetracycline and MLS_B resistome, as well as on culturable anaerobes, over the entire course of the swine production cycle. We found that the feeding of tylosin-supplemented feed at subtherapeutic levels from weaning (3 wk) to finishing (19 wk) resulted in a significant and rapid increase not only in the percentage of tylosin-resistant anaerobes but also in the concentrations of several MLS_B genes. Importantly, no benefit in terms of growth rate could be detected in pigs that were fed tylosin. Chlortetracycline, meanwhile, at recommended growth-promoting levels had no measurable effect on either the percentage of chlortetracycline-resistant anaerobes or on the abundance of any resistance genes.

It was notable that tylosin had no effect on the total number of anaerobic bacteria, rather only the tylosin-resistant fraction was altered. This finding is supported by Dawson et al. (1984) who determined that it was the proportion of resistant anaerobes that changed in response to antibiotic treatment and not the total number of anaerobes. The trends for the control and chlortetracycline cohorts are similar to what has been reported by our laboratory recently, with a decreasing percentage of tylosin-resistant anaerobes over the production cycle of the pig in the absence of tylosin (Pakpour et al., 2012a).

Other studies have indicated a high incidence of erythromycin-resistant *Enteroccocus* spp. on swine farms that used tylosin as a growth promoter in the finishing phase and in pigs

given tylosin-supplemented feed from growing to finishing (Aarestrup and Carstensen, 1998; Jackson et al., 2004). Comparable to our findings, Aaerestrup and Carstensen (1998) observed an immediate increase in erythromycin-resistant enterococci in pigs given 30 mg tylosin kg^{-1} feed, a resistance level which was stable throughout the duration of their work. Other researchers have reported a very high rate of tylosin-resistant anaerobes in the gut of conventionally raised swine even in the absence of tylosin, albeit using a lower minimum inhibitory concentration (5 $\mu g\ ml^{-1}$) than the present study (Kalmokoff et al., 2011).

In Canada there is no withdrawal period required for in-feed tylosin prior to shipping pigs to the slaughterhouse (CFIA, 2012), however, we wanted to investigate whether a two-week withdrawal period prior to shipping would have any significant impact on tylosin resistance. The finding of no significant decrease in tylosin-resistance after this period suggests that any attempt to significantly lower the level of resistance in pigs fed tylosin via a change in feed would have to start much earlier during swine rearing. This is of significance from a food safety and public health perspective since this means pigs that are fed tylosin on a continuous basis for several months are shipped to slaughter with a high level of tylosin-resistant bacteria in their gut.

Our results indicate that even at subtherapeutic concentrations, tylosin can alter the MLS_B resistome in the swine gut microbiota. The detection of *erm*(A) in all starter (9 wk) pigs in the tylosin group when all other groups including the sows were negative, suggests that tylosin treatment may have had a role in the emergence of this gene. Similar results were observed for *erm*(F) and *erm*(G), with these genes detected more often in the tylosin group. Based on this work and on several other studies, *erm*(B) appears to be among the most widely distributed macrolide resistant genes in swine (Jackson et al., 2004; Kalmokoff et al., 2011; Patterson et al., 2007). The concentrations of *erm*(B) and *erm*(A) (when detected) are in agreement with other

studies of swine manure and feces, although the abundance of *erm*(F) appears to be lower in the present study (Chen et al., 2007; Kalmokoff et al., 2011).

The elevated proportion of chlortetracycline-resistant anaerobes seen in the suckling (3 wk), weanling (6 wk), and starter (9 wk) phases, even in pigs not exposed to chlortetracycline, correlates well with other studies of chlortetracycline or tetracycline resistance in post-weaning swine (Dunlop et al., 1998; Langlois et al., 1988; Wagner et al., 2008). Recent work by our laboratory at the same swine complex yielded a similar high background of chlortetracycline-resistance in pigs not exposed to any antibiotics (Pakpour et al., 2012a). The extensive use of antibiotics such as chlortetracycline in swine husbandry since the 1950s is likely responsible for this high level of background resistance (Looft et al., 2012). This baseline resistance may also make detecting changes in culturable bacteria difficult, particularly when growth-promoting levels of antibiotics are used.

Our finding of a large diversity of tetracycline and MLS_B resistance genes in the swine gut, even in pigs not directly exposed to antibiotics, is consistent with previous studies in swine (Aminov et al., 2001; Barkovskii and Bridges, 2011; Chee-Sanford et al., 2001; Looft et al., 2012; Stanton et al., 2011). Earlier work at this site had indicated a wide dispersal of *tet*(O), *tet*(L), as well as *erm*(B) (Pakpour et al., 2012a). In other studies of swine waste lagoons and/or fecal samples, *tet*(O), *tet*(Q), *tet*(L), and *tet*(M) have all been detected with high frequency (Aminov et al., 2001; Chee-Sanford et al., 2001; Chénier and Juteau, 2009; Jindal et al., 2006). In addition, when *tet*(G) has been screened, it has often been detected in swine (Chen et al., 2010). The concentrations for *tet*(O) obtained in the present study (1.2×10^4 to 6.6×10^4 copies ng^{-1} DNA) are also comparable to a prior study at this swine complex (4.7×10^4 to 5.3×10^5 copies ng^{-1} DNA) (Pakpour et al., 2012a).

All of the genes detected in this study have been previously identified as residing on mobile genetic elements, e.g. plasmids and transposons, which may be horizontally transferred between bacteria. Many of these genes may also be linked together on the same plasmid (Chopra and Roberts, 2001; Roberts et al., 1999; Schwarz et al., 1998). This clearly could pose a potential public health threat if commensal bacteria carrying these genes on mobile genetic elements transfer them to opportunistic or pathogenic bacteria. *erm*(B) for example, is found on both conjugative and non-conjugative transposons and has been linked with a number of genes including *tet*(M), *tet*(O), *tet*(Q), and *erm*(G) (Roberts, 2008; Tremblay et al., 2011). In this study we identified a significant positive correlation between the abundance of *tet*(O) and *tet*(Q), although neither was correlated with *erm*(B) concentrations. The PCR detection of several *tet* and *erm* genes were, however, significantly correlated with each other.

Interestingly, we did not detect any improvement in weight gain in the pigs fed either tylosin or chlortetracycline. Although the benefits of using subtherapeutic antibiotics in swine production have been well documented (Cromwell, 2002; Dritz et al., 2002; Zimmerman, 1986), the majority of this research is older and may no longer hold true under modern swine practices (McEwen and Fedorka-Cray, 2002). In addition, the swine complex used in the present study had a low stocking density and a high level of sanitation. Previous reports have indicated that antibiotics have a greater impact on farms with lower levels of sanitation and higher bacterial loads (Dibner and Richards, 2005; Dritz et al., 2002; NRC, 1998). Van Lunen (2003) followed a nearly identical dosing schedule for tylosin as the present study and did not observe any difference in weight gain between treated and untreated swine at any production phase. A recent study by Holt et al. (2011) reported an improvement in average-daily-gain only in the first week post-weaning for 55 mg chlortetracycline kg^{-1} feed. Given that growth promotion is still the

number one reason for using antibiotics in livestock, it might be time to revisit this issue on a larger scale.

In conclusion, this study demonstrates that feeding subtherapeutic concentrations of tylosin (11 to 44 mg kg^{-1} feed) to pigs on a continuous basis results in a significant and rapid increase in the proportion of tylosin-resistant anaerobes as well as in the concentration and prevalence of MLS_B resistance genes. This alteration is stable throughout the production cycle of the pig, even following a two-week withdrawal period. Furthermore, the swine gut constitutes a large reservoir of tetracycline and MLS_B resistance determinants even in the absence of antibiotic treatment. Chlortetracycline, however, appears to have no effect on the measurable chlortetracycline resistance when given at a concentration of 5.5 mg kg^{-1} feed. Neither tylosin nor chlortetracycline was able to increase the growth rate of the pigs in this study. These findings suggest that in swine production, the impact of antibiotics used for the purpose of growth promotion on resistance levels depends on the type of antibiotic used as well as the concentration.

3.6 Acknowledgements

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3.7 References

Aarestrup FM, Carstensen B (1998). Effect of tylosin used as a growth promoter on the occurrence of macrolide-resistant enterococci and staphylococci in pigs. *Microb Drug Resist* **4:** 307-312.

Alekshun MN, Levy SB (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell* **128:** 1037-1050.

Aminov R, Garrigues-Jeanjean N, Mackie R (2001). Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl Environ Microbiol* **67:** 22-32.

Aminov R, Chee-Sanford J, Garrigues N, Teferedegne B, Krapac I, White B et al. (2002). Development, validation, and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria. *Appl Environ Microbiol* **68:** 1786.

Apley MD, Bush EJ, Morrison RB, Singer RS, Snelson H (2012). Use estimates of in-feed antimicrobials in swine production in the United States. *Foodborne Pathog Dis* **9:** 272-279.

Aubry-Damon H, Grenet K, Sall-Ndiaye P, Che D, Cordeiro E, Bougnoux ME et al. (2004). Antimicrobial resistance in commensal flora of pig farmers. *Emerg Infect Dis* **10:** 873-879.

Barkovskii AL, Bridges C (2011). Persistence and profiles of tetracycline resistance genes in swine farms and impact of operational practices on their occurrence in farms' vicinities. *Water Air Soil Pollut***:** 1-14.

Barza M (2002). Potential mechanisms of increased disease in humans from antimicrobial resistance in food animals. *Clin Infect Dis* **34:** S123-S125.

Bibbal D, Dupouy V, Ferré JP, Toutain PL, Fayet O, Prère MF et al. (2007). Impact of three ampicillin dosage regimens on selection of ampicillin resistance in *Enterobacteriaceae* and excretion of blaTEM genes in swine feces. *Appl Environ Microbiol* **73:** 4785-4790.

CFIA (2012). Compendium of Medicating Ingredient. Compendium of Medicating Ingredient. Canadian Food Inspection Agency. http://www.inspection.gc.ca/english/anima/feebet/mib/mibtoce.shtml. Accessed July 2012.

Chee-Sanford JC, Aminov RI, Krapac I, Garrigues-Jeanjean N, Mackie RI (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol* **67:** 1494-1502.

Chen J, Yu Z, Michel Jr FC, Wittum T, Morrison M (2007). Development and application of real-time PCR assays for quantification of erm genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. *Appl Environ Microbiol* **73:** 4407-4416.

Chen J, Michel FC, Sreevatsan S, Morrison M, Yu Z (2010). Occurrence and persistence of erythromycin resistance genes (*erm*) and tetracycline resistance genes (*tet*) in waste treatment systems on swine farms. *Microbial Ecol* **60:** 479-486.

Chénier MR, Juteau P (2009). Impact of an aerobic thermophilic sequencing batch reactor on antibiotic-resistant anaerobic bacteria in swine waste. *Microbial Ecol* **58:** 773-785.

Chopra I, Roberts M (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* **65:** 232-260.

Cromwell GL (2002). Why and how antibiotics are used in swine production. *Anim Biotechnol* **13:** 7-27.

Dawson KA, Langlois BE, Stahly TS, Cromwell GL (1984). Antibiotic resistance in anaerobic and coliform bacteria from the intestinal tract of swine fed therapeutic and subtherapeutic concentrations of chlortetracycline. *J Anim Sci* **58:** 123.

Deckert A, Gow S, Rosengren L, Léger D, Avery B, Daignault D et al. (2010). Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) Farm Program: Results from Finisher Pig Surveillance. *Zoonoses Public Health* **57:** 71-84.

Dibner J, Richards J (2005). Antibiotic growth promoters in agriculture: History and mode of action. *Poult Sci* **84:** 634-643.

Dritz SS, Tokach MD, Goodband RD, Nelssen JL (2002). Effects of administration of antimicrobials in feed on growth rate and feed efficiency of pigs in multisite production systems. *J Am Vet Med Assoc* **220:** 1690-1695.

Dunlop R, McEwen S, Meek A, Clarke R, Black W, Friendship R (1998). Associations among antimicrobial drug treatments and antimicrobial resistance of fecal Escherichia coli of swine on 34 farrow-to-finish farms in Ontario, Canada. *Prev Vet Med* **34:** 283-305.

Gevers D, Danielsen M, Huys G, Swings J (2003). Molecular characterization of *tet*(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. *Appl Environ Microbiol* **69:** 1270-1275.

Holt J, van Heugten E, Graves A, See M, Morrow W (2011). Growth performance and antibiotic tolerance patterns of nursery and finishing pigs fed growth-promoting levels of antibiotics. *Livest Sci* **136:** 184-191.

Jackson CR, Fedorka-Cray PJ, Barrett JB, Ladely SR (2004). Effects of tylosin use on erythromycin resistance in enterococci isolated from swine. *Appl Environ Microbiol* **70:** 4205-4210.

Jindal A, Kocherginskaya S, Mehboob A, Robert M, Mackie RI, Raskin L et al. (2006). Antimicrobial use and resistance in swine waste treatment systems. *Appl Environ Microbiol* **72:** 7813-7820.

Kalmokoff M, Waddington LM, Thomas M, Liang KL, Ma C, Topp E et al. (2011). Continuous feeding of antimicrobial growth promoters to commercial swine during the growing/finishing phase does not modify faecal community erythromycin resistance or community structure. *J Appl Microbiol* **110**:1414-25.

Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ et al. (2012). Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. *Proc Natl Acad Sci USA* **109:** 15485-15490.

Koike S, Krapac I, Oliver H, Yannarell A, Chee-Sanford J, Aminov R et al. (2007). Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year period. *Appl Environ Microbiol* **73:** 4813-4823.

Lamendella R, Santo Domingo JW, Ghosh S, Martinson J, Oerther DB (2011). Comparative fecal metagenomics unveils unique functional capacity of the swine gut. *BMC Microbiol* **11:** 103.

Langlois BE, Dawson KA, Leak I, Aaron DK (1988). Antimicrobial resistance of fecal coliforms from pigs in a herd not exposed to antimicrobial agents for 126 months. *Vet Microbiol* **18:** 147-153.

Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD et al. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proc Natl Acad Sci USA* **109:** 1691-1696.

Malorny B, Hoorfar J, Bunge C, Helmuth R (2003). Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Appl Environ Microbiol* **69:** 290-296.

Marshall BM, Levy SB (2011). Food animals and antimicrobials: Impacts on human health. *Clin Infect Dis* **24:** 718-733.

Mathew A, Upchurch W, Chattin S (1998). Incidence of antibiotic resistance in fecal *Escherichia coli* isolated from commercial swine farms. *J Anim Sci* **76:** 429-434.

Mathew AG, Cissell R, Liamthong S (2007). Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production. *Foodborne Pathog Dis* **4:** 115-133.

McEwen SA, Fedorka-Cray PJ (2002). Antimicrobial use and resistance in animals. *Clin Infect Dis* **34:** S93.

Molbak K, Baggesen DL, Aarestrup FM, Ebbesen JM, Engberg J, Frydendahl K et al. (1999). An outbreak of multidrug-resistant, quinolone-resistant *Salmonella enterica* serotype typhimurium DT104. *N Engl J Med* **341:** 1420-1425.

Ng LK, Martin I, Alfa M, Mulvey M (2001). Multiplex PCR for the detection of tetracycline resistant genes. *Mol Cell Probes* **15:** 209-215.

NRC (1998). Nutrient requirements of swine, 10th ed. National Academies Press: Washington, DC, USA.

Pakpour S, Jabaji S, Chénier MR (2012a). Frequency of antibiotic resistance in a swine facility 2.5 years after a ban on antibiotics. *Microbial Ecol***:** 1-10.

Pakpour S, Milani AS, Chénier MR (2012b). A multi-criteria decision-making approach for comparing sample preservation and DNA extraction methods from swine feces. *Am J Mol Biol* **2:** 159-169.

Patterson AJ, Colangeli R, Spigaglia P, Scott KP (2007). Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by macroarray detection. *Environ Microbiol* **9:** 703-715.

Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J, Seppala H (1999). Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob Agents Chemother* **43:** 2823-2830.

Roberts MC (2008). Update on macrolide–lincosamide–streptogramin, ketolide, and oxazolidinone resistance genes. *FEMS Microbiol Lett* **282:** 147-159.

Schwarz S, Roberts MC, Werckenthin C, Pang Y, Lange C (1998). Tetracycline resistance in *Staphylococcus* spp. from domestic animals. *Vet Microbiol* **63:** 217-227.

Shoemaker N, Vlamakis H, Hayes K, Salyers A (2001). Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. *Appl Environ Microbiol* **67:** 561-568.

Silbergeld EK, Graham J, Price LB (2008). Industrial food animal production, antimicrobial resistance, and human health. *Annu Rev Public Health* **29:** 151-169.

Smith TC, Male MJ, Harper AL, Kroeger JS, Tinkler GP, Moritz ED et al. (2009). Methicillin-resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in midwestern US swine and swine workers. *PLOS ONE* **4:** e4258.

Snoeck V, Cox E, Verdonck F, Joensuu JJ, Goddeeris BM (2004). Influence of porcine intestinal pH and gastric digestion on antigenicity of F4 fimbriae for oral immunisation. *Vet Microbiol* **98:** 45-53.

Spellberg B, Guidos R, Gilbert D, Bradley J, Boucher HW, Scheld WM et al. (2008). The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. *Clin Infect Dis* **46:** 155-164.

Stanton TB, Humphrey SB, Stoffregen WC (2011). Chlortetracycline-resistant intestinal bacteria in organically raised and feral swine. *Appl Environ Microbiol* **77:** 7167-7170.

Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD et al. (1999). Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol* **65:** 4799-4807.

Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L (1996). Detection of erythromycin-resistant determinants by PCR. *Antimicrob Agents Chemother* **40:** 2562-2566.

Tremblay CL, Letellier A, Quessy S, Boulianne M, Daignault D, Archambault M (2011). Multiple-antibiotic resistance of *Enterococcus faecalis* and *Enterococcus faecium* from cecal contents in broiler chicken and turkey flocks slaughtered in Canada and plasmid colocalization of *tet*O and *erm*B genes. *J Food Protect* **74:** 1639-1648.

Van Lunen T (2003). Growth performance of pigs fed diets with and without tylosin phosphate supplementation and reared in a biosecure all-in all-out housing system. *Can Vet J* **44:** 571.

Viola C, DeVincent SJ (2006). Overview of issues pertaining to the manufacture, distribution, and use of antimicrobials in animals and other information relevant to animal antimicrobial use data collection in the United States. *Prev Vet Med* **73:** 111-131.

Wagner BA, Straw BE, Fedorka-Cray PJ, Dargatz DA (2008). Effect of antimicrobial dosage regimen on *Salmonella* and *Escherichia coli* isolates from feeder swine. *Appl Environ Microbiol* **74:** 1731-1739.

Wegener HC (2003). Antibiotics in animal feed and their role in resistance development. *Curr Opin Microbiol* **6:** 439-445.

WHO (2012). Critically Important Antimicrobials for Human Medicine. Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR). 3nd revision. http://apps.who.int/iris/bitstream/10665/77376/1/9789241504485_eng.pdf Accessed October 2014.

Witte W (1998). Medical consequences of antibiotic use in agriculture. *Science* **279:** 996.

Zimmerman DR (1986). Role of subtherapeutic levels of antimicrobials in pig production. *J Anim Sci* **62:** 6-16.

Connecting text

In Chapter 3 we investigated the effect of subtherapeutic tylosin and chlortetracycline on the swine resistome as well as on anaerobic bacterial resistance to these two antibiotics. We observed a rapid increase in the concentration of tylosin-resistant anaerobic bacteria in response to in-feed tylosin as well as an increased detection frequency of several macrolide resistance genes. Although chlortetracycline had no significant effect on chlortetracycline resistance in anaerobes, there was a large variety of tetracycline-resistance genes present in the fecal samples.

In Chapter 4 we wanted to determine what effect these two antibiotics had on the entire gut microbiota and microbial community structure rather than just the culturable fraction. A better understanding of the alterations that occur in response to antibiotics might also aid in the development of antibiotic alternatives. To do this, we used Illumina-based high throughput sequencing of the 16S rRNA gene using DNA from the same samples in Chapter 3.

Chapter 4. Temporal changes and the effect of subtherapeutic concentrations of antibiotics in the gut microbiota of swine

Running title: The effect of antibiotics on the swine gut microbiota

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4.1 Abstract

The use of antibiotics in swine production for the purpose of growth promotion dates back to the 1950s. Despite this long history of use, the exact mechanism(s) responsible for the growth promoting effects of antibiotics in swine remain largely unknown. It is believed, however, that growth promotion is due to antibiotics having a direct impact on the gut microbiota. In this study, the effect of two antibiotics on the swine gut microbiota over a 19-week monitoring period was investigated using Illumina-based sequencing. A shift in the relative abundance of several taxa and in 26 operational taxonomic units (OTUs) was observed in pigs fed subtherapeutic concentrations of tylosin (44 to 11 mg kg^{-1} feed). More minor alterations were noted with the administration of chlortetracycline at 5.5 mg kg^{-1} feed. The most notable changes in the relative abundance of taxa and OTUs were noted between suckling piglets and post-weaned pigs. Diversity was also reduced in the gut microbiota of suckling piglets as measured using the Shannon, Chao1, and phylogenetic diversity indices. These results show that the effect of antibiotics on the swine gut microbiota is variable based on dosage and duration and that the swine gut microbiota exhibits considerable resilience to long-term changes due to antibiotic perturbations.

4.2 Introduction

Antimicrobials have been used for decades in agricultural production to increase the growth rate and feed efficiency of food animals, as well as to treat disease (Cromwell, 2002). Despite this long period of usage, the specific mechanism(s) responsible for the growth promoting benefits of antibiotics remains largely elusive. Currently, it is believed that antibiotics promote animal growth through a direct impact on the gut microbiota related to a reduction in

subclinical disease and harmful metabolites produced by intestinal bacteria or an increase in nutrient absorption and availability in the gut (Dibner and Richards, 2005). The use of antibiotics in livestock, however, selects for antibiotic resistant bacteria and resistance determinants which can be passed to humans through the food chain, the release of animal waste into the environment, or from direct contact with animals (van den Bogaard and Stobberingh, 2000; Marshall and Levy, 2011).

Therefore, the continued use of antibiotics in agriculture, particularly for nontherapeutic reasons (i.e. growth-promotion), is a source of controversy. There is also a question of whether these benefits attributed to antibiotics remain under modern swine production practices (Holt et al., 2011; Holman and Chenier, 2013). A ban on antibiotic use in livestock production has been in place since 2006 in the European Union and it seems probable that similar restrictions may be imposed in Canada and the United States in the future (Mathew et al., 2007; Maron et al., 2013). As a result, there is the need for alternatives to antibiotics in agriculture. The development of an effective replacement for antibiotics in swine production, however, requires a better understanding of the response of the gut microbiota to antibiotic exposure (Allen et al., 2013).

The gut microbiota of the pig is comprised of a large and diverse number of microorganisms which contribute to the health of the animal (Lamendella et al., 2011). The structure of the gut microbiota is largely determined by factors such as diet, age, genetics, and in some cases antibiotic exposure (Scott et al., 2013). High throughput sequencing (HTS) technologies targeting the 16S rRNA gene have revolutionized the way the gut microbiota can be analyzed and allows for a much greater depth of coverage than had been possible with culture-dependent and clone library methods (Hamady and Knight, 2009). To date, only a few studies have examined the impact of antibiotics on the gut microbiota using HTS and none have

monitored the temporal changes in the gut microbiota in farrow-to-finish swine production (Kim et al., 2012; Looft et al., 2012; Poole et al., 2013; Looft et al., 2014a).

Although agricultural usage of antibiotics is not well monitored in Canada or the United States, tylosin and chlortetracycline are two of the most commonly employed antibiotics in swine production (Deckert et al., 2010; Apley et al., 2012). For this reason, in the present study, the impact of the subtherapeutic administration of these two antibiotics on the swine gut microbiota from suckling to finishing was evaluated. Subtherapeutic antibiotic use refers to a dosage that is lower than what would be required for disease treatment (Allen et al., 2013). It is hypothesized that the continuous administration of tylosin and chlortetracycline at subtherapeutic doses in feed reduces the gut microbial diversity in swine and alters the microbial community.

4.3 Materials and Methods

4.3.1 Animals, experimental design, and sample collection

This is a companion study using the same pigs and sows as in Holman and Chénier (2013). The experimental design was also the same with the exception that samples from three males and three females per treatment group were used in the present study (n=6). In addition, the fecal samples used in the current work were taken at suckling (3 weeks), weanling (6 weeks), starting (9 weeks), growing (12 weeks), and finishing (19 weeks). In total, 94 fecal samples (including four samples from the sows) were sent for Illumina sequencing. All other experimental details can be found in Holman and Chénier (2013). Briefly, piglets were weaned at 24 d and randomly assigned to one of three diet groups; control, tylosin, or chlortetracycline. Pigs in the tylosin treatment group received 44 mg tylosin kg^{-1} feed for 21 d beginning at the weanling phase, then 22 mg kg^{-1} feed for 21 d at the starter phase, and finally 11 mg kg^{-1} feed for the remaining 70 d (growing-finishing). Pigs in the chlortetracycline cohort were given feed

supplemented with 5.5 mg chlortetracycline kg^{-1} feed for the entirety of the study beginning at weaning. A control group was included with pigs receiving feed with the same dietary composition as the treatment groups, minus the antibiotics. Antibiotic dosages were based on the Canadian Food Inspection Agency (CFIA) allowance for growth promotion in swine (Canadian Food Inspection Agency, 2014).

4.3.2 DNA amplification and Illumina sequencing

Total DNA was extracted from each fecal sample using the ZR Fecal DNA Miniprep kit (Zymo Research, Irvine, CA, USA) as previously described (Holman and Chenier, 2013). The primers 515-F (GTGCCAGCMGCCGCGGTAA) and 806-R (GGACTACVSGGGTATCTAAT) were used to target the V4 region of the 16S rRNA gene of both Bacteria and Archaea (Caporaso et al., 2011). The forward primer contained a unique 8 bp barcode to allow for pooling of samples prior to sequencing. Amplification and sequencing steps were performed at Molecular Research LP (Shallowater, Texas, USA). Briefly, the 16S amplicons were generated using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA,USA). The PCR program consisted of a 3 min initial denaturation at 94°C followed by 28 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min, with a final extension of 5 min at 72°C. The size and specificity of PCR amplicons were verified using 2% agarose gel electrophoresis and samples were then pooled together in equal proportions and prepared for sequencing according to the standard protocol for Illumina TruSeq DNA library preparation kit (Illumina, San Diego, CA, USA). Sequencing was carried out using a paired-end 2x250 bp Illumina MiSeq following manufacturer's instructions (Illumina).

4.3.3 Data processing and analysis

Sequenced 16S amplicons were processed using the QIIME software package (version 1.8.0) (Caporaso et al., 2010). The two paired end reads were joined together and the sequences were demultiplexed and quality filtered with the removal of sequences containing base calls with a Phred score of <20, all mismatched primer sequences, or a total length of <200 bp. Sequences were assigned de novo to OTUs (operational taxonomic units) at ≥97% similarity with chimera filtering using the USEARCH algorithm (version 6.1.544) within QIIME (Edgar, et al., 2011). Taxonomy was assigned using the naïve Bayesian RDP classifier and the Greengenes 13_5 database and a confidence cutoff of 0.8 (DeSantis et al., 2006; Wang et al., 2007). Low-abundance OTUs (<0.005% of total reads) were removed as recommended by Bokulich et al. (2013) to reduce the number of spurious OTUs. To allow for comparisons between samples and treatment groups, the OTU table was subsampled and rarefied to 12,500 reads per sample for alpha and beta diversity analyses with the loss of one sample from the finishing chlortetracycline group. Sequences were submitted to the NCBI Sequence Read Archive (SRA) under project accession number SRP041290 (http://www.ncbi.nlm.nih.gov/sra).

4.3.4 Statistical analysis

α-diversity was calculated within QIIME using the Shannon index (Shannon, 1948), Chao1 (Chao, 1984), and phylogenetic diversity (PD whole tree) (Faith, 1992). Good's coverage was also measured. β-diversity was calculated using unweighted and weighted UniFrac (Lozupone and Knight, 2005) and displayed using principal coordinate analysis (PCoA). Statistical comparisons of unweighted and weighted UniFrac distances between treatment groups and between different sampling times were done using ANOSIM (analysis of similarities) with 999 permutations.

Due to the fact that the same pigs were sampled repeatedly over the course of the study, a two-way repeated measures ANOVA with post hoc Tukey's honestly significant difference (HSD) comparisons was performed using PROC GLM in SAS (SAS Inst., Inc., Cary, NC, USA) to compare proportions of taxa as well as diversity indices between groups of pigs. Treatment, sex, and sampling time were included in the model, as well as their interactions. Comparisons of OTU abundance were calculated using the non-parametric Kruskal-Wallis test with the false discovery rate (FDR) correction (Benjamini and Hochberg, 1995). All results were considered significant at α=0.05.

4.4 Results

4.4.1 Sequence analysis

A total of 5,880,818 raw sequences were obtained from the Illumina MiSeq sequencing. Following quality filtering and demultiplexing, 5,401,765 sequences remained with an average length of 256 bp. Upon chimera removal, the 4,950,142 sequences left were clustered into OTUs (≥97%) using the de novo method. The removal of low-abundance OTUs (<0.005%) yielded a total of 1281 OTUs for analysis. While the average number of sequences per sample was 31,152, all samples were rarefied at 12,500 sequences per sample in order to retain as many samples as possible (Appendix 2). Good's coverage was > 97.5% for all samples.

4.4.2 Microbial diversity of the swine fecal microbiota

The RDP classifier and Greengenes database was used to assign taxonomy to OTUs from domain to genus level. At the phylum-level, a total of 15 different bacterial phyla and one archaeal phylum were identified in each sample (Fig. 4.1A). On average, *Archaea* accounted for less than 1% of the total sequences per sample (data not shown). The majority of sequences belonged to either *Firmicutes* or *Bacteroidetes*, with these two phyla encompassing greater than

70% of all sequences. *Proteobacteria* and *Spirochaetes* dominated the remaining sequences and only 1.2% of sequences could not be classified at the phylum-level.

The sequences could be definitively assigned into 65 different genera, with an average of 32.4% of sequences being unclassified at the genus level (Fig. 4.1B). In terms of relative abundance, the 14 bacterial genera that accounted for more than 1% of sequences were (in decreasing order of abundance): *Prevotella*, *Treponema*, *Lactobacillus*, *Succinivibrio*, *Bacteroidetes*, *Phascolarctobacterium*, SMB53 (*Clostridiaceae*), *Ruminococcus*, *Blautia*, *Roseburia*, *Streptococcus*, *Faecalibacterium*, *Oscillospira*, and *Campylobacter*. The only two archaeal genera identified were *Methanobrevibacter* and vadinCA11, which is an uncultured archaeal genus from the phylum *Euryarchaeota*, class *Thermoplasmata*, order *Thermoplasmatales* (Godon et al., 1997).

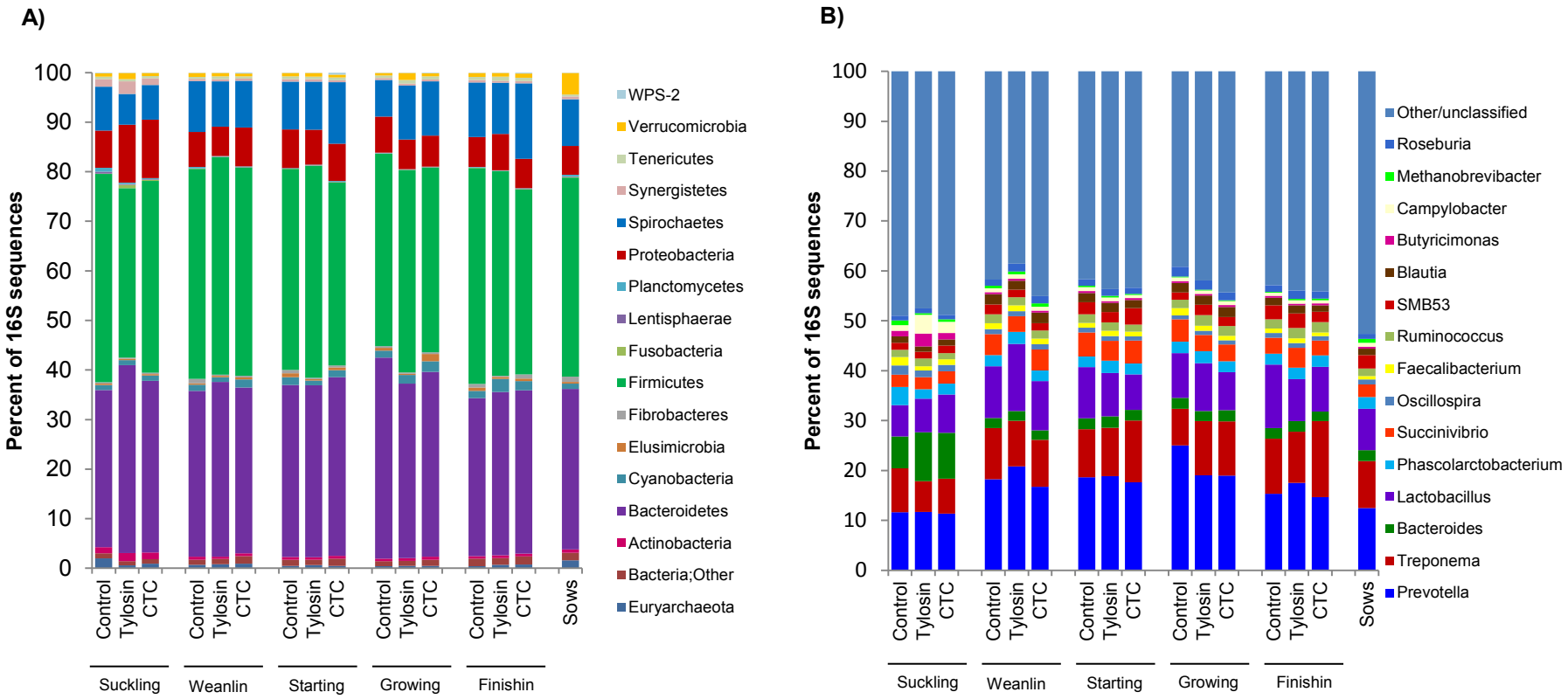

Figure 4.1. Classification of 16S rRNA gene sequences at the A) phylum-level for the control, tylosin, and chlortetracycline-supplemented pigs (n=6) and B) genus-level at each sampling time. In B), only those genera containing ≥1% of sequences are displayed. CTC=chlortetracycline.

Several α-diversity indices were calculated using the samples that had been rarefied at 12,500 sequences to account for unequal numbers of sequences between samples. The overall average of all samples for Chao1, a non-parametric estimator of species (OTU) richness, was 1238.6 ± 14.6 (standard deviation of the mean). The average number of observed unique species (OTUs) was 1064.6 ± 21.0. The average value for the Shannon index, which measures both species (OTU) richness and abundance, was 5.543 ± 0.119. The average phylogenetic diversity (PD whole tree) was 189.40 ± 2.75.

The core microbiota, defined as those OTUs found in all samples at all sampling times (including the sows), was comprised of 284 OTUs. While these OTUs represented only 22.2% of the total OTUs, they contained 80.4% of the total sequences (data not shown). The core microbiota of all treatment groups encompassed 309 OTUs that were found in all treatment groups at all times, excluding suckling piglets and the sows. For each diet group, the core microbiota was comprised of 391, 407, and 394 OTUs for the control, tylosin, and chlortetracycline groups, respectively. Finally, the core microbiota of suckling, weanling, starting, growing, and finishing samples was made up of 392, 429, 441, 431, and 449 OTUs respectively.

4.4.3 Microbial community changes due to the administration of in-feed antibiotics

Unweighted and weighted UniFrac distances were used to estimate β-diversity and to compare all three diet groups. Analysis of similarities (ANOSIM) of unweighted UniFrac distances indicated that while the control, tylosin, and chlortetracycline diet groups ($p=0.022$) were significantly different, the relatively small corresponding R-value (0.0317) suggests that the diet groups are not well separated from each other. The PCoA plot of the unweighted UniFrac distances visually confirmed that the three diet groups do not form distinct clusters when only

microbial community membership is considered (Fig. 4.2A). In contrast, the ANOSIM of weighted UniFrac distances showed a significant difference between treatment groups ($p=0.001$) with a higher R-value (0.212). Weighted UniFrac takes into account the relative abundance of OTUs whereas unweighted UniFrac considers only community membership (i.e. presence/absence of OTUs) (Navas-Molina et al., 2013). A PCoA plot of the weighted UniFrac distances shows greater separation between the tylosin-fed pigs and the control pigs as well as the chlortetracycline group (Fig. 4.2B). The higher R-value indicates that tylosin modulates the relative abundance of OTUs rather than determining their presence or absence. Comparisons of the α-diversity metrics Chao1, phylogenetic diversity (PD whole tree), and Shannon indices for each treatment group revealed no significant differences ($p>0.05$; Table 4.1), thus demonstrating that microbial diversity was not affected by antibiotic treatment.

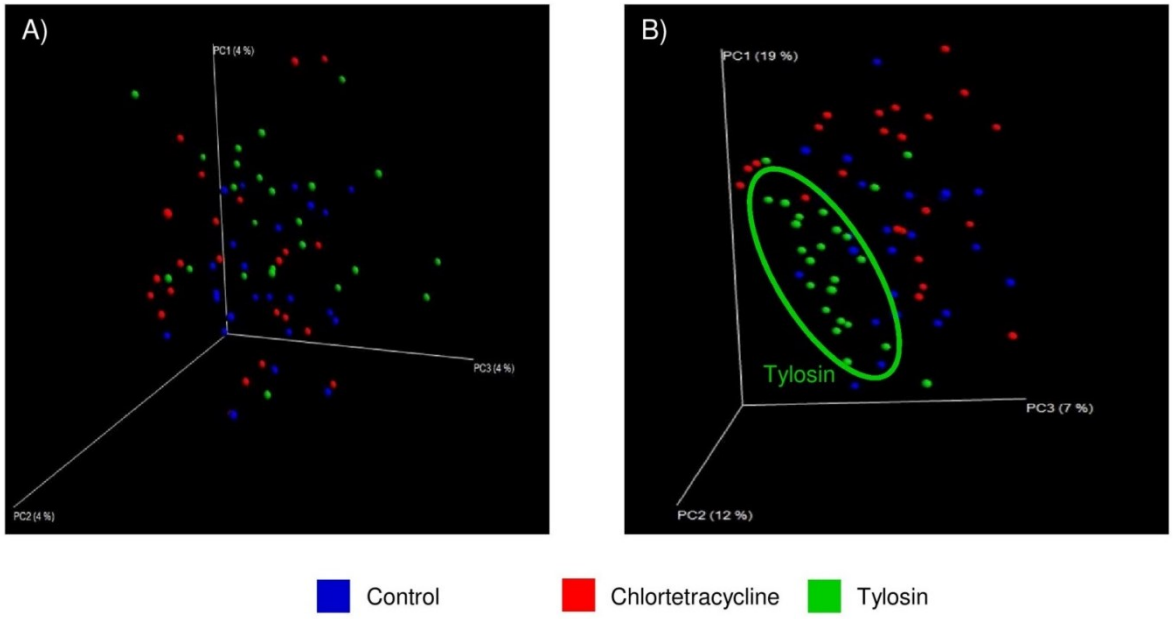

Figure 4.2. Principal coordinate analysis (PCoA) of the A) unweighted UniFrac distances and B) weighted UniFrac distances for each treatment group. Unweighted UniFrac only takes into account the presence or absence of OTUs while weighted UniFrac includes OTU abundance as well. For this analysis, all samples from weanling (6 wk), starting (9 wk), growing (12 wk), and finishing (19 wk) are included together in each diet group (n=24). The percent variation explained by each principal coordinate is indicated on the axes.

Table 4.1. Microbial diversity and abundance estimates of the control, chlortetracycline-, and tylosin-supplemented pigs. All samples from weanling (6 wk), starting (9 wk), growing (12 wk), and finishing (19 wk) are included in each diet group (n=24) ± standard deviation of the mean.

Treatment	Chao1	Observed species	PD whole tree	Shannon
Control	1243.2 ± 13.1	1075.2 ±15.0	190.9 ± 2.0	5.56 ±0.07
Chlortetracycline	1243.1 ± 12.1	1068.8 ± 11.1	190.22 ± 1.90	5.57± 0.09
Tylosin	1237.1 ± 14.8	1073.4 ± 11.3	190.49 ± 1.6	5.54 ±0.08

Phyla and genera with a relative abundance of >0.1% were compared between the antibiotic-supplemented diets and the control diet (Table 4.2). Changes in the relative abundance of taxa between the antibiotic groups and the control group were temporal in nature. At weaning (6 wk), there were significantly more bacterial sequences that were unclassified at the phylum-level in the chlortetracycline-fed pigs. Tylosin-fed swine had significantly fewer sequences classified in the phylum *Fibrobacteres*. In addition, the relative abundance of sequences in the genus *Coprococcus* was significantly higher in the tylosin-fed pigs than in the control cohort.

Several changes in the relative abundance of taxa as a result of antibiotic administration were observed at the starting phase (9 wk). At the phylum-level, pigs treated with tylosin had a significantly lower proportion of *Cyanobacteria* and *Fibrobacteres* sequences than in the control group. WPS-2, a candidate phylum, was significantly enriched in chlortetracycline-fed swine. There were five genera that were differentially abundant at starting. In the tylosin-fed pigs there was a significantly higher proportion of *Streptococcus* and *Coprococcus* compared with the control pigs. However, the relative abundance of *Fibrobacter* was significantly lower in the

tylosin-group. Also at the starting phase, the proportion of SMB53 was significantly increased in pigs on the chlortetracycline diet, while *Lactobacillus* was significantly decreased (Table 4.2).

The sampling of growing phase pigs (12 wk) revealed that tylosin-fed pigs had a significantly lower proportion of sequences in the *Bacteroidetes* phylum when compared with the antibiotic-free pigs. The tylosin group also had a reduced relative abundance of the genera *Succinivibrio* and *Anaerovibrio*. Members of the *Verrucomicrobia* phylum were increased in the tylosin cohort, as were the genera *Coprococcus* and *Akkermansia*. Notably, the relative abundance of *Akkermansia* was enriched almost 10-fold in the tylosin-supplemented pigs in comparison with the control pigs at the growing phase (Table 4.2).

At the finishing phase (19 wk), the tylosin treatment significantly increased the proportion of sequences in the *Cyanobacteria* phylum. Chlortetracycline treatment reduced the relative abundance of *Firmicutes* while increasing the proportion of WPS-2 sequences (Table 4.2). Interestingly, archaeal sequences were also enriched in the feces of the chlortetracycline group in comparison with the control pigs (data not shown). No genus-level differences were detected at finishing, possibly reflecting the lower dosage of tylosin at this time (11 mg kg^{-1} feed for tylosin).

Table 4.2. Effect of tylosin and chlortetracycline on the relative proportion (percentage ±standard deviation of the mean) of the most abundant phyla and genera (>0.1% of taxa) in decreasing order of abundance at various production phases (excluding suckling) when either the tylosin or chlortetracycline group was significantly different ($p<0.05$) from the control (n=6). Different uppercase letters represent significant differences between treatment groups for each taxon ($p<0.05$).

Taxon	Phase	Control	Chlortetracycline	Tylosin
Phylum				
Bacteria (unclassified)	Weanling	1.02A ± 0.19	1.51B ± 0.41	1.06A ± 0.13
Fibrobacteres	Weanling	0.515A ± 0.221	0.323AB ± 0.069	0.247B ± 0.043
Cyanobacteria	Starting	1.58A ± 0.42	1.38AB ± 0.22	0.97B ± 0.12
Fibrobacteres	Starting	0.673A ± 0.345	0.398AB ± 0.247	0.273B ± 0.042
WPS-2	Starting	0.084A ± 0.026	0.420B ± 0.366	0.067A ± 0.034
Bacteroidetes	Growing	40.5A ± 1.3	37.3AB ± 3.9	35.2B ± 2.6
Verrucomicrobia	Growing	0.50A ± 0.08	0.61A ± 0.18	1.44B ± 0.68
Firmicutes	Finishing	43.5A ± 4.2	37.6B ± 1.0	41.3AB ± 1.4
Cyanobacteria	Finishing	1.48A ± 0.46	1.86AB ± 0.27	2.63B ± 0.74
WPS-2	Finishing	0.087A ± 0.041	0.173B ± 0.121	0.097A ± 0.032
Genus				
Coprococcus	Weanling	0.99A ± 0.15	1.18AB ± 0.26	1.40B ± 0.25
Lactobacillus	Starting	10.2A ± 2.4	7.2B ± 0.39	8.7AB ± 1.2
SMB53	Starting	2.42A ± 0.66	3.26B ± 0.91	2.07A ± 0.42
Streptococcus	Starting	1.14A ± 0.25	1.00A ± 0.49	3.35B ± 2.22
Coprococcus	Starting	1.00A ± 0.17	1.05A ± 0.08	1.42B ± 0.40
Fibrobacter	Starting	0.673A ± 0.354	0.399AB ± 0.247	0.273B ± 0.042
Succinivibrio	Growing	4.46A ± 0.70	3.36AB ± 0.47	3.20B ± 1.01
Coprococcus	Growing	0.87A ± 0.12	0.84A ± 0.02	1.02B ± 0.05
Anaerovibrio	Growing	0.830A ± 0.305	0.577AB ± 0.096	0.465B ± 0.090
Akkermansia	Growing	0.13A ± 0.03	0.19A ± 0.14	1.03B ± 0.67

The differences in OTU abundance were also calculated between the antibiotic treated pigs and the control group. This analysis grouped all pigs in each treatment diet together (weanling to finishing) for comparisons. A total of 26 OTUs were differentially abundant between the tylosin-fed pigs and the control group ($p<0.05$ FDR; Table 4.3). Eighteen of these

OTUs were more abundant in the control pigs and the remaining 8 OTUs were more abundant in the tylosin cohort. Exactly half of these OTUs were classified as members of the *Bacteroidetes* phylum. None of the OTUs were differentially abundant between the control and chlortetracycline-supplemented pigs ($p>0.05$ FDR).

Table 4.3. Differentially abundant OTUs between the tylosin-supplemented pigs and the control group (n=24). False discovery rate (FDR) <0.05.

OTU Name	Control (mean abundance)	Tylosin (mean abundance)	FDR	RDP Classifier Consensus Lineage
More abundant in the control group				
denovo22	88.0	50.4	0.0020	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__RF16
denovo32	60.2	39.4	0.029	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__RF16
denovo46	57.8	34.5	0.0082	Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
denovo49	48.5	23.9	0.00033	Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
denovo133	31.2	18.8	0.0020	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales
denovo130	31.1	18.0	0.023	Bacteria
denovo110	28.3	9.25	0.015	Bacteria;p__Fibrobacteres;c__Fibrobacteria;o__Fibrobacterales;f__Fibrobacteraceae;g__Fibrobacter
denovo104	24.5	10.1	0.0011	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7
denovo100	23.6	11.9	0.024	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__[Prevotella]
denovo108	21.9	13.2	0.0020	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales_
denovo156	14.6	8.58	0.028	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae]
denovo229	13.1	8.04	0.035	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales
denovo240	11.5	5.92	0.023	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales
denovo208	10.6	4.17	0.0027	Bacteria;p__Fibrobacteres;c__Fibrobacteria;o__Fibrobacterales;f__Fibrobacteraceae;g__Fibrobacter
denovo237	10.2	3.54	0.015	Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__RF32
denovo211	9.71	4.71	0.0036	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae
denovo447	9.00	2.50	0.0024	Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
denovo507	7.17	3.54	0.0049	Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Megasphaera
More abundant in tylosin group				
denovo5	148	308	0.023	Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
denovo145	13.8	28.0	0.0075	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides
denovo95	12.9	33.625	0.0013	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__[Prevotella]
denovo269	5.50	10.4	0.023	Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo273	5.00	10.4	0.042	Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
denovo618	3.96	9.42	0.023	Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
denovo456	2.89	6.62	0.028	Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo98336	1.46	4.17	0.015	Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae]

4.4.4 Temporal changes in the swine fecal microbiota

Temporal changes in the microbial communities of the pigs at each sampling time were evaluated using unweighted and weighted UniFrac distances. A significant difference between sampling times was observed based on the ANOSIM of unweighted UniFrac distances ($p=0.001$). While the R-value (0.0983) was relatively small, the suckling (3 wk) samples were clearly separated from the other sampling times based on the PCoA plot of the unweighted UniFrac distances (Fig. 4.3A). The ANOSIM of the weighted UniFrac distances was also significant ($p=0.001$) but the R-value (0.403) was much higher (Fig. 4.3B), indicating differences between sampling times are likely a result of alterations in the relative abundances of OTUs rather than the presence or absence of specific OTUs.

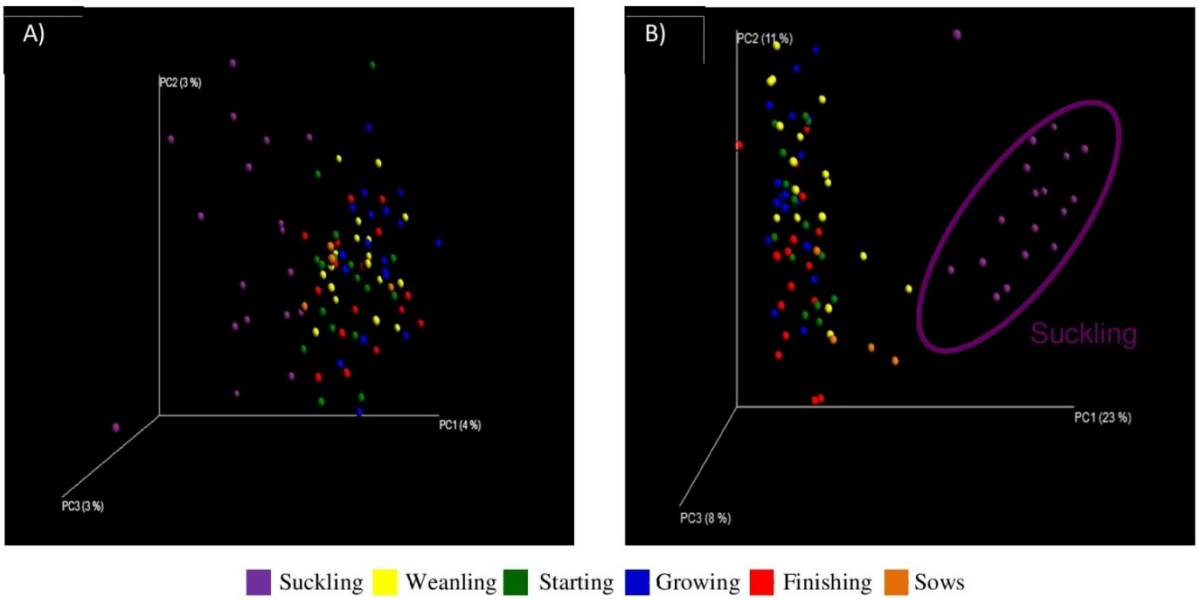

Figure 4.3. Principal coordinate analysis (PCoA) of the A) unweighted UniFrac distances and B) weighted UniFrac distances for each sampling period: suckling (3 wk), weanling (6 wk), starting (9 wk), growing (12 wk), and finishing (19 wk). Unweighted UniFrac only takes into account the presence or absence of OTUs while weighted UniFrac includes OTU abundance as well. All samples from each sampling period are included together for this analysis (n=18). The percent variation explained by each principal coordinate is indicated on the axes.

The α-diversity in the feces of pigs at each sampling period was also compared over the duration of the study using repeated measures ANOVA (Table 4.4). At the suckling phase, the piglets had a significantly lower phylogenetic diversity (PD whole tree) compared with all other sampling times ($p<0.0001$) and a lower number of observed OTUs ($p<0.0001$). The Shannon

index in suckling samples was also significantly lower than the starting (9 wk), growing (12 wk), and finishing (19 wk) samples ($p<0.05$; Table 4.4).

Table 4.4. Diversity and abundance estimates at each sampling time (±standard deviation of the mean). At each phase (n=18) and for the sows (n=4). Different uppercase letters indicate significant differences ($p<0.05$).

Phase	Chao1	Observed OTUs	PD whole tree	Shannon index
Suckling (3 wk)	1240.3AB ± 15.1	1033.0A ± 19.1	185.39A ± 2.38	5.45A ± 0.18
Weanling (6 wk)	1236.1AB ± 11.3	1070.2B ± 10.8	190.24B ± 1.69	5.55ABC ± 0.12
Starting (9 wk)	1243.0A ± 14.2	1075.7C ± 10.7	190.94B ± 1.43	5.57B ± 0.05
Growing (12 wk)	1234.5B ± 12.6	1066.6B ± 15.0	189.76B ± 2.18	5.52C ± 0.08
Finishing (19 wk)	1236.9AB ± 17.6	1074.7BC ± 12.8	190.47B ± 1.75	5.58B ± 0.05
Sows	1247.1 ± 9.6	1078.4 ± 7.5	190.69 ± 0.94	5.70 ± 0.06

Taxonomic-based analysis yielded a number of significant differences in the relative-abundance of taxa between suckling and all other sampling times. Among the most abundant genera (>1% of sequences), the proportion of *Prevotella*, *Treponema*, *Lactobacillus*, *Succinivibrio*, SMB53, *Ruminococcus*, *Roseburia*, *Streptococcus*, and *Coprococcus* were all significantly reduced at suckling (Table 4.5). The proportion of archaeal sequences in the suckling fecal samples was also significantly lower than all other sampling times ($p<0.05$), with the exception of weaning (data not shown). The phyla *Cyanobacteria* and *Spirochaetes* both

accounted for significantly fewer sequences in suckling samples in comparison to every other sampling time.

Suckling samples also had significantly greater proportions of the genera *Bacteroides*, *Campylobacter*, *Butyricimonas*, *Oscillospira*, *Desulfovibrio*, and *Parabacteroides* (Table 4.5). At the phylum level, *Proteobacteria* were significantly enriched in suckling samples ($p<0.05$). Interestingly, suckling samples had a significantly higher proportion of *Enterobacteriaceae*, a bacterial family from the phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Enterobacteriales* that includes pathogens such as *Salmonella* and *Escherichia* coli. The percentage of *Enterobacteriaceae* sequences at suckling was 3.1% ± 0.80 (SEM) while all subsequent sampling periods had less than 1% (data not shown).

When OTU abundance was compared between suckling (3 wk) and weanling (6 wk) samples, a total of 215 OTUs were observed to be differentially abundant ($p<0.05$ FDR; Appendix 3). Meanwhile, there were no significant differences in the abundance of OTUs between the weaning and starting (9 wk) periods ($p>0.05$ FDR; data not shown). There were four differentially abundant OTUs between starting and growing and three OTUs between growing and finishing ($p<0.05$ FDR; data not shown).

Samples taken at the growing phase (12 wk) had a greater relative abundance of *Bacteroidetes* when compared with every other time period (Table 4.5). Along with this increase in *Bacteroidetes*, pigs at the growing phase had significantly lower proportions of *Firmicutes* than every other sampling time with the exception of suckling (Table 4.5).

Table 4.5. Effect of sampling time on the relative proportion (percentage ±standard deviation of the mean) of the most abundant phyla and genera (>1% of taxa) in decreasing order. Only abundant phyla or genera with significant differences between suckling (3 wk) and other sampling times are shown. Different uppercase letters represent significant differences between sampling times ($p<0.05$). For each sampling time all pigs were included together (n=18).

Taxon	Suckling	Weanling	Starting	Growing	Finishing
Phylum					
Firmicutes	38.2AC ± 5.4	42.8B ± 3.7	40.1AB ± 3.5	39.0C ± 2.3	40.9AB ± 3.6
Bacteroidetes	34.3ABC ± 7.7	33.9A ± 3.5	35.4C ± 2.4	37.5B ± 3.8	33.0D ± 2.5
Proteobacteria	10.3A ± 4.5	6.92BC ± 1.52	7.44C ± 1.21	6.49B ± 1.16	6.44B ± 1.13
Spirochaetes	7.35A ± 2.09	9.63B ± 2.48	10.59BC ± 3.29	9.74BC ± 2.96	12.03C ± 3.42
Synergistetes	1.82A ± 1.52	0.44B ± 0.05	0.46B ± 0.07	0.43B ± 0.07	0.44B ± 0.06
Cyanobacteria	1.01A ± 0.16	1.27B ± 0.49	1.31B ± 0.37	1.75C ± 0.57	2.00C ± 0.72
Genus					
Prevotella	11.6A ± 2.3	18.6B ± 3.6	18.4B ± 2.5	21.0C ± 4.6	15.9D ± 2.6
Bacteroides	8.44A ± 4.27	1.99B ± 0.19	2.22BC ± 0.23	2.13C ± 0.18	2.06BC ± 0.22
Treponema	7.32A ± 2.08	9.59B ± 2.47	10.6C ± 3.28	9.69BC ± 2.95	12.0C ± 3.42
Lactobacillus	6.41A ± 1.74	11.2B ± 3.95	8.70C ± 1.96	8.74ABC ± 2.20	10.12BC ± 4.03
Succinivibrio	2.45A ± 0.33	3.85BC ± 1.38	4.46BC ± 1.14	3.67B ± 0.92	3.43B ± 0.86
Campylobacter	1.66A ± 1.14	0.84B ± 0.22	0.71BC ± 0.15	0.72BC ± 0.17	0.65C ± 0.12
Butyricimonas	1.68A ± 1.14	0.39B ± 0.05	0.37C ± 0.06	0.36C ± 0.06	0.35BC ± 0.05
Oscillospira	1.50A ± 0.76	1.03ABC ±0.27	0.93BC ± 0.18	0.84C ± 0.16	0.94B ± 0.11
SMB53	1.42A ± 0.19	1.67B ± 0.40	2.58C ± 0.83	1.78AB ± 0.56	2.63C ± 0.99
Ruminococcus	1.42A ± 0.30	1.66AB ± 0.27	1.61AB ± 0.26	1.91BC ± 0.38	1.98C ± 0.25
Desulfovibrio	1.08A ± 0.58	0.40B ± 0.23	0.32BC ± 0.11	0.27C ± 0.08	0.30BC ± 0.05
Parabacteroides	1.00A ± 0.78	0.46BC ± 0.18	0.55AB ± 0.11	0.52BC ± 0.11	0.47BC ± 0.48
Roseburia	0.92A ± 0.07	1.42B ± 0.46	1.31B ± 0.17	1.77C ± 0.38	1.46B ± 0.34
Streptococcus	0.77A ± 0.13	1.17B ± 0.57	1.83C ± 1.66	0.75A ± 0.20	1.21B ± 0.73
Coprococcus	0.64A ± 0.07	1.19B ± 0.27	1.16B ± 0.31	0.91C ± 0.11	0.94C ± 013

4.5 Discussion

We evaluated the effect of the continuous administration of subtherapeutic concentrations of tylosin and chlortetracycline on the fecal microbiota of swine. We monitored changes in the fecal microbiota for the entire duration of the swine production cycle using 16S rRNA gene Illumina-based sequencing. At present, this is the longest monitoring period and the largest number of pigs from one study location that have been used to examine the swine gut microbiota using high throughput sequencing techniques. To our knowledge, this is also the first time that high throughput sequencing has been used to compare the gut microbiota of the suckling vs. weanling piglet. Our results demonstrate that tylosin causes shifts in the relative abundance of specific taxa and OTUs, and that the microbiota of suckling piglets is significantly different from that of post-weaning swine.

The large majority (>70%) of sequences were classified as either *Firmicutes* or *Bacteroidetes*, a finding that is in agreement with several other studies. Similarly, as in previous reports, *Prevotella* was the most abundant genus in the present study (Kim et al., 2011; Kim et al., 2012; Looft et al., 2012). The microbial diversity of the fecal microbiota as measured with the Shannon index was similar to the values previously reported by Kim et al. (2011) of 5.74 to 6.17. Our finding that the core microbiota of all samples constitutes a minority of the OTUs (22.2%) but a majority of the sequences (80.4%) is congruent with the observation of Kim et al. (2012) that the core OTUs (6.88 to 7.68% of total OTUs) encompasses 82.4% to 85.4% of the sequences.

We hypothesized that antibiotics added to the swine diet would decrease diversity and alter the gut microbiota. While diversity was not affected according to several α-diversity metrics (Table 4.1), the addition of tylosin to the swine diet shifted the overall microbial

community structure when OTU abundance was taken into account using the weighted UniFrac and PCoA (Fig. 4.2B). This indicates that tylosin changed the proportion of specific taxa in the swine gut microbiota rather than altering community membership. This observation is in accordance with the work of Kim et al. (2012) who reported that the changes in the gut microbiota of commercially-raised swine in response to 40 mg kg^{-1} feed of tylosin (from 10 wk to 22 wk), occurred only at certain times and in specific genera and OTUs. These authors also did not record any significant differences in α-diversity indices, including the Shannon index and observed OTUs, between tylosin and no-tylosin pigs (Kim et al., 2012). Poole and others (2013) also found no significant effect on α-diversity when pigs were fed 50 mg kg^{-1} feed of chlortetracycline for 28 days beginning three-weeks post-weaning. In contrast, Looft et al. (2014b) observed significant decreases in total OTUs and the Shannon index in the early period (up to 4 days) of administration of carbadox at 50 mg kg^{-1} feed in six week old piglets. Also of note, these authors found that changes observed in *Prevotella* were relative rather than absolute (Looft et al. 2014b).

We detected changes in the relative abundance of taxa at several taxonomic levels and at various sampling times as a result of tylosin supplementation (Table 4.2). Interestingly, these alterations were observed less often in weanling (6 wk) samples, particularly when compared with starting (9 wk) and growing (12 wk) samples. Pigs were weaned at 24 days and started immediately on their experimental diets. It may be that tylosin takes time to cause observable changes in the gut microbiota. In contrast, we reported a rapid increase in tylosin-resistance in anaerobic bacteria in a previous report using these same samples, although the concentration of total anaerobes remained unchanged (Holman and Chenier, 2013).

Of particular interest is the finding of a decreased proportion of *Bacteroidetes* at growing (12 wk) in the tylosin group compared with the control pigs. A decrease in the relative abundance of *Bacteroidetes* has been associated with weight gain in swine (Guo et al., 2008), although in the present study the tylosin-group did not exhibit any differences in weight gain compared with the other diet groups (Holman and Chenier, 2013). Similarly, Looft and colleagues (2012) also described a decrease in the abundance of *Bacteroidetes* in swine exposed to ASP250, a mixture of 100 mg kg^{-1} feed of chlortetracycline, 100 mg kg^{-1} feed of sulfamethazine, and 50 mg kg^{-1} feed of penicillin. Furthermore, these authors noted a decrease in the genus *Succinivibrio* in antibiotic-treated pigs, a result that was also observed in the current study using tylosin.

Although both antibiotics have different mechanisms of action, given the lower dose of chlortetracycline (5.5 mg kg^{-1} feed) used in comparison to tylosin (44 to 11 mg kg^{-1} feed), it was not unexpected that fewer changes were seen in the microbiota of chlortetracycline-supplemented pig samples. In the latter pigs, the overall community structure did not appear markedly different from the control samples when visualized using PCoA plots of either weighted or unweighted UniFrac distances and none of the OTUs were differentially abundant when compared with the control group. However, some alterations were evident in the chlortetracycline group, most notably a significantly reduced proportion of *Firmicutes* at finishing (19 wk). The relative abundance of *Lactobacillus* sequences also decreased with chlortetracycline-supplementation at starting (9 wk) (Table 4.2).

It should be noted that most of the shifts in the relative abundance of individual taxa due to either antibiotic were temporary rather than permanent. The fact that tylosin was progressively halved from weaning to starting to growing may have played a role or it could be

due to the resiliency of the gut microbiota to long term changes. Kim et al. (2012) suggested that the development of the "mature" gut microbiota in swine is accelerated by the addition of tylosin, although the gut microbiota of untreated pigs eventually reaches this state as well. Therefore, it may be that once this climax community is attained, the gut microbiota is increasingly more resistant to dietary perturbations, including antibiotics (Carney-Hinkle et al., 2013). In addition, despite using the maximum dosage of tylosin and chlortetracycline allowed in Canada for growth promotion in swine, we did not detect any differences in growth rate between diet groups (Holman and Chenier, 2013). Pigs that exhibit an increase in growth rate in response to antibiotic supplementation may have changes in their gut microbiota that are different from the current study. Unfortunately, the growth rate of the pigs is rarely reported in studies examining the effect of antibiotic supplementation on the swine gut microbiota (Kim et al., 2012; Looft et al., 2012; Poole et al., 2013; Looft et al., 2014a).

In terms of temporal changes, the suckling piglets (3 wk) had a significantly different fecal microbiota in comparison to subsequent sampling times (Fig. 4.3; Table 4.5; Appendix 3). One of the more notable observations was that suckling piglets had a significantly greater proportion of *Enterobacteriaceae*, a family that includes potentially pathogenic bacteria such as *Escherichia coli* and *Salmonella* (Schierack et al., 2007). In agreement with this finding, culture-based studies have reported decreasing numbers of *Enterobacteriaceae* 11 days post-weaning (Pieper et al., 2006). Similarly, another group of potentially pathogenic bacteria, *Campylobacter*, was significantly enriched in the feces of suckling piglets. Culture-based methods have found a high prevalence of *Campylobacter* spp. in piglets shortly after birth (Weijtens et al., 1997). At the same time, *Lactobacillus*, a genus associated with beneficial health effects in swine and other mammals (Daly et al., 2014) was reduced in the current study.

The change in the relative abundance of *Bacteroides* from 8.44% at suckling to 1.99% at weanling was also particularly striking. *Bacteroides* is a genus of Gram-negative obligate anaerobes that, along with *Lactobacillus*, comprise a relatively large fraction of the culturable bacteria of the swine gut microbiota and are among the major fermenters of aromatic amino acids (Moore et al., 1987; Gaskins, 2001).

At weaning, piglets are rapidly transitioned from a diet of easily-digestible sow's milk to a lower-digestible diet rich in plant polysaccharides and therefore this is likely the biggest factor driving the changes observed in the current study from suckling to weaning (Lalles et al., 2007). Physiological changes in the piglet gut also occur during the transition from suckling to weaning. For example, the pH of the caecum has been reported to be higher in suckling piglets vs. weanling piglets (Snoeck et al., 2004) and the large intestine is relatively larger in the post-weaning period (Kelly et al., 1991). The immune system of suckling piglets is also relatively immature compared to that of post-weaned pigs, with suckling piglets relying heavily on maternal antibodies for protection (Stokes et al., 2004). Following weaning, maternal antibodies wane and the piglet's immune system must learn to tolerate commensal microorganisms and harmless antigens and yet react appropriately to pathogens. As a result, until the piglet's immune system reaches a mature-state, changes in the gut microbiota are expected (Bailey et al., 2005). The rapidly changing gut ecosystem during the suckling-to-weaning transition may also explain why recent studies of antibiotic growth promoters have indicated a positive effect on growth rate only at this period (Holt et al., 2011).

Fewer major alterations were also observed from weaning through to finishing and so it appears that once established following weaning, the swine gut microbiota undergoes more subtle changes over time. This was clear when OTU abundance was compared between

sampling times. While a total of 215 OTUs were differentially abundant from suckling (3 wk) to weaning (6 wk), there were no significant differences in OTU abundance between weanling and starting (9 wk). Studies using DGGE for example, have demonstrated temporal variation in the gut microbiota of piglets up to 4 weeks following birth with increasing stability beyond this period (Thompson et al., 2008).

The relative abundance of archaeal sequences decreased over time, although the overall proportion remained relatively low at less than 1% of total sequences, with the exception of suckling where piglets carried a higher percentage of archaeal sequences (1.2%). This finding is in accordance with previous estimates of archaeal abundance in the swine gut microbiota (Lamendella et al., 2011). Archaeal diversity was also relatively low, with only one previously cultured genus of *Archaea* identified. This genus, *Methanobrevibacter*, is comprised of methanogens commonly found in swine feces (Mao et al., 2011; Su et al., 2014).

4.5 Conclusion

Understanding how and when the swine gut microbiota changes in response to antibiotics will aid in the development of alternatives to antibiotics. In the current study the administration of tylosin at subtherapeutic concentrations resulted in changes in the fecal microbiota that were identifiable at the phylum through genus-levels. Chlortetracycline had relatively minor effects in comparison but alterations were noticeable in specific taxa. The swine gut microbiota also demonstrated considerable resilience to antibiotic perturbation as most changes to the relative abundance of specific taxa were temporary. Suckling piglets had a microbial community which was very different from that of the post-weaning phase and once established, the gut microbiota of post-weaning swine was significantly more stable in terms of community membership.

Therefore, dietary manipulations beginning around the weaning period are likely to have the most impact on the swine gut microbiota.

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4.7 References

Allen HK, Levine UY, Looft T, Bandrick M, Casey TA (2013). Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends Microbiol* **21**: 114-119.

Apley MD, Bush EJ, Morrison RB, Singer RS, Snelson H (2012). Use Estimates of In-Feed Antimicrobials in Swine Production in the United States. *Foodborne Pathog Dis* **9**: 272-279.

Bailey M, Haverson K, Inman C, et al. (2005). The development of the mucosal immune system pre- and post-weaning: balancing regulatory and effector function. *Proc Nutr Soc* **64**: 451-457.

Benjamini Y & Hochberg Y (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Roy Stat Soc B Met* **57**: 289-300.

Bokulich NA, Subramanian S, Faith JJ et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods* **10**: 57-59.

Canadian Food Inspection Agency (2014). Compendium of medicating ingredients. http://www.inspection.gc.ca/animals/feeds/medicating-ingredients/mib/eng/330705207970/1330714849837. Accessed on April 16, 2014.

Caporaso JG, Lauber CL, Walters WA et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* **108**: 4516-4522.

Caporaso JG, Kuczynski J, Stombaugh J et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335-336.

Carney-Hinkle EE, Tran H, Bundy JW, Moreno R, Miller PS, Burkey TE (2013). Effect of dam parity on litter performance, transfer of passive immunity, and progeny microbial ecology. *J Anim Sci* **91**: 2885-2893.

Chao A (1984) Nonparametric-Estimation of the Number of Classes in a Population. *Scand J Stat* **11**: 265-270.

Cromwell GL (2002) Why and how antibiotics are used in swine production. *Anim Biotechnol* **13**: 7-27.

Daly K, Darby AC, Hall N, Nau A, Bravo D, Shirazi-Beechey SP (2014). Dietary supplementation with lactose or artificial sweetener enhances swine gut *Lactobacillus* population abundance. *Br J Nutr* **111 Suppl 1**: S30-35.

Deckert A, Gow S, Rosengren L, et al. (2010). Canadian integrated program for antimicrobial resistance surveillance (CIPARS) farm program: results from finisher pig surveillance. *Zoonoses Pub Health* **57**: 71-84.

DeSantis TZ, Hugenholtz P, Larsen N, et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72**: 5069-5072.

Dibner JJ & Richards JD (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poult Sci* **84**: 634-643.

Edgar RC, Haas BJ, Clemente JC, Quince C & Knight R (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**: 2194-2200.

Faith DP (1992). Conservation evaluation and phylogenetic diversity. *Biol. Cons.* **61**: 1-10.

Gaskins H (2001). Intestinal bacteria and their influence on swine growth. p. 585-608. *In* A. J. Lewis and L. L. Southern (ed.), Swine nutrition. CRC Press, Boca Raton, Fla.

Godon JJ, Zumstein E, Dabert P, Habouzit F, Moletta R (1997). Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. *Appl Environ Microb* **63**: 2802-2813.

Guo X, Xia X, Tang R, Zhou J, Zhao H, Wang K (2008). Development of a real-time PCR method for *Firmicutes* and *Bacteroidetes* in faeces and its application to quantify intestinal population of obese and lean pigs. *Lett Appl Microbiol* **47**: 367-373.

Hamady M, Knight R (2009). Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. *Genome Res* **19**: 1141-1152.

Holman DB, Chenier MR (2013). Impact of subtherapeutic administration of tylosin and chlortetracycline on antimicrobial resistance in farrow-to-finish swine. *FEMS Microbiol Ecol* **85**: 1-13.

Holt JP, van Heugten E, Graves AK, See MT, Morrow WEM (2011). Growth performance and antibiotic tolerance patterns of nursery and finishing pigs fed growth-promoting levels of antibiotics. *Livest Sci* **136**: 184-191.

Kelly D, Smyth J, McCracken K (1991). Digestive development of the early-weaned pig. *Br J Nutr* **65**: 169–180.

Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ, Isaacson RE (2011). Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Vet Microbiol* **153**: 124-133.

Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ, Isaacson RE (2012). Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. *P Natl Acad Sci USA* **109**: 15485-15490.

Lalles JP, Bosi P, Smidt H, Stokes CR (2007). Nutritional management of gut health in pigs around weaning. *The Proceedings of the Nutrition Society* **66**: 260-268.

Lamendella R, Domingo JW, Ghosh S, Martinson J, Oerther DB (2011). Comparative fecal metagenomics unveils unique functional capacity of the swine gut. *BMC Microbiol* **11**: 103.

Looft T, Allen HK, Cantarel BL, et al. (2014a). Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. *ISME J*. **8**:1566-76.

Looft T, Allen HK, Casey TA, Alt DP, Stanton TB. (2014b) Carbadox has both temporary and lasting effects on the swine gut microbiota. *Front Microbiol*. **10**;5:276.

Looft T, Johnson TA, Allen HK, et al. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *P Natl Acad Sci USA* **109**: 1691-1696.

Lozupone C & Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* **71**: 8228-8235.

Mao SY, Yang CF & Zhu WY (2011) Phylogenetic analysis of methanogens in the pig feces. *Curr Microbiol* **62**: 1386-1389.

Maron DF, Smith TJ, Nachman KE (2013). Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. *Global. Health* **9**: 48.

Marshall BM, Levy SB (2011). Food animals and antimicrobials: impacts on human health. *Clin Microbiol Rev* **24**: 718-733.

Mathew AG, Cissell R, Liamthong S (2007). Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production. *Foodborne Pathog Dis* **4**: 115-133.

Moore WE, Moore LV, Cato EP, Wilkins TD, Kornegay ET (1987). Effect of high-fiber and high-oil diets on the fecal flora of swine. *Appl Environ Microbiol* **53**: 1638-1644.

Navas-Molina JA, Peralta-Sanchez JM, Gonzalez A et al. (2013). Advancing Our Understanding of the Human Microbiome Using QIIME. *Method Enzymol* **531**: 371-444.

Pieper R, Janczyk P, Schumann R, Souffrant W (2006). The intestinal microflora of piglets around weaning with emphasis on lactobacilli. *Archiv Zootech* **9**: 28-40.

Poole T, Suchodolski J, Callaway T, Farrow R, Loneragan G, Nisbet D (2013). The effect of chlortetracycline on faecal microbial populations in growing swine. *J Glob Antimicrob Resist* **1**: 171-174.

Schierack P, Walk N, Reiter K, Weyrauch KD, Wieler LH (2007). Composition of intestinal Enterobacteriaceae populations of healthy domestic pigs. *Microbiology* **153**: 3830-3837.

Scott KP, Gratz SW, Sheridan PO, Flint HJ, Duncan SH (2013) .The influence of diet on the gut microbiota. *Pharmacol Res* **69**: 52-60.

Shannon C (1948) A mathematical theory of communication, Part I. *Bell Syst Tech J* **27**: 379-423.

Snoeck V, Cox E, Verdonck F, Joensuu JJ, Goddeeris BM (2004), Influence of porcine intestinal pH and gastric digestion on antigenicity of F4 fimbriae for oral immunisation. *Vet Microbiol* **98**: 45-53.

Stokes CR, Bailey M, Haverson K et al. (2004). Postnatal development of intestinal immune system in piglets: implications for the process of weaning. *Anim Res* **53**: 325-334.

Su Y, Bian G, Zhu Z, Smidt H, Zhu W (2014). Early methanogenic colonisation in the faeces of Meishan and Yorkshire piglets as determined by pyrosequencing analysis. *Archaea* **2014**: 547908.

Thompson CL, Wang B, Holmes AJ (2008). The immediate environment during postnatal development has long-term impact on gut community structure in pigs. *ISME J* **2**: 739-748.

van den Bogaard AE, Stobberingh EE (2000). Epidemiology of resistance to antibiotics: links between animals and humans. *Int J Antimicrob Agents* **14**: 327-335.

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microb* **73**: 5261-5267.

Weijtens MJ, van der Plas J, Bijker PG, Urlings HA, Koster D, van Logtestijn JG, Huis in't Veld JH (1997). The transmission of campylobacter in piggeries; an epidemiological study. *J Appl Microbiol* **83**: 693-698.

Connecting text

In Chapters 3 and 4, we evaluated the impact of antibiotics on the culturable fraction of the swine gut microbiota as well as on the entire gut microbiota using high throughput sequencing, PCR, and real-time PCR. In Chapter 5 we examined the effect that a potential antibiotic alternative, flaxseed, has on the swine gut microbiota. We used DGGE, real-time PCR, and sequencing to assess this effect.

Chapter 5. Temporal analysis of the effect of extruded flaxseed on the swine gut microbiota

Running title: Extruded flaxseed and the swine gut microbiota

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5.1 Abstract

Flaxseed is a rich source of α-linolenic acid, an essential ω-3 fatty acid reported to have beneficial health effects in humans. Feeding swine a diet supplemented with flaxseed has been found to enrich pork products with ω-3 fatty acids. However, the effect of flaxseed supplementation on the swine gut microbiota has not been assessed to date. The purpose of this study was to investigate if extruded flaxseed has any impact on the bacterial and archaeal microbiota in the feces of growing-finishing pigs over a 51 day period using denaturing gradient gel electrophoresis (DGGE) and real-time PCR. Bacterial DGGE profile analysis revealed major temporal shifts in the bacterial microbiota with only minor ones related to diet. The archaeal microbiota was significantly less diverse than that of *Bacteria*. The majority of bacterial DGGE bands sequenced belonged to the *Firmicutes* phylum while the archaeal DGGE bands were found to consist of only two species, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*. The abundance of *Bacteroidetes* decreased significantly from day 0 to day 21 in all diet groups while the abundance of *Firmicutes* was relatively stable across all diet cohorts and sampling times. There was also no significant correlation between pig weight and the ratio of *Firmicutes* to *Bacteroidetes*. While the addition of extruded flaxseed to the feed of growing-finishing pigs was beneficial for improving ω-3 fatty acid content of pork, it had no detectable impact on the fecal bacterial and archaeal microbiota, suggesting that extruded flaxseed may be used to improve meat quality without adverse effect on the swine gut microbiota or animal performance.

5.2 Introduction

Flaxseed is one of the richest plant sources of α-linolenic acid (18:3 *n*-3), an essential ω-3 fatty acid associated with beneficial health effects in humans such as a reduced risk of cardiovascular and inflammatory diseases (Barcelo-Coblijn and Murphy, 2009). The oil fraction of flaxseed ranges from 35 to 46%, 45 to 58% of which is α-linolenic acid (Puvirajah, 2012; Singh et al., 2011). As a result, flaxseed has been successfully added to swine feed to increase the ω-3 fatty acid content of pork (Kouba et al., 2003; Matthews et al., 2000; Romans et al., 1995; Turner et al., 2014). α-Linolenic acid and oil extracted from flaxseed have also been demonstrated to have bactericidal and anti-adhesion properties *in vitro* (Kankaanpaa et al., 2001). *In vivo*, flaxseed oil has been shown to increase the adhesion and concentration of *Lactobacillus plantarum* in the swine gut while reducing the adhesion of *Escherichia coli* (Nemcová et al. 2012). In addition, water-soluble fibre in the form of mucilage is also an important component of flaxseed and may potentially act as a prebiotic in the gut (Lin et al., 2011).

Swine have a complex and diverse gut microbiota which contributes to the health of the animal (Konstantinov et al., 2006; Lamendella et al., 2011). The gut microbiota helps prevent pathogen colonization, aids in the development of the immune system, and extracts nutrients and energy from non-digestible dietary polysaccharides (Gaskins, 2001). The composition of the gut microbiota depends on several environmental factors, with diet being among the most important (Ley et al., 2008). While *Bacteria* are the predominant and best characterized members of the swine gut microbiota, *Archaea*, in the form of methanogens, are also an important component of this microbial ecosystem since they produce methane (CH_4) by oxidizing hydrogen and reducing carbon dioxide and other single-carbon molecules (Saengkerdsub and Ricke, 2014). Although

methane production in the gut represents a minor energy loss (0.6 to 1.3%) in swine, it is a greenhouse gas of environmental concern with growing pigs producing up to 6.5 L CH_4 day^{-1} (Jørgensen et al., 2007; Monteny et al., 2001). The concentration of fibre in the diet has been shown to be positively correlated with the level of methane produced in swine (Jensen and Jørgensen 1994).

Antibiotics, which are frequently added to feed to enhance swine growth and feed efficiency, are believed to increase growth through a direct impact on the gut microbiota (Dibner and Richards, 2005). Although the exact mechanism for this effect is unknown, it is believed to be due to a reduction in the total bacterial load, inhibition of pathogens, promotion of beneficial bacteria, or immunomodulatory effects in the gut (Allen et al., 2013). Antibiotic use in agriculture, however, also contributes to the reservoir of antibiotic resistance in the environment (Wegener, 2003). As a result, there is a strong need to find alternatives to antibiotics that mimic their effects in the gut and increase feed efficiency (Allen et al., 2013). Flaxseed may offer an alternative to using antibiotics due to the potential bactericidal, anti-adhesion, anti-inflammatory, and prebiotic properties of its various components (Dahiya et al., 2006; Kiarie et al., 2007; Zijlstra and Beltranena, 2013).

While flaxseed has been investigated in numerous studies as a dietary additive for improving pork ω-3 fatty acid content, we are unaware of any research devoted to examining its impact on the gut microbiota in swine. Given the potential number of components in flaxseed that may affect the swine gut microbiota it is important to understand if there is indeed any measurable effect on the swine gut microbiota, particularly if it is to be considered for use as an antimicrobial alternative. Therefore, in the present study we evaluated the effect of extruded

flaxseed on the temporal evolution of the swine gut bacterial and archaeal microbiota using a culture-independent approach.

5.3 Materials and methods

5.3.1 Animals, diets, and experimental design

A total of 80 Duroc x (Landrace x Yorkshire) pigs were fattened from an initial weight of 71.2 ± 8.9 kg to 114.2 ± 8.6 kg over a period of 51 days at the McGill Swine Complex (Montreal, QC, Canada). Pigs were randomly assigned to receive one of four experimental diets with four pens per diet and five pigs per pen (20 pigs per diet). Experimental diets were isocaloric and included a corn-soybean meal supplemented with 0%, 5%, 10%, or 15% of an extruded flaxseed product (Belisle Solution Nutrition Inc, Saint-Mathias, QC, Canada) that consisted of 75% flaxseed and 25% ground alfalfa (Table 5.1). The extrusion process was conducted using an Insta-Pro 2000RC extruder (model 2000RC, Insta-Pro International, Des Moines, IA, USA) that was outfitted with an 8100RC volumetric feeder. The temperature of the extrusion was kept at 122°C. Pigs were given ad libitum access to feed and water and weighed on a weekly basis. Prior to the start of the study, all pigs were fed the control feed during an adaptation period of two weeks (0% flaxseed) and none of the diets included antibiotics. All animal handling procedures were carried out in accordance with McGill University Animal Care and Use Committee guidelines (Protocol # 5711).

Table 5.1. Composition of experimental diets with different levels of extruded flaxseed.

Composition	Extruded flaxseed (g kg^{-1})			
	0	**50**	**100**	**150**
Ingredients (g kg^{-1})				
Corn	683	653	622	592
Soybean meal	182	160	138	116
Extruded flaxseed	0	50	100	150
Dried distillers' grains	100	100	100	100
Soybean hulls	2	5	8	1
Vitamin-mineral premix	9	9	9	9
Calcium	12	12	11	11
Phosphorus	4	4	3	3
Salt	3	3	3	3
Choline chloride	1	1	1	1
Lysine HCl	2.8	3.2	3.6	1.0
Methionine	0.1	0.1	0.1	0.1
Threonine	0	0.1	0.2	0.2
Chemical composition (g kg^{-1})				
Ash	54 ± 7.0	54 ± 1.2	59 ± 1.1	61 ± 0.50
Crude protein	168 ± 2.7	153 ± 9.9	155 ± 4.5	159 ± 2.7
[a] NDF	197 ± 13.4	195 ± 7.2	203 ± 5.7	222 ± 3.7
Ether extract	41 ± 1.9	45 ± 1.6	47 ± 3.4	51 ± 1.7
Starch	464 ± 12.8	445 ± 14.7	426 ± 12.2	408 ± 9.0
Total fatty acids	47 ± 1.3	49 ± 0.5	54 ± 1.8	53 ± 1.7
α-Linolenic acid	25 ± 0.5	70 ± 1.3	109 ± 2.2	116 ± 5.0

[a] NDF= amylase-treated neutral detergent fibre.

5.3.2 Sampling and DNA extraction

Fecal samples were collected from five randomly selected pigs in each treatment group 21 and 51 days after the start of the trial. Fecal samples were also taken at the start of the study (day 0) from five randomly chosen pigs across all groups. Sampling was conducted as described previously (Holman and Chenier, 2013). Total DNA was extracted from each swine fecal sample (100-150 mg) using the ZR Fecal DNA Miniprep kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. The total DNA concentration in each extract was

determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Extracted DNA was stored at -20°C until analysis.

5.3.3 PCR amplification of bacterial and archaeal 16S rRNA genes

The variable region 3 (V3) of the bacterial 16S rRNA gene was targeted using primers 341F (with GC clamp) and 534R (Table 2). Each PCR reaction contained 1X PCR buffer with 1.5 mM Mg^{2+}, 0.5 µM of each primer (Integrated DNA Technologies, Toronto, ON, Canada), 0.2 mM dNTPs (Bioshop Canada Inc., Burlington, ON, Canada), 2 U Hot-Start *Taq* DNA polymerase (Denville Scientific, Saint-Laurent, QC, Canada), 100 ng of template DNA extracted from the fecal sample of each pig, and molecular grade water (Sigma-Aldrich, Oakville, ON, Canada) in a total volume of 50 µl. The PCR conditions were as detailed in Chenier and Juteau (2009).

Archaeal diversity was investigated with the use of the universal archaeal primers ARC787F (with GC clamp) and ARC1059R (Table 5.2) which target the variable region 5 (V5) of the archaeal 16S rRNA gene. Each PCR reaction consisted of 1X PCR buffer with 1.5 mM Mg^{2+}, 0.15 µM of each primer, 0.16 mM dNTPs, 2 U Hot-Start *Taq* DNA polymerase, 50 ng of template DNA extracted from the fecal sample of each pig, and molecular grade water up to 50 µl. The touchdown PCR conditions were as follows: initial denaturation at 94°C for 10 min, followed by 20 cycles at 94°C for 1 min, annealing at 65°C (decreasing 0.5°C per cycle down to 55^{o}C) for 1 min, extension at 72°C for 1 min, then 10 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension time of 3 min at 72°C.

Table 5.2. Oligonucleotide primers used in this study.

Target	Primers	Sequence (5′ → 3′)	Amplicon size (bp)	Reference
Bacteria	341F* 534R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	193	(44)
Archaea	ARC787F* ARC1059R	ATTAGATACCCSBGTAGTCC GCCATGCACCWCCTCT	272	(61)
Bacteroidetes	798cfbF cfb967R	CRAACAGGATTAGATACCCT GGTAAGGTTCCTCGCGTAT	169	(3)
Firmicutes	928FirmF 1040FirmR	TGAAACTYAAAGGAATTGACG ACCATGCACCACCTGTC	112	(3)

*GC-clamp of CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG added to 5' end for DGGE.

5.3.4 DGGE analysis

PCR products were purified using a SpinSmart PCR purification kit (Denville), then 5 µg (*Bacteria*) or 1.5 µg (*Archaea*) of purified PCR product was added to each well in an 8% polyacrylamide gel with a denaturing gradient of 30-70% for *Bacteria* and 45-55% for *Archaea* (100% denaturant was defined as 7 M urea and 40% formamide) in 1X TAE buffer (40 mM Tris-acetate, 0.1 mM EDTA, pH 8.0). For the purposes of normalization, a standard was run on each gel using bacterial strains as per Chenier and Juteau (2009) or a sample-derived standard for *Archaea*. DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad, Mississauga, ON, Canada) as described previously (Chenier and Juteau, 2009).

DGGE gels were imaged using a Red AlphaImager (Proteinsimple, Santa Clara, CA, USA). DGGE gels were normalized and cluster analysis performed using GelCompar II

software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms for both *Bacteria* and *Archaea* were constructed using clustering analysis based on the unweighted pair group method with arithmetic averages (UPGMA) and the Jaccard similarity coefficient.

5.3.5 Sequencing

Selected bands were excised from a DGGE gel consisting of 5 pooled samples from each treatment (diet) and time point using a sterile scalpel. Excised bands were placed in 50 µl of sterile water and DNA eluted at 4°C. For each subsequent PCR reaction, 5 µl of eluted DNA was used as template together with the DGGE primers (either for *Bacteria* of for *Archaea*) minus the GC-clamp. The PCR products were then purified using the SpinSmart purification kit and cloned into a pGEM-T Easy vector (Promega Corp., Madison, WI, USA). Colony PCR was then performed using the high-fidelity Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA, USA) and a single colony as template. At least two colonies were amplified per excised band. PCR products were verified for the correct insert length on a 2% agarose gel (Table 5.2).

Sequencing was done at the McGill University and Genome Québec Innovation Centre (Montreal, QC, Canada) using a 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequences were screened for potential chimeras using DECIPHER (Wright et al. 2012) and chimeras were removed before comparing sequences with those in the Ribosomal Database project (RDP; http://rdp.cme.msu.edu) and BLASTn (http://blast.ncbi.nlm.nih.gov). Sequences were aligned with related sequences in the database using ClustalX version 2.1 (Larkin et al. 2007). Sequences were deposited in the National Center for Biotechnology Information (NCBI) trace repository (http://www.ncbi.nlm.nih.gov/Traces/home) with the following TI (trace archive ID) numbers: 2338273705 to 2338273736.

Phylogenetic analysis was performed using programs found in the PHYLIP software package version 3.695 (Felsenstein, 2010). The aligned sequences were bootstrapped 1000 times using SEQBOOT and a DNA distance matrix calculated with DNADIST using the Kimura 2-parameter model. Phylogenetic trees were then created using the Fitch-Margoliash method in FITCH, and a consensus tree was constructed using CONSENSE. Trees were displayed using TREEVIEW version 1.6.6 (Page 2002).

5.3.6 Real-time PCR

Real-time PCR experiments were carried out as previously described (Holman and Chenier 2013). Primers used to quantify *Bacteria*, *Archaea*, *Firmicutes*, and *Bacteroidetes* are listed in Table 5.2. Concentrations of primers in each 20 µl reaction were as follows: 0.375 µM for 341F and 534R, 0.5 µM for ARC787F and ARC1059R, and 0.625 µM for 798cfbF, cfb967R, 928FirmF, and 1040FirmR. A melt curve analysis from 55°C to 95°C was performed at the end of each run to ensure the amplification of only one product.

5.3.7 Statistical analysis

A one-way ANOVA that included diet in the model was performed for real-time PCR results and DGGE band numbers using PROC GLM in SAS (SAS Inst., Inc., Cary, NC, USA). The Pearson correlation coefficient for the correlation between the relative abundance of *Firmicutes*, *Bacteroidetes*, total *Bacteria*, total *Archaea*, and pig weight was determined using PROC CORR. Results were considered significant at the $\alpha = 0.05$ level.

5.4 Results

The pigs weighed an average of 71.2 ± 8.9 kg at the start of the study and were fattened to 114.2 ± 8.6 kg prior to shipping for slaughter. Extruded flaxseed supplementation had no significant effect on the average daily gain, average daily feed intake, or feed efficiency (data not

shown). Three pigs (one each from the control, 10% flaxseed, and 15 % flaxseed groups) had to be removed from the study due to unrelated illness.

5.4.1 Effect of extruded flaxseed on bacterial community structure

Extruded flaxseed had no apparent effect on the bacterial composition of the swine gut microbiota when DGGE profiles were analyzed using cluster analysis. For the most part, samples tended to cluster together based on the time of sampling rather than diet, although replicate samples of the same diet also tended to cluster together at day 0 and day 51 (Fig 5.1A). The 5 and 10% flaxseed samples, however, formed a distinct cluster at day 51 with less than 40% similarity to all other samples. There was significantly more diversity in terms of the number of bands distinguishable at 21 days than compared to day 0 and day 51 ($p<0.001$) (Fig. 5.2A). Between 11 and 22 bands were detected in all diet groups at all times as indicated in the DGGE profile of pooled samples (Fig. 5.3A).

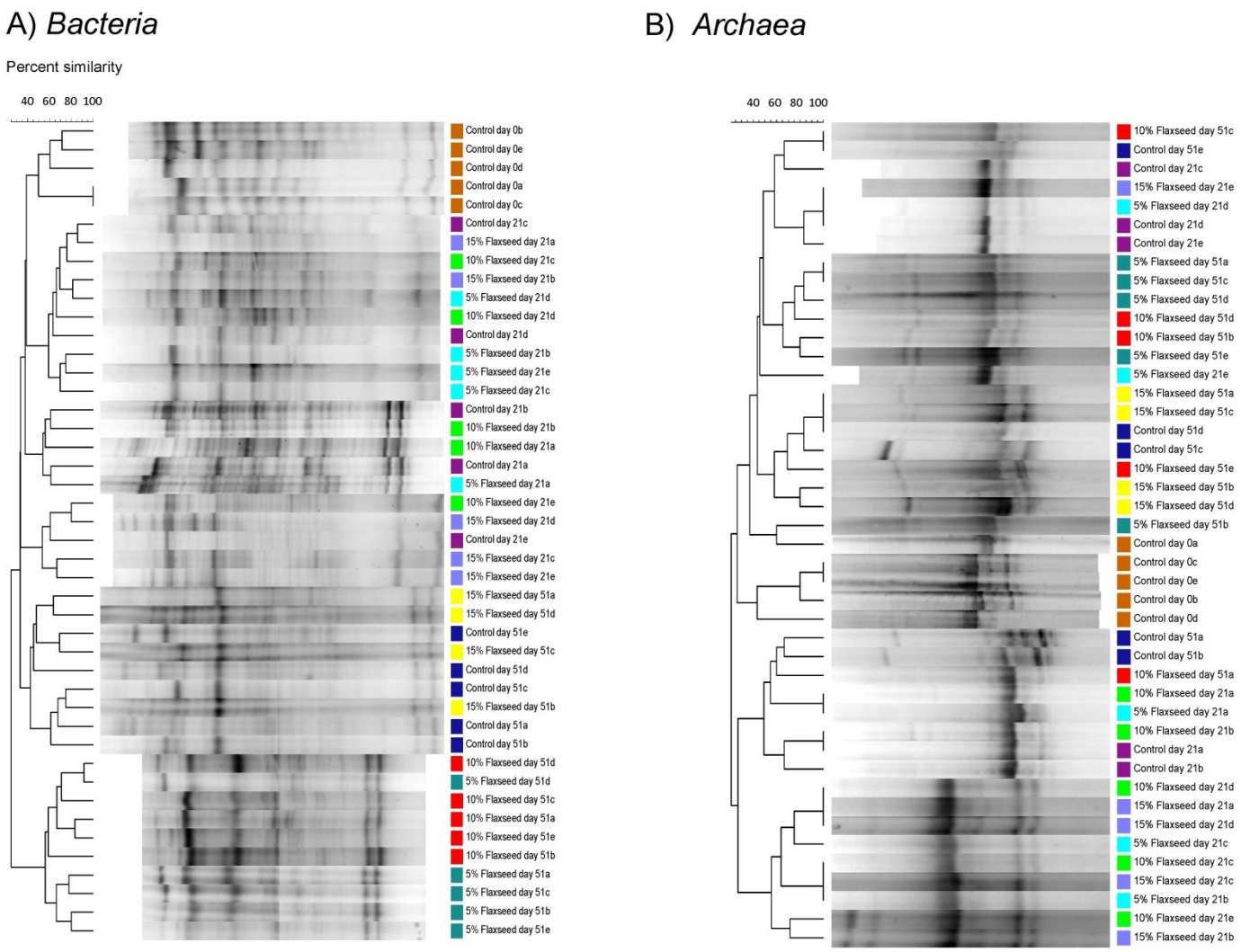

Figure 5.1. Cluster analysis using the UPGMA method and the Jaccard similarity coefficient of A) bacterial 16S rRNA gene (30-70% denaturant) and B) archaeal 16S rRNA gene (45-55% denaturant) DGGE profiles of pigs fed 0, 5, 10, or 15% extruded flaxseed and sampled at days 0, 21, and 51. The percent similarity between samples is shown at the top of each dendrogram.

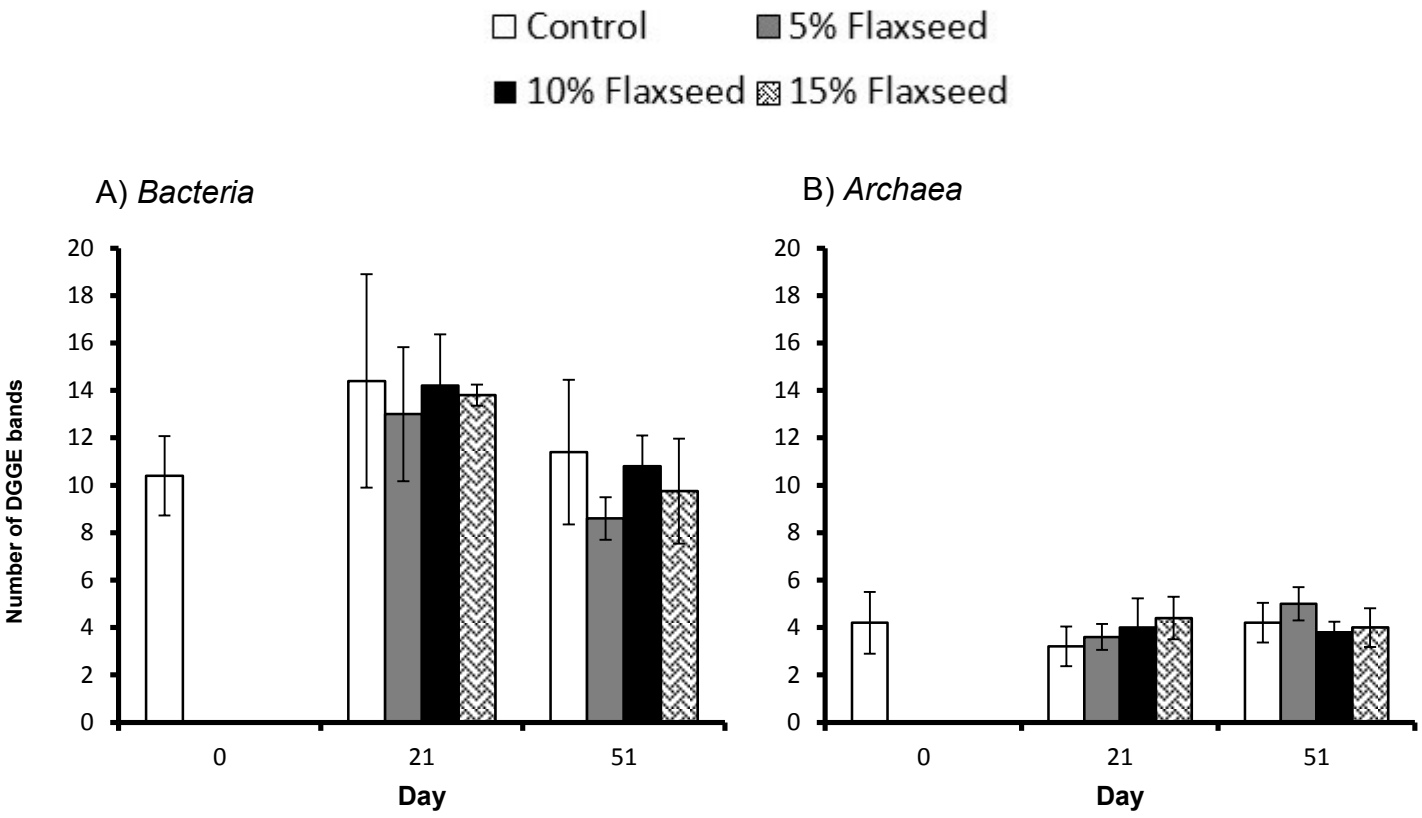

Figure 5.2. Comparison of the number of DGGE bands for A) Bacteria and B) Archaea from fecal samples of pigs fed 0, 5, 10, or 15% extruded flaxseed. Pigs were sampled at days 0, 21, and 51. Error bars represent standard deviation of the mean (n=5).

Fifteen well separated bands were selected for excision from the bacterial DGGE gel. This DGGE gel contained pooled samples from each diet cohort and sampling period and at least one excised band was chosen from each pooled sample (Fig. 5.3A). Due to the fact that 16S amplicons with two different sequences may migrate to the same position on the DGGE gel, it was necessary to perform TA cloning prior to sequencing to ensure that only a single amplicon was sequenced (Gafan and Spratt, 2005). In several instances, more than one sequence was obtained from a single excised band and overall 21 different sequences were obtained from these 15 bands. Of these 21 sequences, 15 were classified as belonging to the *Firmicutes* phylum

while 4 sequences were identified as members of the *Bacteroidetes* phylum. In Figure 5.4A, bands yielding two different sequences are indicted with the suffix "A" or "B".

Bands 3, 4B, 5, 6, and 15 all had sequences that had a high level of sequence similarity with *Clostridium* spp. (Fig. 5.4A) and each migrated to the portion of the gel containing the highest concentration of denaturant, i.e. lowest portion of the gel (Fig. 3A). DGGE bands 1B, 2, 4A, 10B were the only bands with sequences classified as *Bacteroidetes* and all shared >96% identity with *Prevotella* spp. (Fig. 5.3A and 5.4A). In one case, a unique band (band 7) was identified in the 5% flaxseed diet group at 51 days. The sequence of this band was phylogenetically related to *Treponema porcinum*, a spirochaete. One band (13B) that appeared common to all diet cohorts had 97% sequence similarity to *Campylobacter curvus,* a member of the *Proteobacteria* phylum.

5.4.2 Effect of extruded flaxseed on archaeal community structure

Similar to the bacterial microbiota, the archaeal microbiota did not exhibit any identifiable changes as a result of diet based on cluster analysis of the DGGE profiles (Fig 5.1B). However, samples did not cluster by sampling time either. There was significantly less archaeal diversity compared to *Bacteria* in terms of the number of individual bands in each sample; overall, 3 to 6 DGGE bands were detected in each fecal sample ($p<0.001$; Fig 5.2). Unlike bacteria, the number of DGGE bands did not differ by sampling time. Ten of the 11 excised DGGE bands (Fig. 5.3B) were closely related to only two archaeal species, the methanogens *Methanobrevibacter smithii* (bands 19 to 24) and *Methanosphaera stadtmanae* (bands 16, 17, 18 and 25) (Fig. 5.4B). Both of these organisms belong to the same taxonomic family (*Methanobacteriaceae*). Band 26 shared 99% identity with *Methanobrevibacter* sp. WBY1 (data not shown).

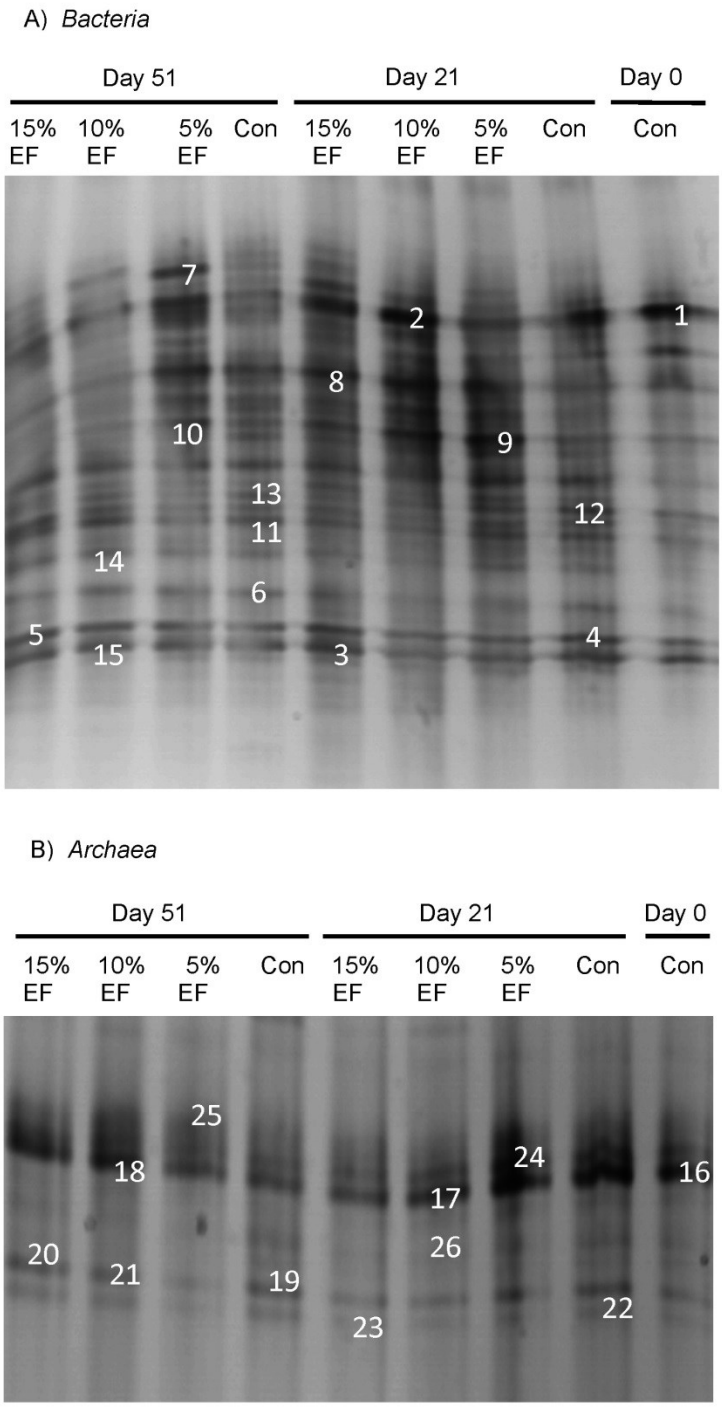

Figure 5.3. Negative DGGE image of pooled samples (n=5) of PCR-amplified 16S rRNA gene fragments from (A) Bacteria and (B) Archaea. Numbers indicate bands that were excised and sequenced. EF= extruded flaxseed, Con=control.

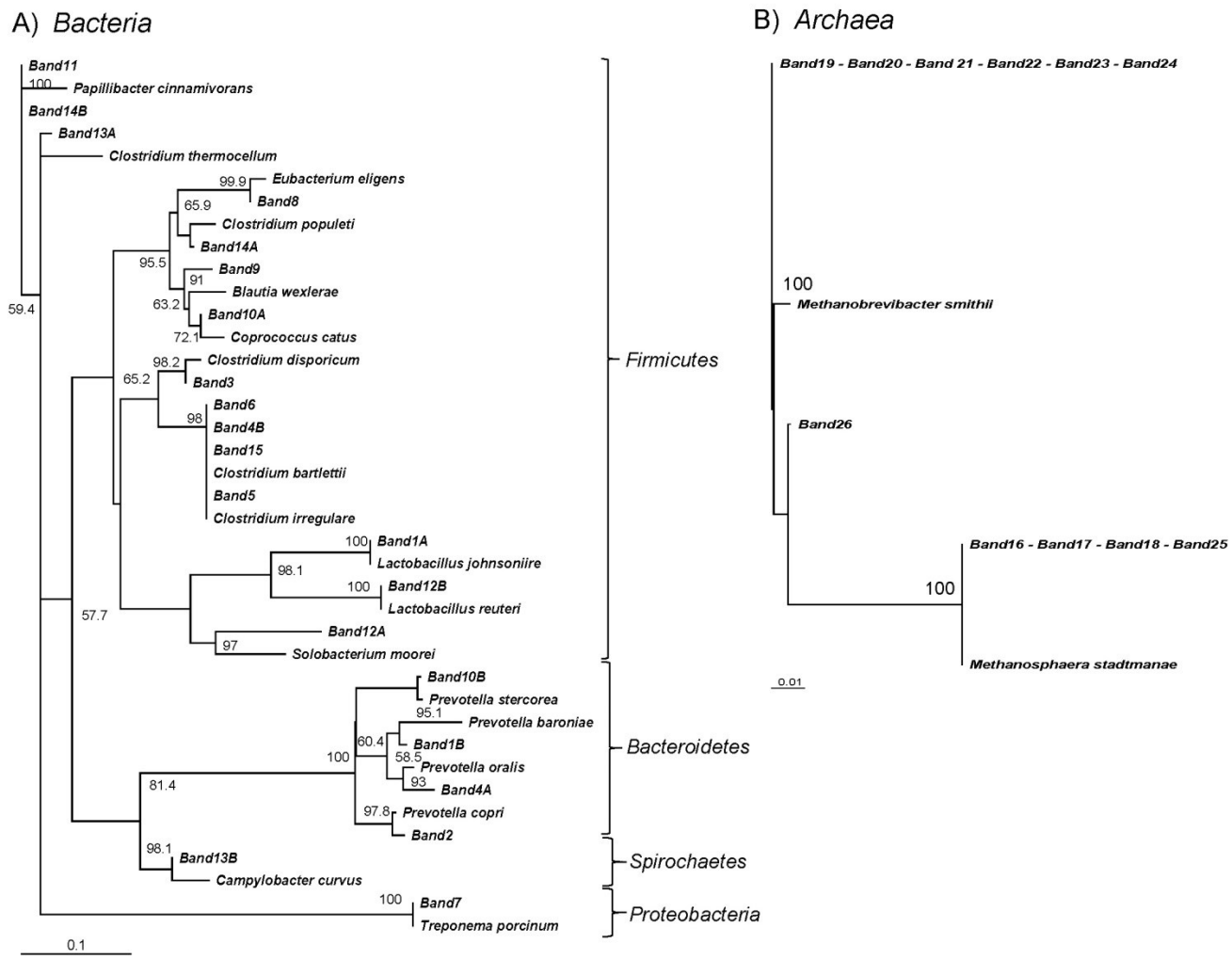

Figure 5.4. Phylogenetic trees showing the relationships of (A) sequenced bacterial 16S rRNA gene DGGE bands and (B) sequenced archaeal 16S rRNA gene DGGE bands. Each tree was inferred using the Fitch-Margoliash method. Bootstrap values greater than 50% are shown at nodes. The scale bar represents substitutions per nucleotide. Bands with the suffix "A" or "B" yielded two distinct sequences from a single band.

5.4.3 Quantification of *Bacteria*, *Archaea*, *Bacteroidetes*, and *Firmicutes*

Total *Bacteria* and total *Archaea* were quantified using real-time PCR and the primers listed in Table 5.2, minus the GC clamp. As the majority of DGGE bands sequenced belonged to two phyla, *Bacteroidetes* or *Firmicutes*, these groups were also quantified. No significant differences were observed for total *Bacteria* or total *Archaea* between the extruded flaxseed diet groups and the control at any sampling time ($p>0.05$; Fig. 5.5A-B). Although diet cohorts did not differ significantly, overall there were significantly more *Bacteroidetes* on day 0 than at days 21 or 51 of the trial ($p<0.05$; Fig. 5.5C). No significant differences were noted for the *Firmicutes* either in terms of diet or time of sampling ($p>0.05$; Fig. 5.5D). The average relative abundance of *Archaea*, *Bacteroidetes*, and *Firmicutes* across all sampling times and diet groups as a percentage of bacterial 16S, was 2%, 14%, and, 16%, respectively (data not shown).

There was no significant correlation between the ratio of *Firmicutes* to *Bacteroidetes* and pig weight ($p>0.05$; data not shown). There was a negative but insignificant correlation between the concentration of *Bacteroidetes* and pig weight ($r=-0.299$; $p=0.0642$). A moderate and significant correlation was noted, however, between the concentration of *Bacteroidetes* and *Archaea* ($r=0.406$ $p=0.0062$). There was no correlation between the concentration of *Bacteria*, *Archaea*, or *Firmicutes* and pig weight.

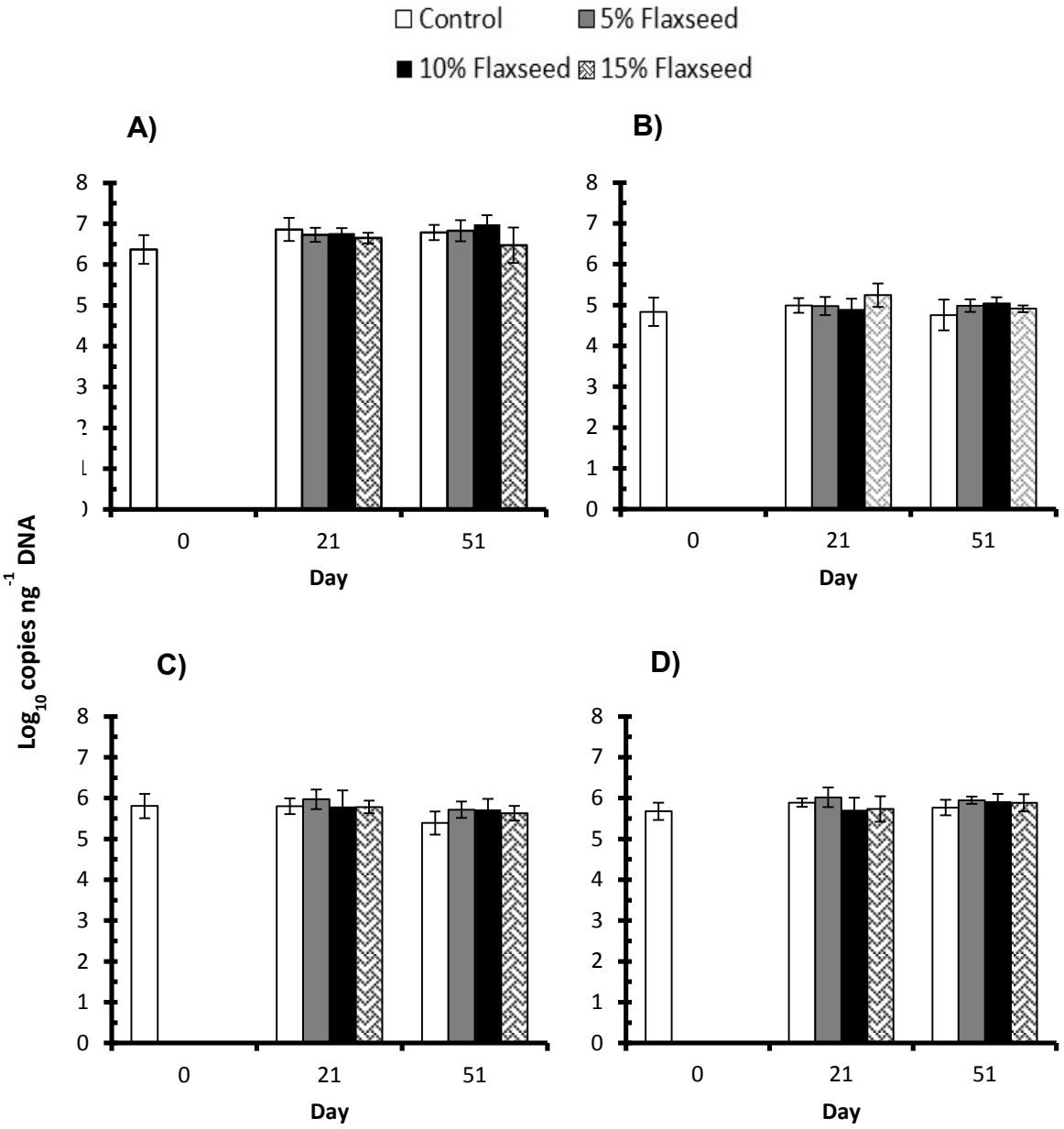

Figure 5.5. Number of copies of 16S rRNA gene per ng of DNA for (A) *Bacteria*, (B) *Archaea*, (C) *Bacteroidetes*, and (D) *Firmicutes* in pigs fed 0, 5, 10, or 15% extruded flaxseed and sampled at day 0, 21, and 51. Error bars represent standard deviation of the mean (n=5)

5.5 Discussion

The present study was part of a larger research project where the ω-3 fatty acid content of pork was significantly increased using extruded flaxseed (B. Baurhoo, Bélisle Solution and Nutrition Inc, personal communication). To our knowledge, this is the first study to examine the impact of an extruded flaxseed diet on the gut microbiota of swine. Despite the changes in the fatty acid content of the pigs, we observed no obvious qualitative or quantitative alteration in the swine gut microbiota as a result of the addition of extruded flaxseed to the diet as measured using DGGE analysis and real-time PCR. Bacterial 16S DGGE profiles tended to cluster together with other samples of the same time and diet cohort. The abundance of *Bacteria*, *Archaea*, *Bacteroidetes*, and *Firmicutes* and were relatively stable throughout the study, with the exception of a decrease in *Bacteroidetes* at day 21. Based on our observations, the age of the pigs at sampling appeared to be the most important factor in shaping the composition of the gut microbiota.

Although a study by Kiarie et al. (2007) found that a diet 12% ground flaxseed reduced anaerobic spore formers in the ileum of piglets we did not detect any antimicrobial effect in the current study using real-time PCR for total 16SrRNA (data not shown). This could be due to a number of factors, such as the fact that the ileal and colonic bacteria are very different in composition, the piglets used by Kiarie and others (2007) were much younger (17 d vs. approx. 16 wk), and that we did not enumerate total anaerobic spore formers as we were more interested in the effect of extruded flaxseed on diversity of the swine gut microbiota.

The bacterial diversity in the swine feces observed in the current study was similar to previously published reports with up to 22 distinct bands detected in fecal samples (Fig. 5.1 to

5.4). Earlier studies of the swine fecal microbiota using DGGE have reported between 19 and 34 DGGE bands for swine feces, although different denaturing gradients and detection criteria for bands were used (Konstantinov et al., 2003; Simpson et al., 1999; Wang et al., 2007). The archaeal diversity, however, was much lower with only 3 to 6 DGGE bands detected in each sample (Fig. 5.1 to 5.4), a finding which is consistent with previous studies of *Archaea* in swine feces (Cao et al., 2012; Luo et al., 2012). This was also reflected in the relative abundance of archaeal total 16S rRNA gene copies to total bacterial 16S rRNA gene copies, where the concentration of total *Archaea* was almost 100 times lower than that of total bacteria (Fig. 5.5B). This finding is in agreement with Lamendella et al. (2011) who reported that *Archaea* comprise less than 1% of the total 16S rRNA gene copies in swine. While a recent report by Li et al. (2012) described a decrease in the abundance of archaeal 16S copies in the rumen of dairy cattle fed ground flaxseed, in the current study we noted no significant differences associated with extruded flaxseed and archaeal 16S copies in swine fecal samples. This may be due to the differences in the rumen of cattle and colon of swine in terms of physiology and the microbiota of each. It could also be related to differences in the texture between ground and extruded flaxseed.

The lack of significant differences in the DGGE profiles of pigs fed different concentrations of flaxseed could be attributable to the age of the pigs (≈ 14 weeks) when the flaxseed diets were introduced. Accordingly, Castillo et al. (2007) found no impact on the abundance of lactobacilli and enterobacteria when different fibre concentrations were included in diets starting at eight weeks of age. In another study, the use of in-feed antibiotics beginning in the growing phase failed to produce a shift in the fecal bacterial community of swine as measured using a clone library (Kalmokoff et al., 2011). Conversely, dietary studies

commencing at weaning (16-28 d) have demonstrated notable changes in bacterial DGGE profiles (Konstantinov et al., 2003; Namkung et al., 2004). Konstaintinov et al. (2003) reported an increase in bacterial diversity in weanling pigs (25-28 days) fed sugar beet pulp and fructooligosaccharides over a 13 day period as measured using DGGE. In addition, the fact that each diet in the present study was designed to be isocaloric may have muted the impact of varied extruded flaxseed concentrations on the gut microbiota.

Bacterial 16S rRNA gene DGGE bands were excised and sequenced, revealing a predominance of *Firmicutes*, a finding that is in accordance with several other culture-independent biodiversity studies on the swine gut microbiota (Kim et al., 2012; Lamendella et al., 2011). In the current study, *Lactobacillus* and *Clostridium* spp. were the most commonly identified members of the *Firmicutes* phylum. Both of these genera are known to be relatively abundant in swine feces (Kim et al., 2011; Leser et al., 2002). Lactobacilli in particular are important members of the swine gut microbiota as they may offer protection against pathogens through the production of volatile fatty acids and bacteriocins. In addition, they may also modulate the immune system (Janczyk et al., 2007; Konstantinov et al., 2008). All bands that had sequences classified as members of the *Bacteroidetes* phylum were closely identified with *Prevotella* spp. (Fig. 5.4A), which is also in agreement with previous research (Kim et al., 2011, 2012; Lamendella et al., 2011; Looft et al., 2012). According to a study by Kim et al. (2011), the *Prevotella* genus accounted for almost 12% of all 16S sequences in swine feces over a 12 week period. Interestingly, a band (13B) common to all diet groups shared 100% sequence homology with *Campylobacter curvus*, a bacterium occasionally isolated from patients with bloody diarrhea, although no association with disease has been observed in swine (Abbott et al., 2005). In terms of unique phylotypes, band 7 appeared only in the 5% flaxseed diet cohort at 51 d and

had 100% identity with *Treponema porcinum*, an obligate anaerobic *Spirochaete* that has recently been isolated from swine feces (Nordhoff et al., 2005).

A link has been suggested between the ratio of *Firmicutes* to *Bacteroidetes* and obesity in humans and mice (Ley et al., 2005; Ley et al., 2006), although this finding is not always consistent (Duncan et al., 2008). Similar results have been observed in minipigs and cloned pigs where a negative correlation between weight gain and the relative abundance of *Bacteroidetes* has been noted (Guo et al., 2008; Pedersen et al., 2013a). In addition, a positive correlation between the ratio of *Firmicutes*:*Bacteroidetes* and weight gain has also been reported by Pedersen et al. (2013b) in Göttingen minipigs, although their findings were not consistent as Ossabaw minipigs had a negative correlation with the *Firmicutes*:*Bacteroidetes* ratio.

As the large majority of DGGE bands sequenced in the present study were also members of these two phyla, we chose to investigate this association using real-time PCR. Overall, the concentration of *Bacteroidetes* decreased significantly over time ($p=0.0196$, Fig 5.5C), which is in agreement with the work of Kim et al. (2011) who observed a decrease in the proportion of *Bacteroidetes* from 10 to 22 weeks in commercial pigs. Our findings for the relative abundance of *Bacteroidetes* to total bacterial 16S are consistent with Guo et al.'s (2008) report of 4-12% in lean and obese swine. Although the relative abundance of *Firmicutes* in the present study was generally lower than the 40% to 70% often reported in swine feces (Guo et al., 2008; Kim et al., 2012; Lamendella et al., 2011), there was considerable variation between individual pigs, ranging from 4% to 68% *Firmicutes*. In addition, the relative abundance of *Bacteroidetes* and *Firmicutes* can depend on the PCR primers used and the region of the 16s rRNA gene that they target (Claesson et al., 2010).

As expected, all of the sequenced bands from the archaeal 16S rRNA gene DGGE profiles shared a high level of relatedness with methanogenic *Archaea*. These two species, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, are common inhabitants of the swine gut microbiota and members of the *Methanobacteriaceae* family (Mao et al., 2011; Su et al., 2014). They are also among the predominant methanogens in the human gut microbiota (Dridi et al., 2009). All of the excised archaeal DGGE bands shared a much higher degree of similarity with each other in comparison to the bacterial DGGE bands, demonstrating the lower level of diversity among *Archaea* in the swine gut. Interestingly, a moderate and significant correlation was found between the concentration of *Bacteroidetes* and *Archaea*. Although the significance of this association is not clear, both groups are obligate anaerobes and therefore may benefit from similar changes in the gut ecosystem. In addition, fermentative end products produced by members of the *Bacteroidetes* phylum such as CO_2, H_2, acetate, and formate, can be metabolized by methanogens to produce methane (Ellis et al., 2008; Zhang et al., 2009).

In conclusion, the addition of extruded flaxseed to the feed of growing-finishing pigs had no measurable impact on the fecal bacterial and archaeal microbiota using DGGE. . Overall, changes in the gut microbiota of the pigs were associated more with time rather than with diet. A similar study with the addition of extruded flaxseed beginning at weaning might yield different results due to the relative instability of the piglet gut microbiota at that age. In addition, the use of high throughput sequencing may reveal differences that aren't visible with DGGE.

5.6 Acknowledgements

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5.7 References

Abbott SL, Waddington M, Lindquist D, Ware J, Cheung W, Ely J, Janda JM (2005). Description of *Campylobacter curvus* and *C. curvus*-like strains associated with sporadic episodes of bloody gastroenteritis and Brainerd's diarrhea. *J Clin Microbiol* **43**: 585-588.

Allen HK, Levine UY, Looft T, Bandrick M, Casey TA (2013). Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends Microbiol* **21**: 114-119.

Bacchetti De Gregoris T, Aldred N, Clare AS, Burgess JG (2011). Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. *J Microbiol Methods* **86**: 351-356.

Barcelo-Coblijn G, Murphy EJ (2009). Alpha-linolenic acid and its conversion to longer chain *n*-3 fatty acids: benefits for human health and a role in maintaining tissue *n*-3 fatty acid levels. *Prog Lipid Res* **48**: 355-374.

Cao Z, Di Liao X, Liang JB, Wu YB, Yu B (2012). Diversity of methanogens in the hindgut of grower and finisher pigs. *Afr J Biotechnol* **11**: 4949-4955.

Castillo M., Martin-Orue SM, Anguita M, Perez JF, Gasa J. (2007). Adaptation of gut microbiota to corn physical structure and different types of dietary fibre. *Livest Sci* **109**: 149-152.

Chénier MR,Juteau P (2009). Impact of an aerobic thermophilic sequencing batch reactor on antibiotic-resistant anaerobic bacteria in swine waste. *Microb Ecol* **58**: 773-785.

Claesson MJ, Wang QO, O'Sullivan O, Greene-Diniz R, Cole JR, Ross RP, O'Toole PW (2010). Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Res* **38**: e200-e200.

Dahiya JP, Wilkie DC, Van Kessel AG, Drew MD (2006). Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. *Anim Feed Sci Tech* **129**: 60-88.

Dibner JJ, Richards JD (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poult Sci* **84**: 634-643.

Dridi B, Henry M, El Khechine A, Raoult D, Drancourt M (2009). High Prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* Detected in the human gut using an improved DNA detection protocol. *PLOS ONE* **4**: e7063.

Duncan SH, Lobley GE, Holtrop G, Ince J, Johnstone AM, Louis P, Flint HJ (2008). Human colonic microbiota associated with diet, obesity and weight loss. *Int J Obes* **32**: 1720-1724.

Ellis JL, Dijkstra J, Kebreab E, Bannink A, Odongo NE, McBride BW, France J (2008). Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle. *J Agr Sci* **146**: 213-233.

Felsenstein J. 2010. PHYLIP (Phylogeny Inference Package) version 3.69, University of Washington, Seattle. http://evolution.genetics.washington.edu/phylip.html Accessed March 24 2014.

Gafan GP, Spratt DA (2005). Denaturing gradient gel electrophoresis gel expansion (DGGEGE)–An attempt to resolve the limitations of co-migration in the DGGE of complex polymicrobial communities. *FEMS Microbiol Lett* **253**: 303-307.

Gaskins H (2001). Intestinal bacteria and their influence on swine growth. In: Southern, AJ (ed). Swine Nutrition. CRC Press LLC: Florida. pp 585-608.

Guo X, Xia X, Tang R, Zhou J, Zhao H, Wang K (2008). Development of a real-time PCR method for *Firmicutes* and *Bacteroidetes* in faeces and its application to quantify intestinal population of obese and lean pigs. *Lett Appl Microbiol* **47**: 367-373.

Holman DB, Chénier MR (2013). Impact of subtherapeutic administration of tylosin and chlortetracycline on antimicrobial resistance in farrow-to-finish swine. *FEMS Microbiol Ecol* **85**: 1-13.

Janczyk P, Pieper R, Smidt H, Souffrant WB (2007). Changes in the diversity of pig ileal lactobacilli around weaning determined by means of 16S rRNA gene amplification and denaturing gradient gel electrophoresis. *FEMS Microbiol Ecol* **61**: 132-140..

Jensen BB, Jørgensen H. 1994. Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl Environ Microbiol* **60**: 1897-1904.

Jørgensen H, Serena A, Hedemann MS, Bach Knudsen KE (2007). The fermentative capacity of growing pigs and adult sows fed diets with contrasting type and level of dietary fibre. *Livest Sci* **109**: 111-114.

Kalmokoff M, Waddington LM, Thomas M, Liang KL, Ma C, Topp E, Dandurand UD, Letellier A, Matias F, Brooks SPJ (2011). Continuous feeding of antimicrobial growth promoters to commercial swine during the growing/finishing phase does not modify faecal

community erythromycin resistance or community structure. *J Appl Microbiol* **110**: 1414-1425.

Kankaanpaa PE, Salminen SJ, Isolauri E, Lee YK (2001). The influence of polyunsaturated fatty acids on probiotic growth and adhesion. *FEMS Microbiol Lett* **194**: 149-153.

Kiarie E, Nyachoti CM, Slominski BA, Blank G (2007). Growth performance, gastrointestinal microbial activity, and nutrient digestibility in early-weaned pigs fed diets containing flaxseed and carbohydrase enzyme. *J Anim Sci* **85**: 2982-2993.

Kim, HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ, Isaacson RE (2011). Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Vet Microbiol* **153**: 124-133.

Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ, Isaacson RE (2012). Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. *Proc Natl Acad Sci USA* **109**: 15485-15490.

Konstantinov, SR, Awati AA, Williams BA, Miller BG, Jones P, Stokes CR, Akkermans AD, Smidt H, de Vos WM (2006). Post-natal development of the porcine microbiota composition and activities. *Environ Microbiol* **8**: 1191-1199.

Konstantinov SR, Smidt H, Akkermans AD, Casini L, Trevisi P, Mazzoni M, De Filippi S, Bosi P, de Vos WM (2008). Feeding of *Lactobacillus sobrius* reduces *Escherichia coli* F4 levels in the gut and promotes growth of infected piglets. *FEMS Microbiol Ecol* **66**: 599-607.

Konstantinov SR, Zhu WY, Williams BA, Tamminga S, de Vos WM, Akkermans ADL (2003). Effect of fermentable carbohydrates on piglet faecal bacterial communities as revealed by

denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. *FEMS Microbiol Ecol* **43**: 225-235.

Kouba M, Enser M, Whittington FM, Nute GR, Wood JD (2003). Effect of a high-linolenic acid diet on lipogenic enzyme activities, fatty acid composition, and meat quality in the growing pig. *J Anim Sci* **81**: 1967-1979.

Lamendella R, Domingo JW, Ghosh S, Martinson J, Oerther DB (2011). Comparative fecal metagenomics unveils unique functional capacity of the swine gut. *BMC Microbiol* **11**: 103.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947-2948.

Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M, Moller K (2002). Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Appl Environ Microbiol* **68**: 673-690.

Ley R.E, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI (2005). Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA* **102**: 11070-11075.

Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Microbiol* **6**:776-788.

Ley RE, Turnbaugh PJ, Klein S, Gordon JI (2006). Microbial ecology: human gut microbes associated with obesity. *Nature* **444**: 1022-1023.

Li L, Schoenhals KE, Brady PA, Estill CT, Perumbakkam S, Craig AM (2012). Flaxseed supplementation decreases methanogenic gene abundance in the rumen of dairy cows. *Animal* **6**: 1784-1787.

Lin B, Gong J, Wang Q, Cui S, Yu H, Huang B (2011). *In-vitro* assessment of the effects of dietary fibers on microbial fermentation and communities from large intestinal digesta of pigs. *Food Hydrocolloid* **25**: 180-188.

Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, Sul WJ, Stedtfeld TM, Chai B, Cole JR, Hashsham SA, Tiedje JM, Stanton TB (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proc Natl Acad Sci USA* **109**: 1691-1696.

Luo YH, Su Y, Wright ADG, Zhang LL, Smidt H, Zhu WY (2012). Lean breed Landrace pigs harbor fecal methanogens at higher diversity and density than obese breed Erhualian pigs. *Archaea* **2012**:605289.

Mao SY, Yang CF, Zhu WY (2011). Phylogenetic analysis of methanogens in the pig feces. *Curr Microbiol* **62**:1386-1389.

Matthews K, Homer D, Thies F, Calder P (2000). Effect of whole linseed (*Linum usitatissimum*) in the diet of finishing pigs on growth performance and on the qualityand fatty acid composition of various tissues. *Brit J Nutr* **83**:637-643.

Monteny GJ, Groenestein CM, Hilhorst MA (2001). Interactions and coupling between emissions of methane and nitrous oxide from animal husbandry. *Nutr Cycl Agroecosys* **60**:123-132.

Muyzer G, de Waal EC, Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**:695-700.

Namkung H, Li M, Gong J, Yu H, Cottrill M, de Lange CFM (2004). Impact of feeding blends of organic acids and herbal extracts on growth performance, gut microbiota and digestive function in newly weaned pigs. *Can J Anim Sci* **84**:697-704.

Nemcová R, Borovská D, Koščová J, Gancarčíková S, Mudroňová D, Buleca V, Pistl J (2012). The effect of supplementation of flax-seed oil on interaction of *Lactobacillus plantarum*–Biocenol™ LP96 and *Escherichia coli* O8: K88ab: H9 in the gut of germ-free piglets. *Res Vet Sci* **93**:39-41.

Nordhoff M, Taras D, Macha M, Tedin K, Busse HJ, Wieler LH (2005). *Treponema berlinense* sp. nov. and *Treponema porcinum* sp. nov., novel spirochaetes isolated from porcine faeces. *Int J Syst Evol Microbiol* **55**:1675-1680.

Page RD 2002. Visualizing phylogenetic trees using TreeView. *Curr Protoc Bioinformatics* 6.2. 1-6.2. 15.

Pedersen R, Andersen AD, Molbak L, Stagsted J, Boye M (2013a). Changes in the gut microbiota of cloned and non-cloned control pigs during development of obesity: gut microbiota during development of obesity in cloned pigs. *BMC Microbiol* **13**:30.

Pedersen R, Ingerslev HC, Sturek M, Alloosh M, Cirera S, Christoffersen BØ, Moesgaard SG, Larsen N, Boye M (2013b). Characterisation of Gut Microbiota in Ossabaw and Göttingen Minipigs as Models of Obesity and Metabolic Syndrome. *PLOS ONE* **8**: e56612.

Puvirajah AS (2012). Quality of western Canadian flaxseed. http://www.grainscanada.gc.ca/flax-lin/harvest-recolte/2012/hqf12-qrl12-eng.pdf. Accessed April 18, 2014.

Romans JR, Wulf DM, Johnson RC, Libal GW, Costello WJ (1995). Effects of ground flaxseed in swine diets on pig performance and on physical and sensory characteristics and omega-3 fatty acid content of pork: II. Duration of 15% dietary flaxseed. *J Anim Sci* **73**:1987-1999.

Saengkerdsub S, Ricke SC (2014). Ecology and characteristics of methanogenic archaea in animals and humans. *Crit Rev Microbiol* **40**:97-116.

Simpson JM, McCracken VJ, White BA, Gaskins HR, Mackie RI (1999). Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. *J Microbiol Methods* **36**:167-179.

Singh KK, Mridula D, Rehal J, Barnwal P (2011). Flaxseed: a potential source of food, feed and fiber. *Crit Rev Food Sci Nutr* **51**:210-222.

Su Y, Bian G, Zhu Z, Smidt H, Zhu W (2014). Early methanogenic colonisation in the faeces of Meishan and Yorkshire piglets as determined by pyrosequencing analysis. *Archaea* **2014:** 547908.

Turner TD, Mapiye C, Aalhus JL, Beaulieu AD, Patience JF, Zijlstra RT, Dugan ME (2014). Flaxseed fed pork: *n*-3 fatty acid enrichment and contribution to dietary recommendations. *Meat Sci* **96**:541-547.

Wang HF, Zhu WY, Yao W, Liu JX (2007). DGGE and 16S rDNA sequencing analysis of bacterial communities in colon content and feces of pigs fed whole crop rice. *Anaerobe* **13**:127-133.

Wegener HC (2003). Antibiotics in animal feed and their role in resistance development. *Curr Opin Microbiol* **6**:439-445.

Wright ES, Yilmaz LS, Noguera DR (2012). DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. *Appl Environ Microbiol* **78**:717-725.

Yu Y, Lee C, Kim J, Hwang S (2005). Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng* **89**:670-679.

Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, Parameswaran P, Crowell MD, Wing R, Rittmann BE, Krajmalnik-Brown R (2009). Human gut microbiota in obesity and after gastric bypass. *Proc Natl Acad Sci USA* **106**:2365-2370.

Zijlstra RT, Beltranena E (2013). Alternative feedstuffs in swine diets. *In*: Sustainable Swine Nutrition. Chiba L., ed. John Wiley & Sons, Hoboken, NJ. p. 231–255.

Chapter 6. General discussion and conclusions

Antibiotics continue to be used in North American swine husbandry for the purpose of growth promotion and disease prevention. This practice continues to cause concern for scientists, medical professionals, and consumers alike for several reasons, but mostly due to their role in creating and maintaining a reservoir of antibiotic-resistant bacteria and resistance determinants (Allen et al., 2013). These resistance determinants may be transferred to pathogenic bacteria leading to treatment failures in human medicine (Levy and Marshall, 2004). This issue is exacerbated by the fact that there are relatively few antibiotics are in the development pipeline and therefore existing drugs must be preserved (Bartlett et al., 2013).

Despite over 60 years of use in agriculture, the exact mechanism(s) responsible for the growth promoting effects of antibiotics remains poorly understood. However, it is generally believed that it is due to a direct impact on the gut microbiota and/or indirect effects related to increased nutrient availability, as germ-free animals do not exhibit growth-rate increases when given antibiotics (Dibner and Richards, 2005). A better understanding of the mechanisms responsible for growth promotion and how antibiotic resistance developments in food animals should help lead to the development of alternatives (Thacker, 2013).

6.1 Experimental approach

The development of culture-independent methods in the past 20 to 25 years has allowed for a broader characterization of the gut microbiota of swine and many other animals (Inglis et al., 2012). In addition, resistance determinants can be detected, monitored, and quantified using molecular methods rather relying solely on phenotypic measures of antibiotic resistance. Instead, complementary studies that combine culture-dependent and -independent methods can give a more comprehensive view of antibiotic resistance in swine. More recently, the

introduction of so-called high throughput sequencing techniques has revolutionized the manner in which the gut microbiota can be characterized (Mardis, 2008). In a single run for example, millions of sequences from a given sample or environment can be generated and analyzed using bioinformatics software (Logares et al., 2012; Shendure and Ji, 2008).

Although several studies have investigated the impact of antibiotics on the swine gut microbiota or on the level of antibiotic resistance determinants or resistant bacteria, none of them have used a monitoring period that includes repeated sampling over the whole duration of the swine production cycle (Kalmokoff et al., 2011; Kim et al., 2012; Looft et al., 2012; Rettedal et al., 2009). To fill this gap in the literature we undertook a 21-week study following and sampling pigs from birth until they were sent for slaughter. We fed pigs either tylosin or chlortetracycline, alongside a control group, at growth promoting (subtherapeutic) levels during this period beginning when the pigs were weaned at four weeks of age. Fecal samples were then taken at times corresponding to specific production phases in the commercial swine industry.

6.2 Effect of subtherapeutic antibiotics on antibiotic resistant anaerobes and antibiotic resistance genes

In our first study, we examined the effect of subtherapeutic tylosin and chlortetracycline on the phenotypic resistance in total anaerobes as well as on the detection of 18 resistance genes commonly found in swine. We also used real-time PCR to quantify changes in the number of copies of some of these genes of interest. We found that pigs that were given a diet that was supplemented with subtherapeutic tylosin exhibited a rapid increase in the proportion of tylosin-resistant anaerobes at weaning (6 wk) as well as in the concentration of *erm*(B), a macrolide resistance gene. Tylosin-fed pigs also had a greater frequency of PCR detection of *erm*(A), *erm*(F), and *erm*(G). Furthermore, despite the dosage of tylosin being halved at week 9 and

again at week 12, the proportion of tylosin-resistant anaerobes did not decrease significantly. Even a two-week drug withdrawal period prior to shipping had no significant effect on tylosin-resistance. The current recommended withdrawal times for specific antibiotics are focused on removing antibiotic residues from meat products rather than reducing antibiotic-resistant bacteria and resistance genes. This is an issue that requires more investigation if the goal is also to reduce antibiotic resistant bacteria and resistance determinants in pork.

In contrast to the results observed with the tylosin-supplemented pigs, chlortetracycline given at 5.5 mg kg^{-1} feed had no significant effect on either chlortetracycline resistance in anaerobes or on the detection frequency of tetracycline resistance genes. Even in pigs that had never been exposed to antibiotics we detected several antibiotic resistance genes and a relatively large proportion of chlortetracycline resistant anaerobes. This likely reflects the long period of use in swine husbandry for chlortetracycline (Viola and DeVincent 2006). All diets also contained at least 110 mg Copper or Zinc kg^{-1} feed in the weaning and starting periods and therefore may have provided sufficient selective pressure for the maintenance of some antibiotic resistance genes which are physically linked with copper and zinc resistance genes. Interestingly, neither of the two antibiotic groups experienced an increase in growth rate at any time, despite this being the number one reason that antibiotics are given to livestock (Deckert et al 2010). This may have been due to the fact that the McGill swine complex is relatively clean compared to larger, commercial operations or possibly that pigs that are grown under the conditions of modern farming practices don't respond the same way to antibiotics (McEwen and Fedorka-Cray 2002). Also the pigs in this study were healthy without any subclinical disease or other ailments.

6.3 Impact of subtherapeutic antibiotics on the swine gut microbiota

We also used these same samples to perform a high throughput sequencing study of the effect of subtherapeutic levels of antibiotics on the swine gut microbiota. It was determined that subtherapeutic tylosin causes significant changes in the relative abundance of specific taxa as well as OTUs. Most of the major shifts in taxa abundance tended to be transient rather than permanent. It is therefore possible that pigs eventually attain a climax community regardless of antibiotic or dietary perturbations (Carney-Hinkle et al., 2013). Similar to our findings in the first study, chlortetracycline did not cause as many alterations in the gut microbiota as tylosin did. This may have been dose dependent, as only 5.5 mg chlortetracycline kg^{-1} feed was administered compared to 44 mg tylosin kg^{-1} feed at weaning.

Suckling piglets were found to have significantly different microbial community structures in terms of taxa and OTU abundance when compared with weanling piglets and subsequent sampling times. This finding was somewhat expected given the large differences in the type of diet they were consuming (milk vs. plant-based feed), as well as physiological factors that are unique to the suckling piglet such as an immature immune system and a lower pH in certain parts of the intestinal tract (Bailey et al., 2005).

6.4 Effect of extruded flaxseed on the swine gut microbiota

We also looked at the effect of extruded flaxseed on the swine gut microbiota using a different group of pigs from the antibiotic experiments. Flaxseed is one of the richest sources of α-linolenic acid, which is an essential ω-3 fatty acid with reported human health benefits (Barceló-Coblijn and Murphy 2009). For this reason it has been used by researchers to increase the ω-3 fatty acid content of pork (Kouba et al., 2003; Matthews et al., 2000). Due to the issues surrounding antibiotics discussed in this thesis, there is a desire to find antibiotic alternatives that mimic the effect they have in the gut. Flaxseed has been suggested as a viable alternative in

swine feed (Zijlstra and Beltranena, 2013). When we analyzed the impact of three different concentrations of extruded flaxseed on the swine gut microbiota using DGGE, we could not detect any differences in the band patterns. The samples tended to cluster together by the time of sampling rather than by diet. Furthermore, the proportion of the two major phyla, *Firmicutes* and *Bacteroidetes*, were not altered by the extruded flaxseed inclusion. This again demonstrates the difficulty in causing large shifts in the swine gut microbiota through dietary modification. There were no negative effects on swine performance in this study so extruded flaxseed may have a role in creating value added pork with increased ω-3 fatty acid content.

6.5 Conclusions

In conclusion, it was demonstrated that tylosin at subtherapeutic levels not only increases the proportion of phenotypic tylosin resistance in culturable anaerobes but also increases the number and quantify of macrolide resistance genes. Tylosin also causes identifiable shifts in the entire gut microbiota at both the taxa and OTU levels but many of these changes are temporary. Once established, the swine gut microbiota demonstrates resilience to dietary changes and appears to form a relatively stable community post-weaning.

6.6 Future study

Understanding how antimicrobial resistance develops in swine production is important from a public health perspective. This knowledge is also necessary for the swine industry as antimicrobial withdrawal times and dosing schedules may be altered based on this information. In this thesis, the effect of two antibiotics at subtherapeutic levels on antibiotic resistance and on the gut microbiota was determined, as was the effect of a two-week withdrawal period. Future work could instead measure the impact of therapeutic doses of these antibiotics given for a shorter period of time. While a two-week withdrawal period was insufficient to significantly

reduce tylosin-resistant bacteria and the concentration of *erm*(B), it remains to be seen if a longer withdrawal period for subtherapeutic tylosin would result in a greater decrease in tylosin resistance. Furthermore, a metagenomic study of pigs given subtherapeutic doses of tylosin and chlortetracycline might also reveal other changes in antibiotic resistance genes that weren't investigated in this thesis. Lastly, high throughput sequencing techniques similar to those employed in chapter 4 of this thesis could reveal more subtle changes caused by extruded flaxseed supplementation.

Allen HK, Levine UY, Looft T, Bandrick M, Casey TA (2013). Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends Microbiol* **21:**114-119.

Bailey M, Haverson K, Inman C, Harris C, Jones P, Corfield G et al. (2005). The development of the mucosal immune system pre- and post-weaning: balancing regulatory and effector function. *Proc Nutr Soc* **64:**451-457.

Barceló-Coblijn G, Murphy EJ (2009). Alpha-linolenic acid and its conversion to longer chain *n*-3 fatty acids: Benefits for human health and a role in maintaining tissue *n*-3 fatty acid levels. *Prog Lipid Res* **48:**355-374.

Bartlett JG, Gilbert DN, Spellberg B (2013). Seven ways to preserve the miracle of antibiotics. *Clin Infect Dis* **56:**1445-1450.

Carney-Hinkle EE, Tran H, Bundy JW, Moreno R, Miller PS, Burkey TE (2013). Effect of dam parity on litter performance, transfer of passive immunity, and progeny microbial ecology. *J Anim Sci* **91:**2885-2893.

Deckert A, Gow S, Rosengren L, Leger D, Avery B, Daignault D et al. (2010). Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) Farm Program: results from finisher pig surveillance. *Zoonoses Public Health* **57 Suppl 1:**71-84.

Dibner JJ, Richards JD (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poult Sci* **84:**634-643.

Inglis GD, Thomas MC, Thomas DK, Kalmokoff ML, Brooks SP, Selinger LB (2012). Molecular methods to measure intestinal bacteria: a review. *J AOAC Int* **95:**5-23.

Kalmokoff M, Waddington L, Thomas M, Liang KL, Ma C, Topp E et al. (2011). Continuous feeding of antimicrobial growth promoters to commercial swine during the growing/finishing phase does not modify faecal community erythromycin resistance or community structure. *J Appl Microbiol* **110:**1414-1425.

Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ et al. (2012). Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. *Proc Natl Acad Sci USA* **109:** 15485-15490.

Kouba M, Enser M, Whittington FM, Nute GR, Wood JD (2003). Effect of a high-linolenic acid diet on lipogenic enzyme activities, fatty acid composition, and meat quality in the growing pig. *J Anim Sci* **81:** 1967-1979.

Levy SB, Marshall B (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med* **10:** S122-129.

Logares R, Haverkamp TH, Kumar S, Lanzén A, Nederbragt AJ, Quince C et al. (2012). Environmental microbiology through the lens of high-throughput DNA sequencing: synopsis of current platforms and bioinformatics approaches. *J Microbiol Methods* **91:** 106-113.

Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD et al. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proc Natl Acad Sci USA* **109:** 1691-1696.

Mardis ER (2008). The impact of next-generation sequencing technology on genetics. *Trends Genet* **24:** 133-141.

Matthews K, Homer D, Thies F, Calder P (2000). Effect of whole linseed (Linum usitatissimum) in the diet of finishing pigs on growth performance and on the qualityand fatty acid composition of various tissues. *Br J Nutr* **83:** 637-643.

McEwen SA, Fedorka-Cray PJ (2002). Antimicrobial use and resistance in animals. *Clin Infect Dis* **34:** S93-S106.

Rettedal E, Vilain S, Lindblom S, Lehnert K, Scofield C, George S et al. (2009). Alteration of the ileal microbiota of weanling piglets by the growth-promoting antibiotic chlortetracycline. *Appl Environ Microbiol* **75:** 5489-5495.

Shendure J, Ji H (2008). Next-generation DNA sequencing. *Nat Biotechnol* **26:** 1135-1145.

Thacker PA (2013). Alternatives to antibiotics as growth promoters for use in swine production: a review. *J Anim Sci Biotechnol* **4:** 35.

Viola C, DeVincent S (2006). Overview of issues pertaining to the manufacture, distribution, and use of antimicrobials in animals and other information relevant to animal antimicrobial use data collection in the United States. *Prev Vet Med* **73:** 111-131.

Zijlstra RT, Beltranena E (2013). Alternative feedstuffs in swine diets. *In*: Sustainable Swine Nutrition. Chiba L., ed. John Wiley & Sons, Hoboken, NJ. p. 231–255.

Appendices

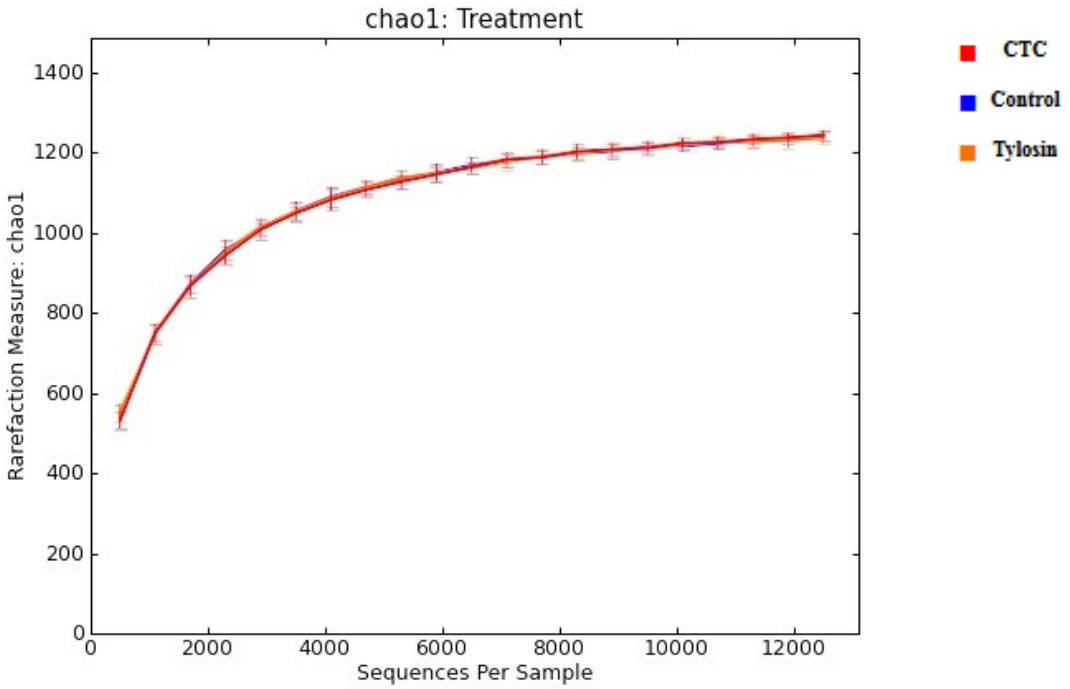
Appendix 1.

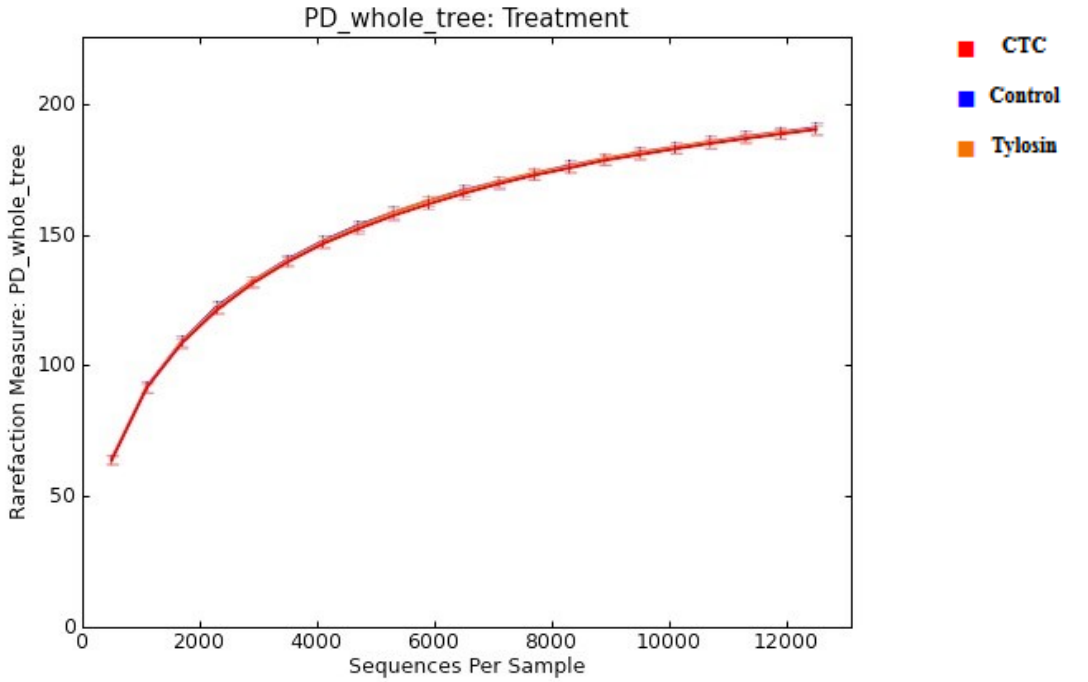
Composition of feeds used in Chapters 3 and 4, not including antibiotics. Weanling pigs received approximately 0.6 kg feed/d, starter pigs 1.0 kg feed/d, and growing-finish pigs 2.0 kg feed/d (Agribrands-Purina, St-Hubert, QC, Canada).

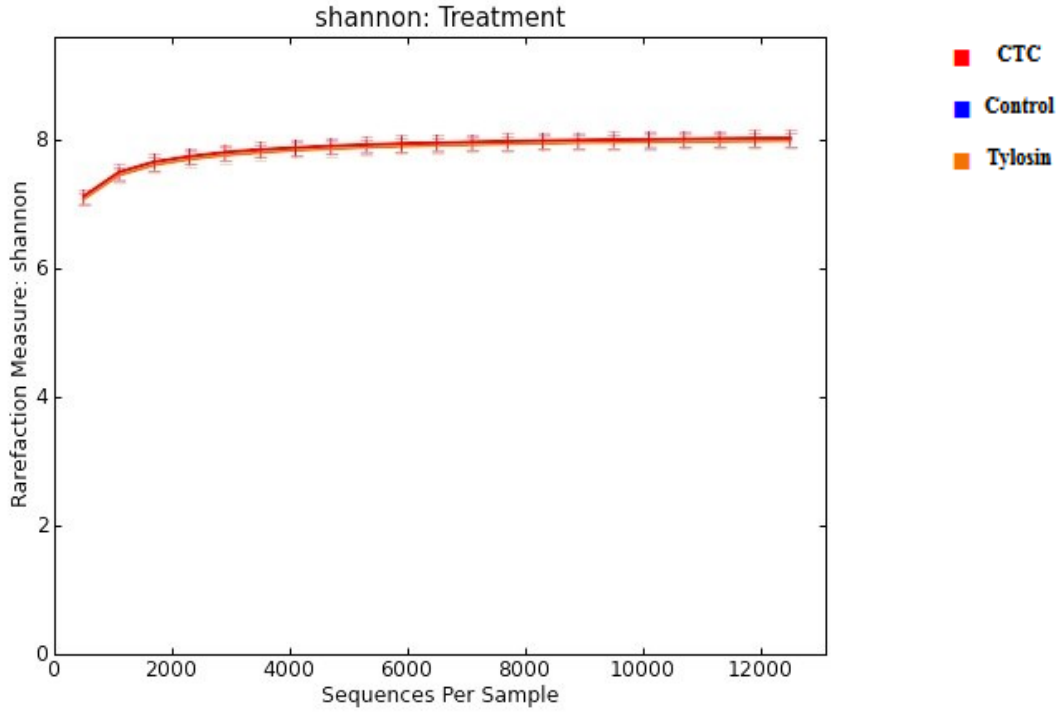
Ingredient	Weaning	Starting	Growing-finishing
Proximate composition (%)			
Crude protein	≥ 16.0	≥ 15.0	≥ 15.5
Crude fat	≥ 2.0	≥ 5.0	≥ 2.0
Crude fibre	≤ 8.0	≥ 5.0	≥ 7.0
Minerals (mg kg^{-1})			
Copper	125	125	20
Zinc	200	110	100
Selenium	0.26	0.29	0.26
Calcium	0.60	0.63	0.65
Phosphorous	0.50	0.50	0.50
Sodium	0.20	0.20	0.20
Vitamin (IU kg^{-1})			
A	≥ 9000	≥ 5400	≥ 4500
D_3	≥ 1500	≥ 1200	≥ 1000
E	≥ 60	≥ 36	≥ 30

Appendix 2. Rarefaction curves for chao1, PD whole tree, and Shannon indices for A) treatment and B) production phase. For A) treatment n=6. CTC = chlortetracycline. B) Phase n=18, sows: n=4 (each sow was sampled once prior to farrowing and after farrowing). Suckling (3 wk), weanling (6 wk), starting (9 wk), growing (12 wk), and finishing (19 wk). Please note that for the Shannon index the values presented are log base 2 values rather than the natural log values used in the text of the thesis.

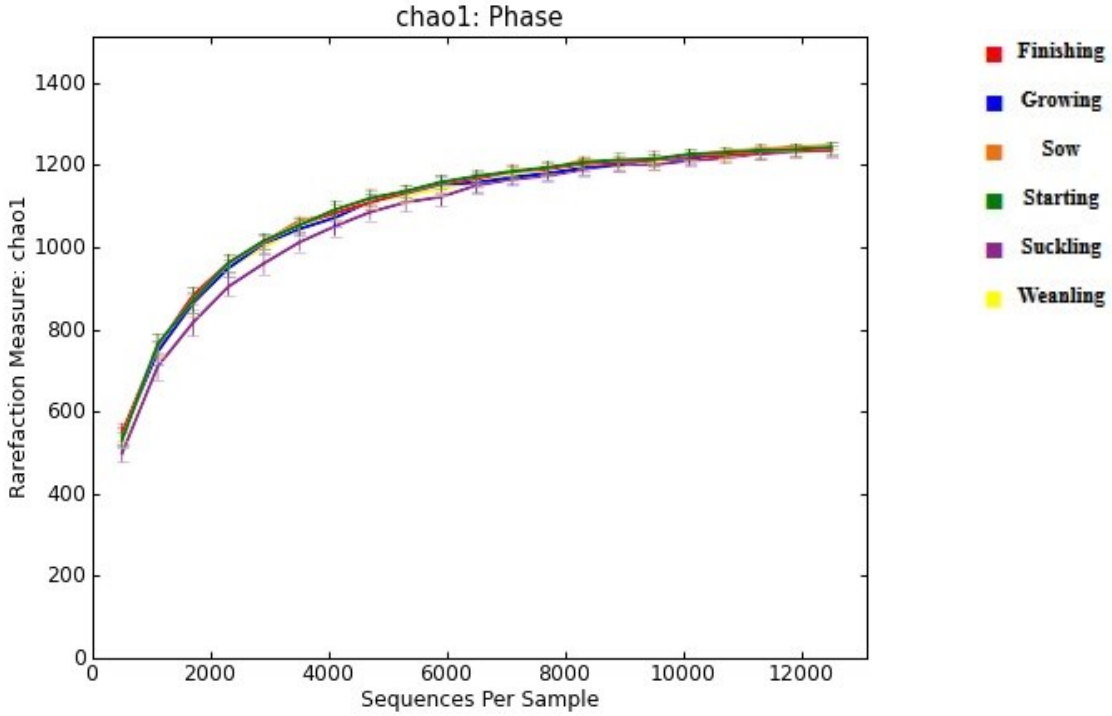
A)

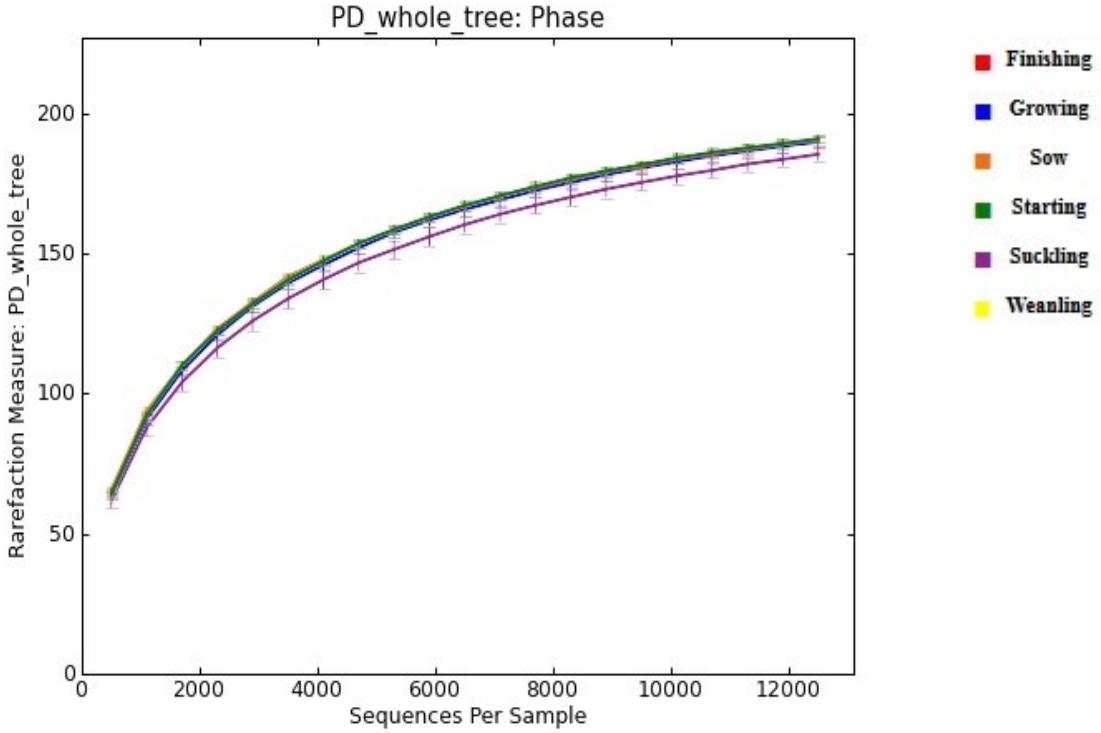



PD_whole_tree: Treatment
Rarefaction Measure: PD_whole_tree
Sequences Per Sample
0
50
100
150
200
0
2000
4000
6000
8000
10000
12000
CTC
Control
Tylosin


shannon: Treatment
Rarefaction Measure: shannon
Sequences Per Sample
0
2
4
6
8
0
2000
4000
6000
8000
10000
12000
CTC
Control
Tylosin

B)


chao1: Phase
Rarefaction Measure: chao1
Sequences Per Sample
0
200
400
600
800
1000
1200
1400
0
2000
4000
6000
8000
10000
12000
Finishing
Growing
Sow
Starting
Suckling
Weanling


PD_whole_tree: Phase
Rarefaction Measure: PD_whole_tree
Sequences Per Sample
0
50
100
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4000
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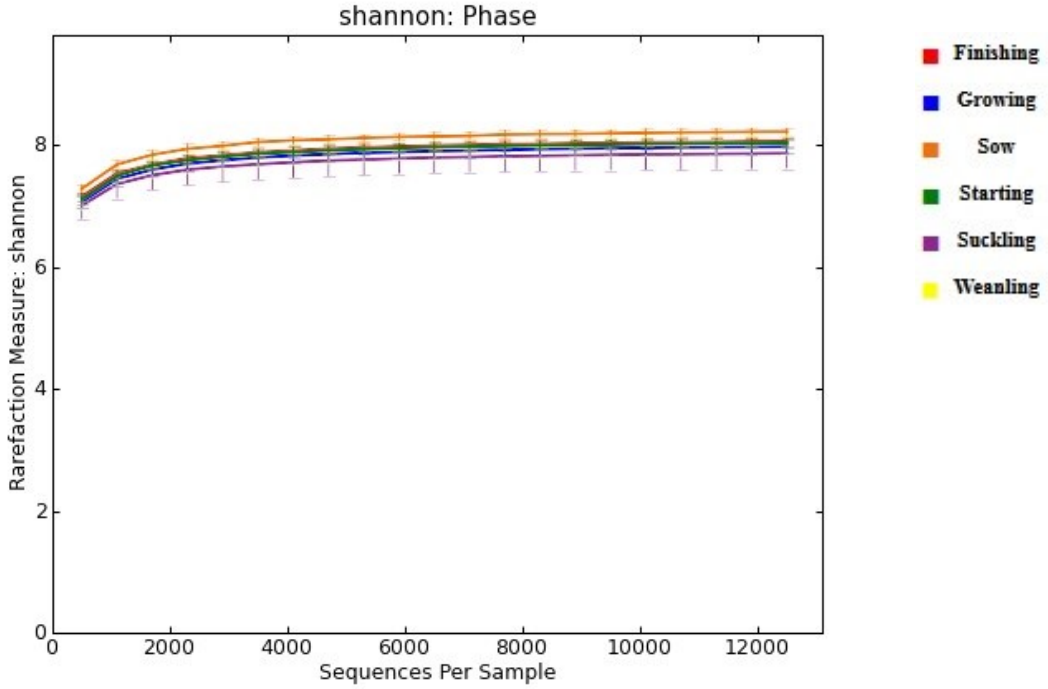
8000
10000
12000
Finishing
Growing
Sow
Starting
Suckling
Weanling


shannon: Phase
Rarefaction Measure: shannon
Sequences Per Sample
0
2
4
6
8
0
2000
4000
6000
8000
10000
12000
Finishing
Growing
Sow
Starting
Suckling
Weanling

Appendix 3. Differentially abundant OTUs between suckling and weanling piglets (n=18). False discovery rate (FDR) <0.05.

OTU Name	Control (mean abundance)	Tylosin (mean abundance)	FDR	RDP Classifier Consensus Lineage
More abundant in the suckling piglets				
denovo8	417.44	100.67	0.0022	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;sfragilis
denovo15	313.28	62.89	0.0022	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
denovo40	184.06	40.11	0.0003	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Odoribacteraceae];g_Butyricimonas_
denovo24	175.39	34.94	0.0022	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo29	164.11	36.44	0.0005	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo34	161.44	33.39	0.0007	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo37	132.72	30.50	0.0474	k__Bacteria;p__Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Phascolarctobacterium;
denovo45	131.17	46.28	0.0006	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Desulfovibrio
denovo50	121.89	25.50	0.0151	k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__Campylobacter
denovo96	117.61	25.44	0.0003	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae
denovo117	113.28	23.22	0.0006	k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae;g__Synergistes
denovo56	81.11	26.17	0.0067	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo69	78.67	18.56	0.0235	k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae;g__Pyramidobacter;s__piscolens
denovo90	64.06	13.44	0.0024	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyromonadaceae;g_Parabacteroides
denovo101	45.06	10.94	0.0022	k__Bacteria;p__Firmicutes;c__Clostridia;o_Clostridiales
denovo172	44.11	8.28	0.0005	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Bilophila
denovo114	43.61	11.56	0.0183	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae
denovo126	38.17	7.78	0.0052	k__Archaea;p__Euryarchaeota;c_Thermoplasmata;o_E2;f_[Methanomassiliicoccaceae];g_vadinCA11
denovo154	34.50	7.22	0.0283	k__Bacteria;p__Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae;g_Sutterella;
denovo135	30.17	10.83	0.0240	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo204	28.61	7.78	0.0005	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides;s__distasonis

denovo169	27.44	8.06	0.0362	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo242	25.94	6.22	0.0005	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus];s__gnavus
denovo176	24.61	11.72	0.0183	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo173	22.78	6.78	0.0141	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo184	22.00	6.17	0.0290	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
denovo179	21.89	7.78	0.0111	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo199	20.83	5.78	0.0047	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
denovo260	20.78	4.17	0.0024	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo215	16.83	3.94	0.0039	k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Victivallales;f__Victivallaceae
denovo230	16.00	5.00	0.0018	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
denovo354	15.22	2.28	0.0005	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__uniformis
denovo271	13.06	2.89	0.0416	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo289	12.94	2.33	0.0207	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo282	12.50	2.61	0.0019	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae];g_Odoribacter
denovo677	12.00	5.50	0.0463	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
denovo21923	11.56	3.06	0.0284	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
denovo338	10.67	4.17	0.0102	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo3545	10.28	1.61	0.0237	k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae;g__Methanobrevibacter
denovo308	10.00	3.11	0.0085	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira
denovo263361	8.83	1.39	0.0070	k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__Campylobacter
denovo313	8.78	3.39	0.0343	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
denovo573	8.50	1.44	0.0086	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo331	8.33	2.11	0.0068	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo7837	8.11	0.83	0.0005	k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae
denovo359	7.67	2.44	0.0329	k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Helicobacteraceae;g__Flexispira;s__rappini
denovo439	7.50	1.56	0.0029	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira
denovo369	7.39	2.22	0.0112	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo485	7.11	1.67	0.0063	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo462	6.67	1.28	0.0029	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo256317	6.56	1.50	0.0064	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo154733	6.44	1.11	0.0031	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o_Bacteroidales;f__[Odoribacteraceae];g_Butyricimonas
denovo705	6.39	1.28	0.0023	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo3744	6.33	1.72	0.0365	k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae;g__Synergistes

denovo495	5.89	1.33	0.0024	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Actinobacillus
denovo165847	5.89	1.61	0.0141	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo515	5.67	0.78	0.0041	k__Bacteria;p__Fusobacteria;c_Fusobacteriia;o_Fusobacteriales;f_Fusobacteriaceae;g_Fusobacterium
denovo1254	5.28	1.17	0.0060	k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae
denovo316607	5.11	1.11	0.0104	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo459	5.06	1.28	0.0204	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira
denovo751	5.00	1.28	0.0283	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo448	4.72	1.06	0.0463	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o_Bacteroidales;f_[Odoribacteraceae];g_Butyricimonas
denovo275795	4.56	1.00	0.0204	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo134939	4.50	1.56	0.0284	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
denovo1645	4.44	1.28	0.0156	k__Bacteria;p__Firmicutes;c__Clostridia;o_Clostridiales;f__Lachnospiraceae;g__[Ruminococcus];s__gnavus
denovo475	4.39	1.11	0.0450	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo265065	4.28	0.89	0.0141	k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae;g__Synergistes
denovo1435	4.22	1.67	0.0253	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__fragilis
denovo192266	4.17	1.06	0.0134	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo504	4.11	1.11	0.0039	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae
denovo2817	3.94	0.44	0.0022	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
denovo308126	3.94	0.89	0.0029	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;__Bacteroidales;f_[Odoribacteraceae];g_Butyricimonas
denovo1098	3.83	0.50	0.0022	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
denovo1059	3.61	1.06	0.0126	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo163418	3.61	1.00	0.0283	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides;s__distasonis
denovo219347	3.56	0.72	0.0013	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae
denovo1310	3.56	1.83	0.0467	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo582	3.11	0.61	0.0116	k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Victivallales;f__Victivallaceae
denovo727	3.11	0.72	0.0407	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo816	2.78	0.39	0.0025	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides__
denovo30083	2.67	0.78	0.0493	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
denovo600	2.56	1.22	0.0260	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo2284	2.39	0.94	0.0467	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o_Bacteroidales;f__Bacteroidaceae;g_Bacteroides
denovo7168	1.94	0.72	0.0463	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
denovo8	417.44	100.67	0.0022	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_fragilis
denovo15	313.28	62.89	0.0022	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
denovo40	184.06	40.11	0.0003	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o_Bacteroidales;f_[Odoribacteraceae];g_Butyricimonas

denovo24	175.39	34.94	0.0022	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo29	164.11	36.44	0.0005	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo34	161.44	33.39	0.0007	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
denovo37	132.72	30.50	0.0474	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Phascolarctobacterium
More abundant in weanling piglets				
denovo0	306.17	577.72	0.0283	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g_Lactobacillus
denovo2	311.50	550.06	0.0005	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s__copri
denovo1	243.94	374.78	0.0308	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o_Aeromonadales;__Succinivibrionaceae;g_Succinivibrio
denovo13	181.00	275.50	0.0063	k__Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus;s__reuteri
denovo6	157.22	271.94	0.0006	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo4	171.17	221.11	0.0077	k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
denovo7	127.06	199.67	0.0005	k__Bacteria;p__Firmicutes;c_Clostridia;o_Clostridiales;f__Veillonellaceae;g_Phascolarctobacterium
denovo48	85.22	127.44	0.0022	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia
denovo16	79.83	120.33	0.0379	k__Bacteria;p__Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus;s_luteciae
denovo17	59.28	115.11	0.0005	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo43	81.72	113.61	0.0156	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo19	58.28	104.83	0.0146	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo25	50.44	95.17	0.0134	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia;s__producta
denovo23	48.61	80.94	0.0015	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__CF231
denovo44	40.67	67.67	0.0283	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales
denovo53	27.61	63.94	0.0116	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo82	30.78	60.94	0.0064	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o_Bacteroidales;f_[Paraprevotellaceae];g_[Prevotella]
denovo78	28.89	59.94	0.0024	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Anaerovibrio
denovo64	15.39	54.94	0.0234	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo65	29.67	49.11	0.0077	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia
denovo102	21.72	49.00	0.0072	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo47	26.67	45.06	0.0051	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo120	24.28	43.83	0.0022	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium;s__prausnitzii
denovo70	24.22	41.28	0.0017	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Dorea
denovo60	29.89	40.11	0.0216	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo85	12.83	39.33	0.0052	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo109	26.00	38.83	0.0283	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia
denovo52	19.39	35.00	0.0031	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo54	20.78	33.83	0.0350	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella

denovo94	20.72	32.72	0.0086	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
denovo1149	19.17	30.83	0.0012	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
denovo76	15.94	30.11	0.0283	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo136	16.61	27.67	0.0012	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo75	16.22	27.50	0.0284	k__Bacteria;p__Spirochaetes;c_Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
denovo214	18.67	27.06	0.0029	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
denovo107	11.17	26.33	0.0006	k__Bacteria;p__Firmicutes;c__Clostridia;o_Clostridiales;f__Peptostreptococcaceae
denovo146	9.11	24.94	0.0017	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f_Lachnospiraceae;g_Coprococcus;s_catus
denovo95	13.11	22.67	0.0283	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g_[Prevotella]
denovo348	15.78	21.28	0.0210	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
denovo123	15.00	20.22	0.0463	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira
denovo10217	11.67	20.11	0.0252	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
denovo180	9.67	18.89	0.0028	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus
denovo884	11.83	18.39	0.0216	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
denovo161	6.83	16.50	0.0467	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
denovo140	10.22	16.39	0.0357	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
denovo138	8.72	15.28	0.0308	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
denovo431	8.67	14.67	0.0183	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s__copri
denovo129	9.56	14.28	0.0293	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales
denovo222	4.11	13.67	0.0141	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium; s__prausnitzii
denovo297	3.06	13.39	0.0141	k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
denovo323	3.44	12.44	0.0237	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus
denovo217	7.22	11.89	0.0072	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae
denovo299	6.83	10.94	0.0401	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
denovo290	5.83	10.61	0.0156	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia
denovo616	4.22	10.17	0.0401	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
denovo227	5.72	9.94	0.0077	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus;s_ _bromii
denovo253	3.61	9.94	0.0066	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia
denovo212	6.56	9.83	0.0383	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Lachnospira
denovo245	4.94	9.17	0.0051	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus
denovo249	3.50	9.11	0.0012	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae
denovo150	29.89	9.00	0.0034	k__Bacteria;p__Firmicutes;c_Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__p-75-a5
denovo264	5.94	9.00	0.0401	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
denovo487	5.28	8.83	0.0283	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo356	4.11	8.78	0.0201	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
denovo386	2.89	8.39	0.0141	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae

denovo452	3.94	7.56	0.0225	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae
denovo526	3.83	7.50	0.0071	k__Bacteria;p__Proteobacteria;c_Gammaproteobacteria;o_Aeromonadales;f_Succinivibrionaceae;g__Succinivibrio
denovo269	3.22	7.22	0.0044	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo336	2.94	7.17	0.0204	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
denovo277	3.11	6.67	0.0308	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo976	4.11	6.44	0.0336	k__Bacteria;p__Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus;s_vaginalis
denovo392	3.06	6.33	0.0416	k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
denovo528	2.61	6.22	0.0060	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
denovo595	1.89	6.22	0.0095	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae
denovo481	1.28	6.17	0.0032	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus
denovo446	2.72	6.11	0.0024	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
denovo404	2.22	6.06	0.0024	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo401	3.28	5.89	0.0357	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
denovo353	2.67	5.89	0.0216	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira
denovo281	2.83	5.78	0.0459	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
denovo599	1.56	5.33	0.0094	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo503	1.39	4.89	0.0051	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
denovo508	2.50	4.83	0.0450	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
denovo992	2.50	4.78	0.0151	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
denovo399	2.17	4.78	0.0260	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo345	2.06	4.72	0.0463	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae]
denovo418	1.39	4.56	0.0183	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo805	2.33	4.44	0.0407	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Anaerovibrio
denovo295	1.72	4.33	0.0202	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo683	1.17	4.33	0.0006	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
denovo601	2.22	4.00	0.0401	k__Bacteria;p__Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus;s_vaginalis
denovo377	1.67	3.72	0.0401	k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae
denovo8451	1.72	3.61	0.0141	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o_Bacteroidales;f__Prevotellaceae;g_Prevotella;s_copri
denovo31189	1.72	3.61	0.0379	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo454	0.78	3.61	0.0024	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Succinivibrionaceae
denovo876	1.72	3.39	0.0401	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__SMB53
denovo505	1.50	3.39	0.0283	k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
denovo98336	1.22	3.28	0.0401	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae]
denovo686	1.78	3.00	0.0497	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
denovo1859	1.06	3.00	0.0454	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia;s__faecis
denovo453	1.00	3.00	0.0094	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo1327	0.67	3.00	0.0031	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales

denovo45146	1.56	2.94	0.0329	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f_Veillonellaceae;g_Phascolarctobacterium
denovo868	0.94	2.89	0.0084	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia;s__producta
denovo1887	1.33	2.83	0.0497	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
denovo2082	1.11	2.83	0.0191	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo603	1.39	2.67	0.0279	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo840	1.22	2.61	0.0283	k__Bacteria;p__Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae
denovo663	0.72	2.61	0.0250	k__Bacteria
denovo1058	0.61	2.56	0.0373	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia
denovo1834	1.11	2.44	0.0416	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
denovo1439	0.72	2.44	0.0401	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo651	1.00	2.39	0.0362	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
denovo2238	0.78	2.39	0.0035	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__CF231
denovo652	0.61	2.33	0.0296	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
denovo2041	0.72	2.17	0.0047	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__CF231
denovo885	0.83	2.11	0.0308	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
denovo308995	0.56	2.11	0.0116	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
denovo593	0.78	2.06	0.0416	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium
denovo636	0.39	2.06	0.0024	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g__Prevotella;s__copri
denovo3066	0.94	1.94	0.0382	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae]
denovo259624	0.61	1.94	0.0295	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Anaerovibrio
denovo1315	0.44	1.83	0.0183	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium
denovo1519	0.78	1.78	0.0497	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f_Veillonellaceae;g_Phascolarctobacterium
denovo1097	0.50	1.78	0.0060	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7
denovo74331	0.61	1.72	0.0213	k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
denovo12483	0.39	1.72	0.0131	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacill
denovo1543	0.56	1.67	0.0348	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira
denovo2373	0.56	1.50	0.0459	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium;s__prausnitzii
denovo157837	0.44	1.39	0.0463	k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
denovo397336	2.78	0.61	0.0283	k__Bacteria;p__Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Odoribacteraceae];g_Butyricimonas

Appendix 4.

The concentration of the three most abundant resistance genes in Chapter 3 were also measured using the samples in Chapter 5 and the real-time PCR conditions found in section 3.3.9. It was determined that extruded flaxseed had no significant effect ($p>0.05$) on the concentration of A) *tet*(O), B) *tet*(Q), or C) *erm*(B). CON=control, FLAX5= 5% extruded flaxseed, FLAX10= 10% extruded flaxseed, and FLAX15= 15% extruded flaxseed. Error bars represent standard deviation of the mean (n=5) and different lowercase letters indicate significant differences between groups.