THE ROLE OF ANTIBIOTICS METAPHYLAXIS ON DEVELOPMENTAL DYNAMICS OF FECAL MICROBIOTA AND PERSISTENCE OF ANTIMICROBIAL RESISTOME IN PIGLETS

BY

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THESIS

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ABSTRACT

The swine gastrointestinal microbiota is comprised of a diverse and complex microbial population that coexists in a coordinated, complex mucosal ecosystem that contributes to host gastrointestinal and immunological development. While antimicrobial are cost-effective tools for prevention and treatment of infectious diseases, the impact of their use on potentially beneficent mucosal microbial communities has not been widely explored. Optimization of antimicrobial use in swine management systems requires full understanding of antimicrobial-induced changes on developmental dynamics of gut microbiota and prevalence of antimicrobial resistance genes (ARGs). While the antibiotic resistance profiles of pathogens have been characterized, the antimicrobial resistance bacteria and ARGs from the whole gut microbiota have received far less attention.

The objective of this study was to characterize the impact of parenteral antibiotics administration on composition and diversity of the resident fecal microbiota in pigs. In commercial swine farm, five antimicrobial treatment groups, each consisting of four, eight-week old piglets, were administered one of the antimicrobial; Tulathromycin (TUL), Ceftiofur Crystalline free acid (CCFA), Ceftiofur hydrochloride (CHC), Oxytetracycline (OTC), and Procaine Penicillin G (PPG) at label dose and route. Individual fecal swabs were collected immediately before antimicrobial administration (control = day 0), and again on days 1, 3, 7, and 14 after dosing. Additionally, a randomized complete block design was used to study the impacts of early-life antimicrobial intervention on fecal microbiota structure, and their accompanying antimicrobial resistome in neonatal piglets. Forty-eight litters were blocked to one of six treatments; Control (CONT), TUL, CCFA, CHC, OTC and PPG. Two piglets per litter were individually identified and weights and deep fecal swabs were collected at days 0 (prior to treatment), 5, 10, 15 and 20. All fecal swabs were processed for DNA extraction. Sequencing analysis of the V3-V4 hypervariable region of 16S rRNA gene and the selected ARGs were performed using Illumina Miseq platform. Moreover, whole genome, metagenomics sequencing approach was performed on subset of samples from the CONT and TUL groups, to assess the fecal microbiota structure and their accompanying antimicrobial resistome.

In growing piglets, the most abundant phyla were *Firmicutes*, *Bacteroidetes,* and *Proteobacteria.* Linear discriminant analysis, showed a pronounced, antimicrobial-dependent

shift in the composition of fecal microbiota over time from day 0. By day 14, the fecal microbial compositions of the groups receiving CHC and TUL had returned to a distribution that closely resembled that observed on day 0, but differences were still evident. In contrast, animals that received PPG, OTC and CCFA, showed a tendency towards a normalized microbiota structure on day 7, but appeared to deviate away from the day 0 composition by day 14.

In neonatal piglets, our results show that, while early-life antibiotics prophylaxis had no effect on individual weight gain, or mortality, it was associated with noticeable changes in the prevalence of selected ARGs, and minor shift in the composition of the fecal microbiota during this developmental stage. Relative to CONT, only TUL treated piglet exhibited significant decline in chao1 richness index at day 20. Compared to the CONT, the PPG treated piglets exhibited a significant increase in the prevalence of *ermB* and *tetW* at day 20 of life. Tulathromycin intervention was also resulted in significant increase in the abundance of *tet W* at days 10 and 20, and *erm B* at day 20. Using whole genome metagenomics sequencing on subset of samples from the CONT and TUL groups, a total of 127 antimicrobial resistome related to 19 different classes of antibiotics were identified across all samples. The majority of identified antimicrobial resistome were observed in both experimental groups and at all-time points. The magnitude and extent of differences in microbial composition, and antimicrobial resistome, between the TUL and CONT groups were statistically insignificant. However, both the fecal microbiota composition and antimicrobial resistome were changed significantly between the sampling days.

Based on our results, the observed changes in fecal microbiota in growing piglets showed antimicrobial-specific variation in both duration and extent. While in the perinatal piglets, the shifts in fecal microbiota structure caused by perinatal antimicrobial intervention are modest and limited to particular groups of microbial taxa. However, early life PPG and TUL intervention could promote selection of ARGs in herds. While additional investigations are required to explore the consistency of these findings across larger populations, these results could open the door to new perspectives on the utility of early life antimicrobial administration to healthy neonates in swine management systems.

This dissertation is dedicated to: ✍

My mother, my father, my brother, my sisters, my wife Radwa and my lovely daughters,

Nouran and Sandy

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CHAPTER 1
LITERATURE REVIEW

1.1 INTRODUCTION

The swine GI microbiota is composed of rich and diverse ecosystem that harbors thousands of different microbial species belonging to *Firmicutes*, *Bacteroidetes,* and *Proteobacteria* (Holman et al., 2017). Importantly, swine GI microbiota has a wide range of functions including; providing colonization resistance against potential pathogens, absorbing different kinds of nutrients, modulation of host immune system, metabolizing indigestible polysaccharides, and regulating host metabolism (Venable et al., 2016). The hemostatic steady physiological balance of swine GI microbiota can be disrupted by many factors, namely host genetic, maternal microbiome, age, housing, surrounding environment, dietary habits, pathogen exposure, use of feed additives, and antimicrobial intervention (Holman and Chénier, 2015) (Marchesi et al., 2016).

In modern swine breeding conditions, antimicrobial are commonly prescribed for neonatal and growing populations (Papatsiros, 2013). Despite the ban on antimicrobial, antibiotics are still used in either therapeutic and/or and sub-therapeutic concentration to prevent swine GI infections (Gresse et al., 2017). The antibiotic induced dysbiosis of the resident swine GI microbiota has profound impacts on swine health throughout life (Holman and Chénier, 2014). Equally important, the potential enrichment of antibiotic resistome between the homeostatic resident GI microbiota become one of the most important public health issues that we face currently (Wright, 2007), as it reduces efficacy and increases cost of clinical treatment (Woolhouse et al., 2015). Until recently, the impact of antibiotic intervention on the GI microbiota and their accompanying antibiotic resistome was largely characterized by traditional culture-based techniques or PCR-based approach, which underestimate novel resistance genes (Zhu et al., 2013). Consequently, culture independent platforms (real-time PCR quantification, functional metagenomics or next generation sequencing), have been used to efficiently quantify and assess the antimicrobial resistance and the antibiotic resistome (Gerzova et al., 2015). The response of resident microbiota and their accompanying antibiotic resistome to antibiotics intervention can be affected by many factors, including treatment duration, drug spectrum, dosage, and administration route, as well as resident microbiota composition and antimicrobial

susceptibility (Holman and Chénier, 2014). While it is clear that antibiotics intervention disrupts the GI microbiota, we are just beginning to estimate the relative contribution of its use to the resident mucosal communities and their accompanying antibiotic resistome.

In this review, we present an overview of the antibiotics use in swine production and its association with emergence of antimicrobial resistance. Additionally, we review the current understanding of the swine GI microbiota, the factors that influence its development, and its role in swine health. Finally, we explore the effects of antibiotics on the swine GI microbiota and their accompanying antibiotic resistome. The presented data is critical to the development of robust non-antibiotic alternative strategies to restore GI microbiota functionality and guarantee effective continued use of antimicrobial in livestock production.

1.2 ANTIMICROBIAL USE IN SWINE MANGEMENT SYSTEM

Since the discovery of antimicrobial, they have proven to be exceptionally efficacious against a broad range of bacterial populations (Macdonald and Mcbride, 2009). It is therefore not surprising that antimicrobial are the most common prescribed drugs in livestock industry (Dumas et al., 2016). It is estimated that the food-producing animals consume more than 70% of antimicrobial produced worldwide. Usually pigs are raised in groups in close proximity to one another, with many production systems using all-in-all- out management to control and prevent infectious diseases outbreaks (Dewey et al., 1999). However, high contact rates provide optimal conditions for the spread of infectious disease, many of which require the use of antibiotics to minimize economic losses and welfare concerns. The swine industry uses antimicrobial at all stages of production. Estimates range from 62% of nursery production units and 44% of grower/finisher units (McEwen and Fedorka-Cray, 2002) to 33% of nursery units and 30% of grower/finisher units use antimicrobial for growth promotion (Holman and Chénier, 2015) depending on the survey timing, location and definitions. Even with surveys conducted at the same time and in the same country there are differences. Data collected in 2001 by the USDA for US herds found that 70% used antimicrobial in starter feeds, 59% used them in grower-finisher feeds, and 46% used antimicrobial in sow feeds (Cromwell, 2002), which was higher than the estimates of (McEwen and Fedorka-Cray, 2002) in a similar population. The most widely prescribed antimicrobial in conventional swine farms are tetracycline, penicillin, macrolides, sulfonamides cephalosporin, bacitracin and other betalactams (McEwen and Fedorka-Cray,

2002). The routes of antibiotics administration is oral (drinking water or feed) or parenteral injection (Holman and Chénier, 2015). For disease prevention and control in swine, the duration of antimicrobial administration ranges from 20-40 days (Stone et al., 2009). Alternatively for growth promotion, antimicrobial are generally used over a long period of time at relatively low concentration (Dewey et al., 1997). In growing-finishing piglets, the average duration of antimicrobial inclusion for growth promotion is ranged from 22.7 to 76.8 days (Dewey et al., 1997). It is remarkable that using antibiotics as a growth promoters in younger piglets is more efficacious with little to no response seen in older piglets (Cromwell, 2002)(Skinner et al., 2014). This early exposure at below therapeutic concentrations provides ample opportunity for antimicrobial resistance to develop, especially when compared to therapeutic use (Holman and Chénier, 2014). Therefore, the European Union has prohibited the use of antimicrobial that are important in human medicine as growth promoters in food producing animals (Holman and Chénier, 2015). While the mode of action for growth promotion by antibiotics remains poorly characterized, several potential mechanisms have been proposed. These include: 1) decrease the detrimental metabolites produced by gastrointestinal microbiota (metabolic effect), 2) increase absorption of available dietary nutrients (nutritional effect), and 3) control of endemic subclinical disease (disease control effect) (Dibner and Richards, 2005). However, it seems inevitable that antimicrobial administration as a growth-promoters will diminish in the future. Additionally market limitations and consumer pressure are building to make using such antibiotics commercially impractical (Sommer et al., 2017). Therefore, finding alternative to antibiotics will play a key role in maintaining animal productivity in the absence of antimicrobial.

1.3 ASSOCIATION BETWEEN ANTIMICROBIAL USE AND ANTIMICROBIAL RESISTANCE

Antibiotics are compounds with antimicrobial properties that can be synthetic, semi-synthetic, or found naturally in the environment (Phillips et al., 2004). The goal of antibiotics administration in swine management systems are to eliminate pathogenic microbes thereby facilitating the growth and restoration of beneficial microbial communities (Holman and Chénier, 2015). However, these goals are routinely complicated by the presence and dissemination of antimicrobial resistance genes (ARGs) among microbes (McEwen and Fedorka-Cray, 2002). Resistance to antimicrobial is a natural occurrence, developed by microbes to help

in their survival against other antibiotic-producing microorganisms in the environment (Phillips et al., 2004). Traditionally, the impact of antibiotic treatment on the emergence of antibiotic-resistant bacteria has focused only pathogenic bacteria (e.g., *Salmonella, E. coli, Shigella,* and *Enterobacter*) (Founou et al., 2016). Many researches have started to investigate the association between antimicrobial use in livestock and the development of antibiotic resistance across a wider range of bacteria (Holman and Chénier, 2015). In many cases the detection of clinical signs for a disease in individual animals provokes the prophylactic treatment for the whole herd (Founou et al., 2016). This approach can increase abundance of resistant bacterial strains and elevate the expression of ARGs (Langdon et al., 2016). The prevalence of such antimicrobial-resistant bacteria and ARGs of livestock origin are widely reported, with great differences among different geographic locations (Founou et al., 2016).

When an antibiotic is administered, it eliminates the susceptible microbial populations, leaving behind unusual strains that continue to evolve and multiply in its number (Founou et al., 2016). The antibiotic exposure exacerbates the innate immune response and the selective pressure is exploited by antibiotic resistant microbes providing them with an evolutionary advantage (Brandl et al., 2008). The resistant population, in the presence of antibiotics, has a competitive advantage which facilitates their spread among the microbial population in surrounding ecosystem (Holmes et al., 2016). The resistant bacteria transmit their genetic resistance pools to their progeny, through vertical evolution, or to other adherent bacterial species, through horizontal evolution (acquired resistance) (Holmes et al., 2016). The vertical gene transfer occurs during cell division where the resistant genes either on the chromosomes or plasmids transfer to progeny cells, leading to an intrinsic natural resistance (Lawrence, 2004). Alternatively, horizontal gene transfer involves the exchange the genetic pool within and between microbial populations, where the genetic density and complexity of the commensal microbial community stimulate the spread of resistance genes among microbes through horizontal transmission (Founou et al., 2016). The resistance genetic material is transferred either through conjugation, transformation, and or transduction (Holmes et al., 2016). Additionally, the transmission of antibiotic resistance can occur in commensal, saprophytic transient, and pathogenic microbes in both animals and humans (Sommer et al., 2017).

The reservoirs of antibiotic resistant bacteria are ubiquitous, and can merge with the GI resident microbiota through two different mechanisms (Holman and Chénier, 2015). First, the

resistant bacteria can be acquired directly by the host and colonize the GI mucosal epithelium; secondly, a previously susceptible bacterial species become resistant through induction of antibiotic-resistant mutants or through resistant gene transfer events (Holmes et al., 2016). While, there is a clear association between the use of antibiotics and emergence of the antibiotics resistance, this relationship is complex and is under the influence of multiple confounding factors (e.g. pathogen-host interactions, pathogen–drug interactions, rate of mutation, rate of transmission, cross-resistance, and co-selection of resistance to unrelated drugs) (Holmes et al., 2016).

1.4 SWINE GASTROINTESTINAL MICROBIOTA

The term microbiome is widely used to describe the resident populations of different organisms (bacteria, viruses, fungi, archaea, and protists) that live and/or colonize the body of multicellular host and their genetic material (Turnbaugh et al., 2007). Generally, the GI microbiome harbors the majority of these microbial populations and is critical for proper immune development and digestion of available nutrients (Hollister et al., 2014). Understanding how GI microbiome composition affects livestock health is an emerging area of study (Isaacson and Kim, 2012) (Zeineldin et al., 2017a). The swine GI microbiota is a rich ecosystem that harbors millions of diverse microbial populations (aerobic, facultative anaerobic and strictly anaerobic) dwelling in different location along the GI tract (Metzler and Mosenthin, 2008) (Holman et al., 2017). The swine GI microbiota is not uniform and differs drastically between individuals reared in either same or different management systems. Additionally, the relative abundance of specific bacteria differs with different GI biogeographic locations (Leser et al., 2002) (Yeoman et al., 2018), with a significantly more rich and diverse community in the colon compared to ileum and stomach (Holman and Chénier, 2015) (Henriksson et al., 1995). Recently, many reports have attempted to evaluate the overall proportions of microbial communities in the swine GI tract (Holman et al., 2017). Most of swine GI microbiota research have focused on next generation sequencing of 16S rRNA gene hypervariable regions. While there is a concern that sample level factors (type of sample, extraction methods, sequencing methods) impact the reported microbiome, a recent meta-analyses of GI microbiota revealed that these biological factors have a smaller effect on the reported microbiota composition than the study-level variables (age, sex, GI locations) (Holman et al., 2017) (Holman and Chénier, 2014) (Holman et

al., 2017) (Zeineldin et al., 2018a). The swine GI microbiota composition is influenced by many factors, including how the pig is delivered at birth (Wang et al., 2013a), host genetics, maternal microbiome, age, surrounding environment, housing, diet changes, pathogen exposure, and use of feed additives and antibiotics (Pluske et al., 2018).

The ability of the host to maintain and restore the physiological balance between the GI tract and healthy microbial ecosystem is very important, not only for physiological balance, but also for health-disease status. Many events can disturb the physiological balance and the homeostatic interplay between the GI tract and the resident microbial population that colonize the different segments. The disturbance of the homeostatic resident GI microbiota, results in a loss of protection against colonization by potential pathogens, and subsequently can affect the health status of the host due to increased susceptibility to disease.

1.5 GENOMIC TOOLS USED TO STUDY THE SWINE GASTROINTESTINAL MICROBIOTA

Historically, studies of the swine GI microbial communities have relied primarily on conventional culture-based methods, focusing on the major pathogens that are readily cultured (Isaacson and Kim, 2012). While beneficial, these protocols do not provide information on bacteria that cannot be cultivated, thus, providing only limited understanding of the complexity of this microbial ecosystems (Henriksson et al., 1995). In contrast, several molecular techniques have been employed to quantify the GI microbial community composition in swine (Dowd et al., 2008). For examples, immunohistochemistry, real-time quantitative polymerase chain reaction and fluorescent *in situ* hybridization have been used to demonstrate the presence and characteristics of organisms in clinical specimens (Sanduleanu et al., 2001) (Radaelli et al., 2008) (Sachse et al., 2010). While these techniques identify specific bacterial species and evaluate the similarities and differences between the community compositions, they do not provide direct molecular sequence evidence. In addition, only previously classified bacteria can be identified using these closed reference techniques, since each assay is generally designed to detect a narrow range of bacterial taxa (Suen et al., 2011).

These restrictions have led to widespread use of high-throughput sequencing of the 16S rRNA gene as a common phylogenetic biomarker for profiling bacteria present in a specific host or environment (Winker and Woese, 1991) (Xie et al., 2013). Advance is next generation

sequencing has provided researchers with the tools necessary to dive deeper into microbiome research and discovery. Next generation sequencing allows for sequencing of thousands of DNA molecules in parallel at a reduced cost with less labor compared to the traditional method of Sanger sequencing. Sanger sequencing was first introduced in the late 1970s and was used as the main sequencing method until the early 2000s (Schuster, 2008). Development of more efficient method of sequencing was then driven by the human genome project and become the main platforms used in sequencing (e.g. Illumina, SOLiD, IonTorrent, and Roche 454) (Huws et al., 2016)(Caporaso et al., 2011).

Current sequencing technology, which is used to isolate microbial DNA from a single specimen, generates many short reads sequence in a single run. Additionally, the structure of the bacterial community and its depth can be quantified at this level of sequencing (Schuster, 2008). Interpretation of this sequencing data requires bioinformatics tools such as QIIME, Mothur, kraken and etc. (Caporaso et al., 2011)(Zeineldin et al., 2017c). These tools allow for removal of low quality sequence reads from data sets and taxonomic assignment (Holman et al., 2017). Based on sequences resemblance, these short reads are clustered into operational taxonomic units at the different taxonomic classification levels: kingdom, phylum, class, order, family, genus, and species (Dickson et al., 2016). Recently, whole-genome sequencing (shotgun metagenomics) has superseded 16S metataxonomics in studying microbial populations, because it has the capacity to provide a deeper and more extensive view of microbiota compositions and function (Gaeta et al., 2017).

Regardless of the methodology chosen for sequencing studies, it has become apparent that there is not yet a gold standard for sequencing or analysis, especially when using fecal material as the DNA source. Changes in integrity of the samples can be introduced at several steps of the process and depending on the chosen methodology; different results may be obtained from the same samples. For example, when collecting fecal samples, it is critical to handle them all in the same way, making an effort to freeze them as soon as possible and avoiding multiple freeze thaw cycles. Additionally, the choice of primers and PCR annealing times during DNA extraction protocols may also have effects on the quality of samples and overall diversity profiles. Using widely employed commercial kits for DNA extraction from fecal samples is likely the best way to avoid inconsistencies. The choice of primers depends on the region of interest. Since the genetic information on the 16S gene varies along its length and there is no

described gold standard of the region best suited for study of the GI tract reading different regions can lead to different results from the same sample (Holman et al., 2017). Longer annealing times and use of error-correcting polymerases in PCR may be important to reduce chimera formation and PCR error and improve quality of samples overall. The parallel advances in mass spectrometry platforms have led to improved methods for detecting microbial activity, including protein products (metaproteomics), gene expression (metatranscriptomics) and metabolic profiles (metabolomics) (Gonzalez-Martinez et al., 2018) (Hassa et al., 2018). The strategic use of these advanced techniques in mucosal ecosystem studies may be the cornerstone for improving our understanding of swine GI health in the future.

1.6 DEVELOPMENT OF GASTROINTESTINAL MICROBIAL ECOSYSTEM IN SWINE

The development of GI tract structure and function in swine is a complex multistage process that occur before and after parturition (Pluske, 2016). The growth, functionality and maturation of the GI tract in young piglets is crucial for the piglet's growth rate, efficiency of feed utilization, and ultimately for overall performance (Pluske, 2016). The perinatal anatomic maturation and development of GI tract comprises of three phases including; a prenatal phase, a neonatal phase, and the post-weaning phase (Zabielski et al., 2008). The key distinguisher of the prenatal phase is minimal development of the structures on the mucosal surface of the GI lumen. The growth rate of the fetus is dramatic from day 70 of pregnancy till day of parturition (McPherson et al., 2004), and this is accompanied by noticeable development and growth of the neonatal GI tract (Sangild et al., 2000). In the few weeks before birth, the GI tract develops more quickly than the whole body with rapid change in the gastric function (Sangild et al., 2000). The neonatal phase is characterized by changes which are predominately induced by colostrum and milk intake (Maradiaga et al., 2018). Soon after birth, the GI tract starts to adapt rapidly to the transition from placental nutrition to enteral milk nutrition received per os (Pluske, 2016). During the first four weeks after birth, the weight of the piglets increases more than fivefold, with the GI organs growing faster than many other organs of the body (Zabielski et al., 2008). Finally the post-weaning phase is accompanied by remarkable changes to increase adaptation of GI tract especially small intestine to solid feed (Zabielski et al., 2008) (Pluske, 2016).

In parallel to this sequential of anatomic growth of the GI tract, the gradual establishment of microbial communities takes place throughout gestation and during the peri-parturient period

(Mach et al., 2015). Many intrinsic and extrinsic factors affect the composition, trajectory, and stability of these developing microbiotas which may play a crucial role in GI health (Dou et al., 2017). Contrary to the previous hypothesis that neonates mammals are born sterile, the recent use of next generation sequencing platforms has showed that microbial DNA is present in utero and in amniotic fluid (DiGiulio, 2012) (Aagaard et al., 2014), thus suggesting that that prenatal GI tract development might occurs in the presence of microbiotas (Mach et al., 2015). In swine, the microbiota of piglets following delivery resembles their dam and depends on the route by which the pig is delivered (Wang et al., 2013b). The early- life GI microbiota in neonates that were born vaginally shares microbial communities with the dam's vaginal microbiota, while in neonates born by cesarean section, the early GI microbiotas matches that of skin microbial communities of the mother (Hansen et al., 2014) (Bokulich et al., 2016).

Bacterial colonization succession after birth is a complex process, and is intensely impacted by many host and environmental factors, such as colostrum intake, housing, and dietary composition (Frese et al., 2015)(Koppena et al., 2015). In people, various microorganisms are detectable in the GI tract of healthy neonates during early-life that varies dramatically between the individual (Bosch et al., 2016) (Bokulich et al., 2016). However, the previous studies revealed that the GI microbial communities establishment and succession and its underlying principles can be similar across different animal species (Richards et al., 2005). After birth, the most crucial determinant of shaping the GI microbial colonization is milk feeding (Marcobal et al., 2011). Recently, Bian et al., have revealed that the GI microbiota of sow-fed piglets showed a clear dissimilarity from the GI microbiota in formula-fed piglets (Bian et al., 2016). Understanding which microbiota members are impacted by milk feeding and their fate after weaning provides prospective into the importance of factors that influence the major populations in gastrointestinal tract during early in life (Bian et al., 2016) (Holman et al., 2017) (Pluske et al., 2018).

The variation in GI microbial communities composition between the pre and post-weaning are largely impacted by the enormous diet changes from an easily digestible milk to grain-based feed (Holman and Chénier, 2015). Maintaining a healthy GI microbiota composition during this stage is of extreme importance (Richards et al., 2005). The weaning process, particularly when associated with other environmental stressors, is a pivotal factor impacting the GI microbial composition of piglets (Richards et al., 2005). The weaning phase is often

associated with decreased feed and water intake, dramatic decrease in growth rate, changes in GI barrier capacity, diarrhea, microbial ecological changes and increased disease incidence and mortality rate (Melin et al., 2000) (Orgeur et al., 2001). The GI microbiota in swine appears to change and develop rapidly after weaning (Holman et al., 2017). The swine GI microbiota is filled with nutrient absorbing bacterial mechanisms that digest different substrates. With this logic, one can assume that dietary changes result in microbial composition changes to facilitate appropriate metabolic functions (Isaacson and Kim, 2012). While the mechanistic pathways and the abundance of these alternations have not been fully elucidated in swine, the microbial pattern changes indicated microbial composition shifts occurred to aid in nutrient and food digestion.

1.7 THE ROLE OF GASTROINTESTINAL MICROBIOTA IN SWINE HEALTH

The swine GI tract is dynamic, complex and changeable ecosystem especially throughout life, in which GI tract undergoing marked alteration in morphology, microbiota composition and absorptive performance and local immune response (Pe et al., 2004). According to the definition of (Bischoff, 2011), there are five criteria that defined GI health including: functional digestion and absorption, disease free, hemostatic microbiota, functional immune response and host homeostatic status. Some human data and animal experiments have revealed that the crosstalk and complex interactions between GI mucosal surface, resident microbiota, and the immune function are the key in impacting GI health and maintain physiological homeostasis in the host (Blaut and Clavel, 2007). It is clear that the successful interaction between those factors affect the diversity, activity and microbial succession of the GI microbiota (Pluske et al., 2018). In germ-free mice, the GI resident microbiota impacted not only functions of the GI tract but also play an important role in development of gut- brain axis (Neufeld et al., 2011). The GI microbial populations contribute to immune system development, energy homeostasis, and prevents mucosal infections. Gastrointestinal microbiota play a crucial role in gut barrier function, that prevent emergence of many allergic, infectious, and inflammatory conditions (Bischoff, 2011)(Pluske et al., 2018). However, the precise and exact mechanisms of how the GI microbiota contributes to gut health are still unclear, although there are novel studies endeavoring to increase our understanding about this mechanism (Pluske et al., 2018).

Expectedly, varied and diverse microbes were detected in different segments of the swine GI tract and at various stages of the production cycle throughout life (Holman et al., 2017).

Those microbes represents a compromise between GI tract functionality and the deleterious effects of dysbiotic pathologies (Celi et al., 2017). Moreover, the GI microbial communities are intimately involved in cross talk between the host immune system and GI mucosal surfaces, providing host with the essential component that required in provoking host responses against infectious diseases, particularly in neonates (Metzler and Mosenthin, 2008). This powerful communication leads to different levels of metabolic changes that could lead the host towards health or disease (Celi et al., 2017). In addition, the GI commensal microbiota play an important function in bile salt re-cycling, volatile fatty acid and vitamin K production, cellulose digestion, metabolism of undigested carbohydrates, and nutrient recovery (Bischoff, 2011). Therefore, disturbance of the GI microbial communities' composition may detrimentally influence the host health status and inhibit the colonization of pathogens. Commensal GI microbiota contribute to the resistance against colonization through competition for binding sites, nutrient utilization at mucosal epithelium, and by modifying the local environment (Holman and Chénier, 2015). The presence pathogenic microbes is not enough to induce disease. It requires the number of invading pathogens propagate to such an extent to overcome the GI homeostatic resident microbiota (Knight-Sepulveda et al., 2015).

The associations between the mucosal immune system, mucosal microbiota and the GI tract mucosal epithelium is important when discussing the potential impact of GI microbes in host health (Burkey et al., 2009). The mucosal immune system and epithelial barrier function is continually challenged by internal and external factors (e.g., microbial composition, diet, and environment). Gastrointestinal diseases are usually associated increased permeability of the epithelial barrier that may results in toxin translocation, and increase the potential for invasion by viruses and bacteria (Pluske et al., 2018). A dysbiotic GI microbiota is a characteristic of many GI diseases in swine, although the mechanisms of association are not always obvious. Therefore, understanding the different factors that shape the microbiota composition particularly in early life, are required to promote optimal gastrointestinal development and subsequent host health.

1.8 EFFECT OF ANTIMICROBIAL INTERVENTION ON SWINE GASTROINTESTINAL MICROBIOTA

With the advancement of methodologies to assess the microbiota and appreciation of the crucial role that the mucosal microbiome plays in homeostasis of host health (Zeineldin et al.,

2017b), several considerations have been raised about the impact of antimicrobial administration on the homeostatic resident microbial populations in our bodies (Bokulich et al., 2016). There are several reports and longitudinal studies in both animals and human which attempt to understand the impacts of antimicrobial intervention on the GI microbiota (Holman and Chénier, 2015)(Gerzova et al., 2015) (Bokulich et al., 2016) (Founou et al., 2016) (Langdon et al., 2016). Commonly, antimicrobial are given to wipe out pathogenic microbes during acute infection (Dewey et al., 1999). However, many antimicrobial classes are not specific, and consequently wipe out a wide range of resident GI microbiota that are beneficial, and are pivotal for health (Neuman et al., 2018). Several reviews attempted to integrate all the published literature about the impact of the different categories of antimicrobial on the GI microbial populations (McEwen and Fedorka-Cray, 2002) (Holman and Chénier, 2015) (Founou et al., 2016) (Neuman et al., 2018). However, it is hard to draw clear conclusions from these papers due to heterogeneous data, small sample sizes, and different methods of microbiome analysis (Zeineldin et al., 2018b). Recently, a comprehensive review by Langdon et al. revealed that antimicrobial intervention drastically changes both adult and neonatal microbiota structure (Langdon et al., 2016). Both a single and repeated courses of antimicrobial treatment led to significant shift in beneficial microbiota in the healthy GI tract (Leibovitz et al., 2003). This shift has been associated with an increase the chance of subsequent GI disease (Pettigrew et al., 2012). Although large shifts in microbiota composition occurred following antimicrobial administration, most microbial communities returned to the pretreatment state four weeks after a single course of treatment, while other taxa failed to return to the pretreatment state within six months of treatment (Jernberg et al., 2010). The precise components responsible for GI microbiota recovery after antimicrobial administration are undefined. Therefore, recognition of the factors that promote microbiota recovery after antibiotic administration opens up new opportunities for the development of novel therapies that promote GI health and prevent pathogen colonization. While the class and dosage of the antimicrobial may result in a particular microbial change, short term antimicrobial administration can have long-term impacts on the structure of the GI microbial population (Jakobsson et al., 2010).

Similarly, antibiotic administration temporally influences the swine GI microbiota (Oultram et al., 2015)(Holman et al., 2018)(Zeineldin et al., 2018a). The swine GI microbiota response to antimicrobial intervention has varied across multiple studies possibly due to different

dosages, route of administration, antimicrobial class and other genetic and environmental variables (Kim et al., 2012) (Looft et al., 2014a) (Looft et al., 2014b) (Schokker et al., 2015) (Mu et al., 2017) (Soler et al., 2018). It is important, when quantifying the impact of antimicrobial intervention on the GI microbiota structure of swine, to consider the age of the pig, the method of microbiome analysis (real-time PCR, 16S rRNA, whole genome sequencing, or other sequencing methodologies), in addition to the class and dosage of antibiotics (Neuman et al., 2018).

In most swine production systems, antimicrobial are routinely administered early in life (e.g. prenatal, perinatal, weaning and post-weaning) (Li et al., 2017a) (Li et al., 2017b) (Soler et al., 2018). Generally, antibiotic administration decreases microbial diversity in both neonatal and growing piglets (Kim et al., 2012) (Li et al., 2017a) (Zeineldin et al., 2018a) (Gao et al., 2018a). While there are some similarities between the effects of antimicrobial administration on GI microbiota structure in growing and neonatal piglets, there are also some dissimilarities due to the distinct characteristics of the neonatal microbial composition (Holman and Chénier, 2015). A recent study of 16, 42 day old ileal-cannulated pigs demonstrated that ampicillin, gentamicin, and metronidazole treatment modified the GI microbial population structure and potential metabolic functions (Gao et al., 2018b). More precisely, use of ampicillin, gentamicin, and metronidazole decreased *Lactobacillus* and *Bifidobacterium* abundance, while increasing the abundance of 256 fold *Shigella*, compared to pigs not receiving antibiotics (Gao et al., 2018b). The microbial alterations were associated with a 2.88-fold reduction in acetate concentration. Acetate plays a crucial role in GI tract metabolism. Similarly, in a study by Li et al. where for the first 14 days of life neonatal piglets given a broad-spectrum antimicrobial, antibiotic administration exerted transient impact on the developing microbiota and their metabolite production (Li et al., 2017a). Additionally, Kim et al. conducted a study to assess the impacts of tylosin on the development of the GI microbiota in pigs (Kim et al., 2012). Relative to controls, tylosin induced microbiota compositional changes that resulted in increased microbial succession and microbial maturation rates. Qualitative and quantitative assessment illustrated that tylosin administration induced microbiota shifts in both abundant and less abundant microbial communities. For example, the tylosin treated piglets showed higher relative abundance of *Lactobacillus, Eggerthella, Acetanaerobacterium* and *Sporacetigenium,* species compared to control piglets (Kim et al., 2012). In contrast, in some studies antibiotic administration did not

affect the microbiota composition in growing pigs (Holman and Chénier, 2014). As mentioned previously, different classes of antimicrobial have distinct modes of action and therefore affect the GI microbiota in different ways. For example, when assessing the impacts of sub therapeutic concentration of two common antibiotics, tylosin and chlortetracycline, tylosin administration resulted in major shift in the relative abundance of several taxa (Holman and Chénier, 2014) while, chlortetracycline administration only resulted in minor alterations of GI microbiota composition. Similarly, oral vancomycin and metronidazole have different effects on *Clostridium difficile in* mice where only vancomycin had an obvious impact on microbial community structure (Lewis et al., 2015). In total, the published data suggest that different classes of antimicrobial disrupt the GI microbiota in different ways, which should be included in the decision-making process for the prescription of antibiotics in livestock management systems.

1.9 GASTROINTESTINAL MICROBIOTA AS A RESERVOIR OF ANTIBIOTICS RESISTOME

The concept of an antibiotic resistome was proposed by Gerard Wright in 2007 as a means of describing the collection of all known ARGs in the microbial ecosystem and their precursors at multiple levels (e.g. environment, pathogenic and nonpathogenic microbes) (Wright, 2007). The known ARGs are likely to represent just a small portion of the actual ARG population. It is reasonable to assume that with the explosion of bacterial genome sequencing many new ARGs will be identified (Noyes et al., 2016).

With the generation of more information about ARGs and the antibiotic resistome, the relationship between the resident microbiome and the accompanying resistome has become less clear. Although the resistome is theoretically accessible to all bacteria, GI microbiota harbor a distinct antibiotic resistome (Sundin and Wang, 2018). In parallel with the consecutive development of GI microbiota the antibiotic resistome is established during the first few days of life or perhaps during prenatal phase even without prior exposure to antimicrobial treatment (Wright, 2007). This concept endorses the theory that resistant bacteria are established shortly after birth and are acquired either directly from their dam or through direct contact with resistant bacteria in the surrounding environment (Gonzales-Marin et al., 2012). In healthy children the GI resistome is established early in life and persists throughout childhood (Gibson et al., 2015).

The commensal microbiota of the GI track has a large and diverse genetic pool which utilizes many pathways to transmit resistance between and within the resident commensal species (Sengupta et al., 2013). The effects of different antimicrobial interventions on the structure of GI microbiota and emergence of the antibiotic resistome has been extensively demonstrated (Wright, 2007) (Enwemeka, 2013) (Holmes et al., 2016). There are multiple factors including dose, route, class, and spectrum of an antibiotic that affects the GI microbiota and their associated resistome in different ways. For instance, a long-term study by Yassour et al. revealed that when the infants received antimicrobial in the first three years of life, the GI microbiota expressed high level of antibiotic resistance compare to control (Yassour et al., 2016). Similarly, several reports have assessed the relationship between antibiotic administration and the increased abundance of antimicrobial resistance genes in swine (Holman and Chénier, 2015). In a study by Zhu et al., the abundance of 149 ARGs conferring resistance to different class of antibiotics were detected in swine feces from production unit that used different antibiotics either orally or via intramuscular injection (Zhu et al., 2013). In contrast, many reports have revealed the emergence antibiotic resistance determinants in pigs without prior antimicrobial administration (Pakpour et al., 2012) (Agga et al., 2015) (Holman and Chénier, 2015). For instance, several tetracycline resistance genes (e.g. tetO, tetW, tetM, tetX and tetQ), and the macrolide resistance genes (e.g. ermG, ermF and ermB) are frequently identified in swine production facilities in the absence of antibiotic exposure (Looft et al., 2012). The emergence of these ARGs without direct exposure to antibiotics reveals that the antibiotic resistome present in the swine GI microbiota may not affected by a reduction in antibiotic administration in swine management systems (Holman and Chénier, 2015). A better understanding of the impact of antibiotics on the resistome in livestock production systems is critically important to understand the role that antimicrobial use has in selecting ARGs that could influence human health (Sundin and Wang, 2018).

1.10 ANTIBIOTIC ALTERNATIVES FOR MODULATING THE GASTROINTESTINAL MICROBIOTA

The detrimental impact of antimicrobial on GI microbiota and potential for the spread of multi-drug resistant bacteria, the importance of developing novel non-antibiotic strategies to modulate GI microbial balance and prevent GI infection in food-producing animals cannot be

understated (Allen et al., 2013) (Stanton, 2013). Efforts to define the complex composition of GI microbiota and how that community responds to antibiotics intervention would improve our ability to increase animal productivity and improve overall animal health (Marchesi et al., 2016).

Traditionally, the alternatives approach to antibiotics for the control of dysbiotic microbiota have focused on targeting specific species in the GI microbiota. More recently, several strategies have utilized the delivery of additional exogenous bacterial species to the GI tract with the expressed purpose of modifying its resilience to pathogen colonization. These strategies include the use of feed additives, phage lysins, phage therapy, vaccination, fecal microbiota transplants, bacteriocins, and predatory bacteria (Allen et al., 2014). Broad discussions of possible antibiotic alternatives for animal pathogens have been mentioned elsewhere (Potter et al., 2008)(Allen et al., 2013)(Papatsiros, 2013)(Allen et al., 2014)(Czaplewski et al., 2016). We will therefore consider probiotics and phage therapy as important examples of antibiotics alternatives.

Probiotics are defined as living microorganisms which, when given at a high enough concentration (either inherent or added), modulate the GI microbiota and provide benefit to the overall host health (Collins and Gibson, 1999). Probiotics are used for the treatment of a wide range of GI infections and to restore a dysbiotic microbiota to it normal state (Fallah et al., 2013). The effective probiotic should be nonpathogenic, able to survive in GI environment, able to produce a nutrient, has no ARGs and is able to colonize the host (Allen et al., 2013). Widely used probiotics include *Bifidobacterium, Streptococcus, Lactobacillus, Bacillus*, and *yeasts* (Dunne et al., 2001). Recently, using probiotics as an alternative to antibiotics treatment in swine has increased in frequency with many commercial probiotic products available for the livestock industry (Allen et al., 2013). As an example, supplementing lactic acid bacteria (*Enterococcus faecalis*) increases relative abundance of *Bifidobacterium* and *Lactobacillus* spp., and reduces incidence of diarrhea in newly weaned piglets (Hosseindoust et al., 2017). Orally delivered probiotics (lactic acid bacteria and *Saccharomyces cerevisiae*) have demonstrated the ability to inhibit colonization of weaned piglets by pathogenic E.coli (Trevisi et al., 2017). In addition, a high dose of *Lactobacillus rhamnosus* in pigs with pathogenic E.coli increased the relative abundance of *Lactobacillus* and *Bifidobacterium* with an corresponding decrease in fecal coliform shedding (Li et al., 2012). While the published data clarifies that most of commercially

available probiotics can modulate the GI dysbiosis, it will require more data to determine the probiotic strains that are most effective and their effectiveness in specific age groups.

Another alternative to antibiotics that has received attention recently is bacteriophage (phage) therapy (Parisien et al., 2008). Phage therapy involves the use of bacterial viruses (phages) to attack a specific bacterial species or narrow group of microbes without harming the resident autochthonous microbial communities (Kutateladze and Adamia, 2010). Subsequent research demonstrated that bacteriophage attacks bacteria by attaching to the cell wall and injecting their genetic material into the bacterial cytoplasm with subsequent integration into the bacterial genome. This process results in the rapid production of phage progeny which when released from the bacteria infect other bacteria. In addition to GI microbiota inhabitants, the GI tract harbors diverse viral communities that play a synergistic effect along with the resident microbial communities to maintain gut health (Allen et al., 2013). In a recent study, oral administration of a phage cocktail was employed to alleviate clinical diarrhea, increase the prevalence of beneficial microbes (e.g. *Bifidobacterium* and *Lactobacillus*), and decrease the relative abundance of coliforms and Clostridium spp. in post-weaning piglets (Hosseindoust et al., 2017).

Similar to antibiotic resistance, bacteria can develop phage resistance. Phage-resistant strains are believed to generally be less virulent than the phage susceptible wild type (Kutateladze and Adamia, 2010). Despite this advantage there are many technical limitations in the implementation of a phage therapy for the treatment of infectious diseases among humans and or animals (Allen et al., 2014). The available phages have a limited microbial range, require rapid administration after infection, could be neutralized by the host immune system, and do not target only a limited number of microbial pathogens at the same time, so the effective use of phage therapy necessitates precise recognition of target pathogens (Papatsiros, 2013). Therefore, high- throughput next generation sequencing and genetic engineering will be necessary to create a more reasonable phage design that best enhances its impact and creates the best alternative to antibiotics.

CHAPTER 2

IMPACT OF PARENTERAL ANTIMICROBIAL ADMINISTRATION ON THE STRUCTURE AND DIVERSITY OF THE FECAL MICROBIOTA OF GROWING PIGS

2.1 ABSTRACT

While antimicrobial are cost-effective tools for prevention and treatment of infectious disease, the impact of their use on potentially beneficent mucosal microbial communities has not been widely explored. The objective of this study was to characterize the impact of parenteral antibiotics administration on the composition and diversity of the resident fecal microbiota in pigs. Five antimicrobial treatment groups, each consisting of four, eight-week old piglets, were administered one of the antimicrobial; TUL, CCFA, CHC, OTC, and PPG at label dose and route. Individual fecal swabs were collected immediately before antimicrobial administration (control = day 0), and again on days 1, 3, 7, and 14 after dosing. Genomic DNA was extracted, and the V1-V3 hypervariable region of 16S rRNA gene was amplified and sequenced using Illumina Miseq- based sequencing. Across all groups, the most abundant phyla were *Firmicutes*, *Bacteroidetes,* and *Proteobacteria.* Linear discriminant analysis and stacked area graphs, showed a pronounced, antimicrobial-dependent shift in the composition of fecal microbiota over time from day 0. By day 14, the fecal microbial compositions of the groups receiving CHC and TUL had returned to a distribution that closely resembled that observed on day 0, but differences were still evident. In contrast, animals that received PPG, OTC and CCFA, showed a tendency towards a normalized microbiota structure on day 7, but appeared to deviate away from the day 0 composition by day 14. Based on our results, the observed changes in fecal microbiota showed antimicrobial-specific variation in both duration and extent. Understanding the impact of these important antimicrobial-induced changes will be a critical step in optimizing the use of antimicrobial in health management programs in the swine industry.

2.2 INTRODUCTION

The swine gut microbiota is comprised of a diverse and complex microbial population (Li et al., 2017a) that influences many physiological, nutritional, and immunological processes in the host (Lamendella et al., 2011) (Kim and Isaacson, 2015). Certain components of these microbial communities are always present in huge and approximately constant numbers in the healthy gut. These symbionts aid in colonic fermentation (Hijova and Chmelarova, 2007), and improve nutritional energy harvesting capacity (Turnbaugh et al., 2006). Moreover, these microbiota provide a first line of defense against external invaders, and act as a barrier against colonization by potentially pathogenic microorganisms (Rashid et al., 2012). The fecal microbiota can be considered as a proxy for the diverse microbial communities that exist at different biogeographical locations along the gastrointestinal tract, and can therefore be used as an estimate of a potentially injurious dysbiosis at one or more of these sites (Dou et al., 2017). While the composition and functional profiles of porcine fecal microbiota have been a significant research topic for decades, most of these previous studies were restricted by their dependence on traditional culture-dependent methods, which provides a relatively limited picture of the complexity of these populations (Sears, 2005) (Kim and Isaacson, 2015). The advancements in sequence-based metagenomics and bioinformatics platforms have affords a comprehensive assessment of the microbial communities structure in several animal gut locations (van Dijk et al., 2014).

In commercial swine husbandry systems, antimicrobial have been used for over 60 years in the control, prevention, and treatment of infectious disease (therapeutic), and to improve growth and feed conversion efficiency (non-therapeutic) (Rettedal et al., 2009). While there has been a widespread focus on the role of antibiotics use on the emergence of antibiotics resistance among bacteria in the host (Allen and Stanton, 2013) (Francino, 2016), another important potential consequence of antimicrobial use is a negative impact on the structure and composition of the host microbiota (Bokulich et al., 2016). It is important to understand that the gastrointestinal microbiota is not simply a transient population of microbes involved in nutrient metabolism, but that many microbial taxa coexist in a coordinated, complex mucosal ecosystem that contributes to host gastrointestinal and immunological development, particularly in the growing animal (Clemente et al., 2012) (Hooper et al., 2012). If a healthy and stable microbiome is an important element of host health and development, then it is important to understand how

common management practices, such as antimicrobial administration, might impact this complex host ecosystem in animals raised in intensive production systems. This will in turn, help improve the productivity, and sustainability of livestock production. As we reach the post antibiotic era novel, systematic management tools that reduce the impact of disease without therapy will be needed to meet the nutritional needs of an expanding world population.

The aim of this study was to use next generation Illumina- based sequencing to characterize the impact of antimicrobial administration (TUL, CCFA, CHC, OTC, and PPG) on the structure and diversity of the resident fecal microbial communities in growing piglets.

2.3 MATERIALS AND METHODS

2.3.1 Experimental design and sample collection.

Twenty, eight-week-old piglets, owned by Carthage Innovative Swine Solutions, were used in this study under written consent from the facility owner. The pigs had all been housed together at a commercial research facility in Champaign county- Illinois since weaning, were fed the same diet, and had not taken any antimicrobial in the 7 weeks prior to enrollment in the study. The pigs were each randomly assigned into one of five treatment groups (n=4 pigs per group). Each treatment group was kept in a self-contained pen that was separated from other groups by an open pen. This arrangement prevented direct contact between pigs in different treatment groups. The pigs in each group received a different antimicrobial regime [TUL group - Tulathromycin (2.5 mg/kg IM); CCFA group - Ceftiofur Crystalline free acid (5.0 mg /kg IM); CHC group - Ceftiofur hydrochloride (5 mg/kg IM); OTC group - Oxytetracycline (9 mg/lb IM); PPG group -Procaine Penicillin G (15,000 units/lb IM)]. The antimicrobial dosages and routes of administration were based on the manufacturer label instructions, except for the PPG which was administered in accordance with common US commercial swine practices. Fecal swabs were collected from each pig immediately before antibiotic administration (day 0), and again on days 1, 3, 7, and 14. Following collection, all samples were kept on dry ice, transported to the lab, and stored at -80°C pending further processing and analysis. All animal protocols were approved by Illinois University Institutional Animal Care and Use Committee.

2.3.2 Genomic DNA extraction

Fecal swabs were prepared for total DNA extraction using Power Fecal DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), according to manufacturer recommendations. Before the DNA extraction, the fecal swabs were mixed with 750 µl of Bead Solution (Power Fecal DNA Isolation kit, MO BIO Laboratories, Inc.), heated at 65˚C for 10 minutes, and settled in Bullet Blender 24 Gold tube holder (Next Advance, Inc., Averill Park, NY, USA. The power® Fecal DNA extraction Kit protocol was used to complete the extraction according to manufacturer instructions. For each sample, the total DNA concentration was assessed using a Nanodrop™ spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at wavelengths of 260 and 280 nm, and the DNA integrity was evaluated by agarose gel electrophoresis (Bio-Rad Laboratories, Inc, Hercules, CA, USA).

2.3.3 Fluidigm Access Array amplification and Illumina sequencing

The extracted DNA from each fecal sample was then subjected to Fluidigm Access Array Amplification (Fluidigm Corporation, South San Francisco, CA, USA). Prior to amplification, all DNA samples were evaluated on a Qubit fluorometer (Life technologies, Grand Island, NY) using the High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA) (Zeineldin et al., 2017b) (Zeineldin et al., 2017a)**.**

A CS1 forward tag and CS2 reverse tag was added to each sample according to the Fluidigm protocol. The primer sequences F28-2-for (ACACTGACGACATGGTTCTACA) and R519-2-rev (TACGGTAGCAGAGACTTGGTCT) were used to amplify the V1-V3 region of 16s rRNA gene. PCR reactions were performed on a Fluidigm Biomark HD™ PCR machine (Fluidigm Corporation, South San Francisco, CA, USA) using the default Access Array cycling program without imaging (Table 1). The final product was then assessed on a Qubit fluorometer (Life technologies, Grand Island, NY, USA), and the quality and size of the amplicon was checked using a Fragment Analyzer (Advanced Analytics, Ames, IA, USA). PCR products were then size selected on a 2% agarose E-gel™ (Life technologies, Grand Island, NY, USA), and extracted from the isolated gel slice using a commercial kit (Qiagen, Valencia, CA). Cleaned, size-selected product was then re-evaluated for profile and size on an Agilent Bioanalyzer™. The final, pooled Fluidigm libraries were then transferred to the DNA Services lab at the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois at Urbana-

Champaign, Urbana, IL) for Illumina sequencing. The Illumina MiSeq™ platform (Illumina, San Diego, CA, USA) was used to sequence the V1- V3 region of the 16S rRNA gene. The libraries were sequenced from both ends of the molecules to achieve a total read length of 300nt according to the manufacturer instructions.

2.3.4 Sequence data processing and statistical analysis

The raw sequence data were preprocessed from Illumina base call (bcl) files into compressed paired end read fastq files (2 × 300) using bcl2fastq 1.8.4 (Illumina, San Diego, CA, USA) without demultiplexing, and then sorted by initial PCR-specific primer using a custom in-house pipeline. After preprocessing, the 16S rRNA gene sequences were processed using the open source Quantitative Insights into Microbial Ecology (QIIME) software, version 1.9 (http://qiime.org/) (Caporaso et al., 2010). Sequences were filtered for quality using the default parameters of the split_libraries.py command (Bokulich et al., 2012)**.** Sequences were clustered into operational taxonomic units (OTUs) based on 97% similarity using the UCLUST algorithm (Edgar, 2010)**,** and were taxonomically assigned against the Greengenes reference database (McDonald et al., 2012) using open reference OTU analysis. In an open-reference OTU picking process, sequence reads are first clustered in to known taxonomic groups, and then the remaining reads are subsequently organized into OTUs based on a *de novo* strategy. Chimeric sequences were detected and removed using UCHIME (Edgar et al., 2011). Bacterial diversity was estimated within QIIME using the Chao1, Shannon index and Phylogenetic Diversity (PD) whole tree. Due to uneven sequencing depth between the different samples, and to standardize our analysis, all samples were randomly rarefied to 2347 sequences per sample using QIIME software.

Statistical analyses were performed using JMP 13 software (SAS Institute Inc., North Carolina, USA). The least-square mean of bacterial diversity indices, and the relative abundance of fecal bacterial taxa for each treatment group, were compared using repeated measures ANOVA with a mixed-model's procedure fitted in JMP 13 (SAS Institute Inc. North Carolina, USA). Dunnett's multiple comparisons procedure was used to compare the mean bacterial diversity indices, and the mean relative abundance of fecal bacterial phyla and genera, for each sampling day (1, 3, 7 and 14) after each antimicrobial treatment, against day 0 (pre-treatment). Differences with a value of $p \leq 0.05$ were considered significant. Linear discriminant analysis

models were generated using the MASS package in the R statistical software (http://cran.rproject. org), using the relative abundances of different bacterial taxa in each sample as covariates, and sampling days as the categorical variable (Team, 2013). Canonical loading plots were used to illustrate the microbial shift from day 0 until day 14.

Fastq data obtained in the current study were uploaded to the sequence read archive (SRA) on National Center for Biotechnology Information (NCBI) website (http://www. ncbi.nlm.nih.gov/sra) to make the files publically available (bio-project accession number PRJNA316772).

2.4 RESULTS

2.4.1 Sequencing analysis summary and general taxonomic classification

In total, 597573 sequencing reads remained after the quality filtering steps, with an average of 6097.68 reads per sample (standard deviation, 3487.67; range, 2348–26924 reads). The final dataset was represented by 28,563 OTUs. Taxonomic classification revealed a total of 16 different bacteria phyla among all fecal samples. The most plentiful bacterial phyla across all samples (mean±SEM) were *Firmicutes* (0.481±0.017), *Bacteroidetes* (0.208±0.014), and *Proteobacteria* (0.075±0.012) (Figure 1). All other assigned OTUs related to phyla and averaged less than 1% of the total abundance, were represented as others/unassigned taxa (Figure 1).

At the genus level, taxonomic classification revealed that the most prevalent genera across all groups (mean±SEM) were *Prevotella* (0.168±0.007), *Clostridium* (0.148±0.008), *Bacteroides* (0.113±0.004), *Streptococcus* (0.051±0.005), *Blautia* (0.053±0.003) and *Oscillospira* (0.046±0.001). Other common bacterial genera were *Treponema* (0.039±0.004), *Flavobacterium* (0.032±0.001), *Alkaliphilus* (0.027±0.001), and *Succinivibrio* (0.023±0.003) (Figure 2). All other assigned OTUs that related to bacterial genera but averaged less than 1% of the total abundance, were classified as “other’ or ‘unassigned’ (Figure 2).

2.4.2 Impact of Antimicrobial Treatment on Composition of the Fecal Microbiota

2.4.2.1 Firmicutes/ Bacteroidetes ratio

The *Firmicutes/Bacteroidetes* (*F:B)* ratio is commonly employed as a universal indicator of bacterial community shifts (Zhang et al., 2014). The effect of the different antimicrobial treatments on the fecal *F:B* ratio are summarized in (Figure 3). The TUL group exhibited a five-

fold increase in *F:B* ratio at day 3 (P=0.01) and day 7 (P=0.01), mainly due to an increased abundance of *Firmicutes,* and decreased abundance of *Bacteroidetes*. CCFA pigs showed a two-fold increase in *F:B* ratio at day 14 (P=0.02), mainly due to a reduced proportion of *Bacteroidetes*. There were also observable, but not statistically significant, changes in *F:B* ratio seen in CHC (increased at day 3), OTC (decreased at day 3), and PPG (decreased at days 3 and 7) treated pigs.

2.4.2.2 Relative abundance of certain genera

The temporal pattern of changes in the relative abundance of bacterial taxa on different sampling days varied between antimicrobial groups. In addition, the observed duration for the fecal microbial communities to return to pre-treatment (day 0) proportions was taxa-dependent. In the following results, the post-treatment change in relative abundance of each particular taxa is described, and calculated, with reference to the abundance of the same taxa at the pre-treatment sampling time point (day 0).

In the TUL group, there was a statistically significant post-treatment increase in the proportion of *Succinivibrio* at day 3 (P= 0.048), *Alkaliphilus* (P= 0.024) at day 7, and of *Clostridium* (P= 0.039), and *Treponema* at day 14 (P= 0.036). In contrast, the TUL pigs exhibited a significant reduction in the proportion of *Bacteroides* at day 3 (P= 0.036), and *Streptococcus* at day 1 (P= 0.045), day 3 (P= 0.002), day 7 (P= 0.0012), and day 14 (P= 0.017) (Figure 4).

In the OTC group, there was a significant reduction in the proportion of *Streptococcus* at day 14 (P= 0.048) and *Alkaliphilus* at day 3 (P= 0.043). In contrast, OTC treated pigs showed a significant increase in the proportion of *Oscillospira* at day 3 (P= 0.032) and day 14 (P= 0.013), *Desulfonauticus* at day 14 (P= 0.006) and *Ruminococcus* at day 14 (P= 0.002) (Figure 5).

PPG treated pigs showed a significant decrease in the relative abundance of *Clostridium* at day 3 (P= 0.047), *Streptococcus* at day 1 (P= 0.012), day 3 (P= 0.0002), day 7 (P= 0.0006) and day 14 (P= 0.0005), and *Flavobacterium* at day 14 (P= 0.041). On the other hand, the PPG pigs revealed a significant increase in the proportion of *Blautia* at day 3 (P= 0.002), *Oscillospira* at day 3 (P= 0.006) and day 14 (P= 0.026), *Desulfonauticus* at day 3 (P= 0.027), and *Ruminococcus* at day 3 (P= 0.017) and day 14 (P< 0.0001) (Figure 6).

In the CCFA group, the pigs exhibited a significant reduction in the proportion of *Bacteroides* at day 7 (P= 0.019) and a significant increase in the proportion of *Lactobacillus* at day 7 (P= 0.021) (Figure 7). In the CHC group, there was a significant increase in the proportion of *Blautia* at day 7 (P= 0.041) only (Figure 8).

2.4.2.3 Alpha diversity

The fecal microbiota alpha-diversity was assessed using several metrics namely Chao1, Shannon diversity, and PD whole tree index. The overall average of Chao1 index of all samples (a nonparametric estimate of species richness) was 2528.90±101.97. The average value for the PD whole tree (an estimate of the phylogenetic difference between different species) and the Shannon diversity index (an estimate of microbial species diversity in a community) of all samples was 59.07±1.64 and 6.77±0.18 respectively (Figure 9).

In the CCFA group, the treated pig demonstrated significant decrease in Chao 1 index at day 3 (P=0.033), PD whole tree at day 3 (P=0.041) and Shannon diversity index at day 1 (P=0.041) and day 3 (P=0.04). In the CHC group, there was a significant increase in the Chao 1 index at day 7 (P= 0.018) and day 14 (P= 0.019), PD whole tree at day 3 (P= 0.031), day 7 (P= 0.017) and 14 (P= 0.006). CHC treated pig also showed significant increase in Shannon diversity index at day 3 (P= 0.041), 7 (P== 0.031) and 14 (P= 0.015). At day 3, TUL treated pig showed significant decrease in Cho1 index (P= 0.041), PD whole tree (P= 0.032) and Shannon diversity (P= 0.042). In PPG and OTC groups, comparisons of the bacterial diversity metrics of the fecal microbiota (Chao1, PD whole tree and Shannon diversity index) showed no significant differences after antimicrobial administration (P >0.05).

2.4.2.4 Beta diversity and core microbiota

Linear discriminant analysis showed a pronounced, antimicrobial-dependent shift in the composition of fecal microbiota over time, from the pre-treatment (day 0). By day 14, the fecal microbial compositions of the groups receiving CHC and TUL had returned to a distribution that closely resembled that observed on day 0. In contrast, animals that received PPG, OTC and CCFA, showed a tendency towards a normalized microbiota on day 7, but appeared to deviate away from the day 0 composition by day 14 (Figure 10).

Finally, a Venn diagram was done to describe the unique and common OTUs in each treatment group. Using OTU counts, the data set within all treatment groups were represented by 28563 OTUs. The distribution of OTUs showed that, there were 1234, 604, 903, 1609 and 1452 unique OTUs identified in CCFA, CHC, TUL, OTC and PPG respectively. While, a total of 2339 OTUs were represented core microbiota shared between all the treatment groups.

2.5 DISCUSSION

The swine gastrointestinal microbiota is comprised of a diverse and complex microbial population that coexists in a coordinated, complex mucosal ecosystem that contributes to host gastrointestinal and immunological development (Li et al., 2017a). The structure and stability of the gastrointestinal microbiota depends on many factors, such as diet, genetics, and age (Looft et al., 2014) (Zhang et al., 2016). Homeostasis of gut microbiota is considered to be valuable to the host by modulation of host immune system and contributing to colonization resistance process, while an imbalanced microbiota might induce bacterial overgrowth and development of antibiotic-resistant bacterial infections. Since gastrointestinal microbial communities engage in mutualistic and antagonistic interactions, it is important to understand how common management practices, such as antimicrobial administration, might impact this complex host ecosystem in animals raised in intensive production systems. While the effect of in-feed antimicrobial administration on specific gastrointestinal bacterial taxa (Looft et al., 2012) (Li et al., 2017b)**,** and the emergence of microbial resistance within the gastrointestinal microbiota (Gibbons et al., 2016)**,** has been well established, the impact of parenteral antimicrobial exposure on the composition, and diversity of the swine gut microbiota has not been widely studied. Since antimicrobial agents are an essential tool in the management of intensive swine health and production, a robust understanding of the potential impact of antimicrobial exposure on the composition and diversity of the gut microbiota has important significance for the industry. In this study, exposure to a labeled, parenteral dose of each of several antimicrobial had a significant impact on the taxonomic composition and diversity of the fecal microbiota in a healthy growing population of pigs. The observed changes in fecal microbial communities showed antimicrobial-specific variations in both duration and extent. In line with other studies, our results demonstrated that the fecal microbial communities in all treatment groups were dominated by *Firmicutes* and *Bacteroidetes* at the phylum level (Lamendella et al., 2011) (Looft

et al., 2012), and by *Prevotella*, *Clostridium*, *Bacteroides*, *Streptococcus* at the genus level (Kim et al., 2012) (Looft et al., 2012). All of the antimicrobial agents used in our study were associated with an observable change in the fecal *Firmicutes/Bacteroidetes* (*F:B)* ratio (Figure 2), which has been described as a reliable, universal indicator of bacterial community shifts (Zhang et al., 2014). However, the increase in *F:B* ratio was only statistically significant in the TUL and CCFA groups, mainly due to decreased abundance of *Bacteroidetes*. Similar patterns of *Bacteroidetes* decrease have been described in swine exposed to ASP250 (a combination of chlortetracycline, sulfamethazine and penicillin). In our study, the increase in *F:B* ratio observed on day 3 in the TUL pigs, was accompanied by an increased abundance of the *Succinivibrio* genus microbes, a pattern that was also observed in the ASP250-treated animals. While performance indicators were not measured in our study, a decrease in the proportion of *Bacteroidetes* has been linked to weight gain in growing pigs (Guo et al., 2008). At the genus level, the changes associated with CCFA and TUL administration were manifest as a significant decrease in the abundance of organisms in the *Bacteroides* taxa in fecal samples of pig from these groups.

At the lower taxonomic level, different antimicrobial administration showed, antimicrobial-specific changes in the relative abundance of certain bacterial genera. For example, the relative abundance of *Clostridium* was increased in the TUL-group (day 14) but decreased in the PPG group (day 3). In the CHC group, significant increases in the relative abundance of *Blautia* genera were observed on day 3. A significant decrease in the proportion of *Streptococcus* was observed in pigs from both the OTC (day 14) and PPG (days1-14) groups. Not surprisingly these latter two therapeutics are commonly used to treat *Streptococcal* infections in commercial swine operations (Schwartz et al., 1981) (Stevens et al., 1987) (Darwish et al., 2002). In addition, a significant reduction in the proportion of *Bacteroides* taxa was observed in CCFA (day 7) and TUL (day 3) pigs. While it is difficult to understand whether the short-term changes in composition of gastrointestinal microbiota observed in this study would have any significant long-term impact on the health and production of the growing piglet, the significance of antimicrobial induced microbial shift have been well documented by other researches. High relative abundance of *Lactobacillus* has been associated with beneficial health effects in swine, possibly through the production of antimicrobial bacteriocins (Daly et al., 2014)**.** In people, investigators have shown a mutualistic benefit of *Bacteroides* spp. to the host, possibly through

preventing gut colonization by potential pathogens (Backhed et al., 2005), while others have reported potential negative effects of increased *Bacteroides spp.* in the intestinal tract (Ou et al., 2013). The measured bacterial diversity indices in this study showed that the fecal microbiota in growing pigs were rich and diverse. The change in the bacterial diversity indices after parenteral antimicrobial administration are supported the published studies that have shown that the antimicrobial administration and many other management practices have a long-term impact on the structure and diversity of the healthy gastrointestinal microbial communities (Looft et al., 2012) (Looft et al., 2014).

The investigation and explanation of sequencing results of the consequence generated in our study, are considerable challenge for researchers (Edrington et al., 2012). We used a well-established approach, linear discriminant analysis, to help analyze these data set, and to detect microbial taxa that that significantly discriminated between different sampling days in each treatment group. This approach has been used successfully to visualize metagenomic data generated by next generation sequencing and identify bacterial taxa of interest for further analysis (Zeineldin et al., 2017a). In our study, linear discriminant analyses of the fecal microbial communities demonstrate that antimicrobial administration causes microbial shifts in the relative abundance of identified bacterial taxa within 24 hours of administration, which is a similar to that described by other researches (De La Cochetière et al., 2005)**.** While there were treatment-specific distinctions in the pattern of observed microbial changes, certain data trends were common to all groups. For example, no matter how the treatment affected the fecal microbial community composition, there was always an attempt to correct deviations from baseline over the following 14 days, typically between days 3 and day 7. Three of the treatments (PPG, OTC and TUL) exhibited their maximum association with microbial community disturbance at day 3. The fecal microbial community change in the CCFA and CHC groups was observed on days 1 and 7 respectively. In all treatment groups, except for PPG, the disordered fecal microbial communities showed a distinct trajectory of recovery, over time, towards the day 0 community structure. In each of these instances, the microbial communities on day 14 were still significantly different from this pre-treatment time point. In the CCFA and OTC treatment groups, it was difficult to see a clear trajectory of recovery in the fecal microbial communities over time. In fact, at day 14 the direction of disturbance appeared to continue its deviation away from that

observed on day 0. These findings support the theory that there is a longer lasting impact of these two antimicrobial on the fecal microbiota.

While the results of this study were crucial and could extend our understanding regarding the effect of parenteral antimicrobial administration on the resident fecal microbial communities, the potential limitations of our study should be considered. We used clinically health pigs, which had been reared without any exposure to antimicrobial over the 7 weeks prior to enrollment. While this approach allowed us to explore the impact of antibiotic administration on, what we presume to be, the normal fecal microbiota of 8-week-old pigs, it prevented us from drawing any conclusions regarding sick pigs, which are the individuals that most commonly receive antibiotics. In addition, it would have been interesting to continue to sample the pigs for a longer period to see when, or if, the microbiota would return to normal pretreatment community composition. Finally, in contempt of the fact that fecal microbiota is often presumed to represent the gut microbial populations, there is increasing evidence indicating that this representative of the mucosal populations at any particular biogeographic location in the intestinal tract. Despite these experimental limitation, understanding of the intrinsic and extrinsic factors that impact the gastrointestinal microbiota would be important in determining whether management strategies can be used to encourage a sustain gut microbial ecology during this important and determinative phase of production. The swine gut is a complex ecosystem, where many interactions between host, microbiota and environment occur simultaneously (Schokker et al., 2015). There is significant clue that the immune maturation process, and the maintenance of epithelial health in the intestinal mucosa, is impacted by the microbial populations that colonize the gastrointestinal tract at the beginning of life (Ohnmacht et al., 2011). If early life antimicrobial disrupt the ecology of the developing gastrointestinal tract, then additional studies are required to define how these changes impact the future health and productivity of growing animals. If these concepts are confirmed in larger swine populations from different environments, then these findings could be a crucial for novel trajectories of investigation in to swine gastrointestinal health, and in particular, the development of alternative approaches to antimicrobial administration in these early life stages.

2.6 CONCLUSION

In conclusion, parenteral antimicrobial administration showed significant shifts in the composition and diversity of fecal microbiota in growing pigs. The observed changes in fecal microbiota structure showed antimicrobial-specific variations in both duration and extent. None of the groups exhibited a full return to pre-administration fecal microbial community structure by day 14 post-treatment. Additional studies should be designed to explore the potential long-term impact of these changes on pig health and productivity.

2.7 FIGURES AND TABLE

Figure 1: Stacked bar showing relative abundance % of microbial population at phylum level for different treatment groups according to sampling day. Only the bacterial phyla representing populations that averaged more than 1% of the relative abundance across all samples are displayed. All other classified OTUs comprising less than 1% of the total abundance are represented as others/unassigned taxa.

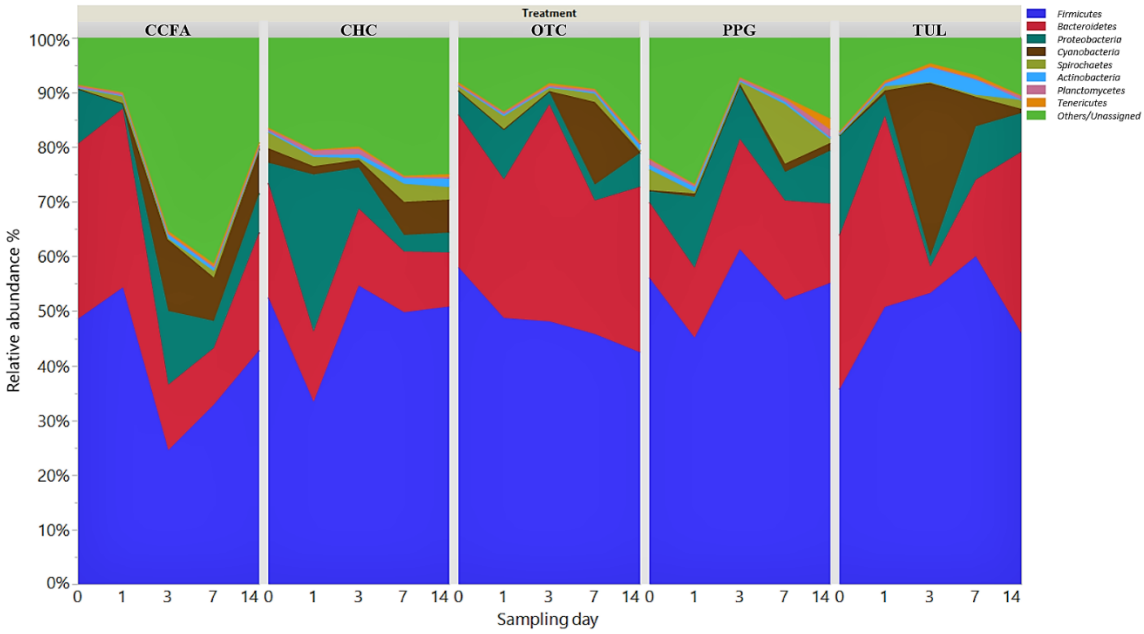


Figure 2: Stacked bar showing relative abundance % of microbial population at genus level for different treatment groups according to sampling day. At genus level, only those bacterial genera representing populations that averaged more than 1% of the relative abundance across all samples are displayed. All other classified OTUs comprising less than 1% of the total abundance are represented as others/unassigned taxa.

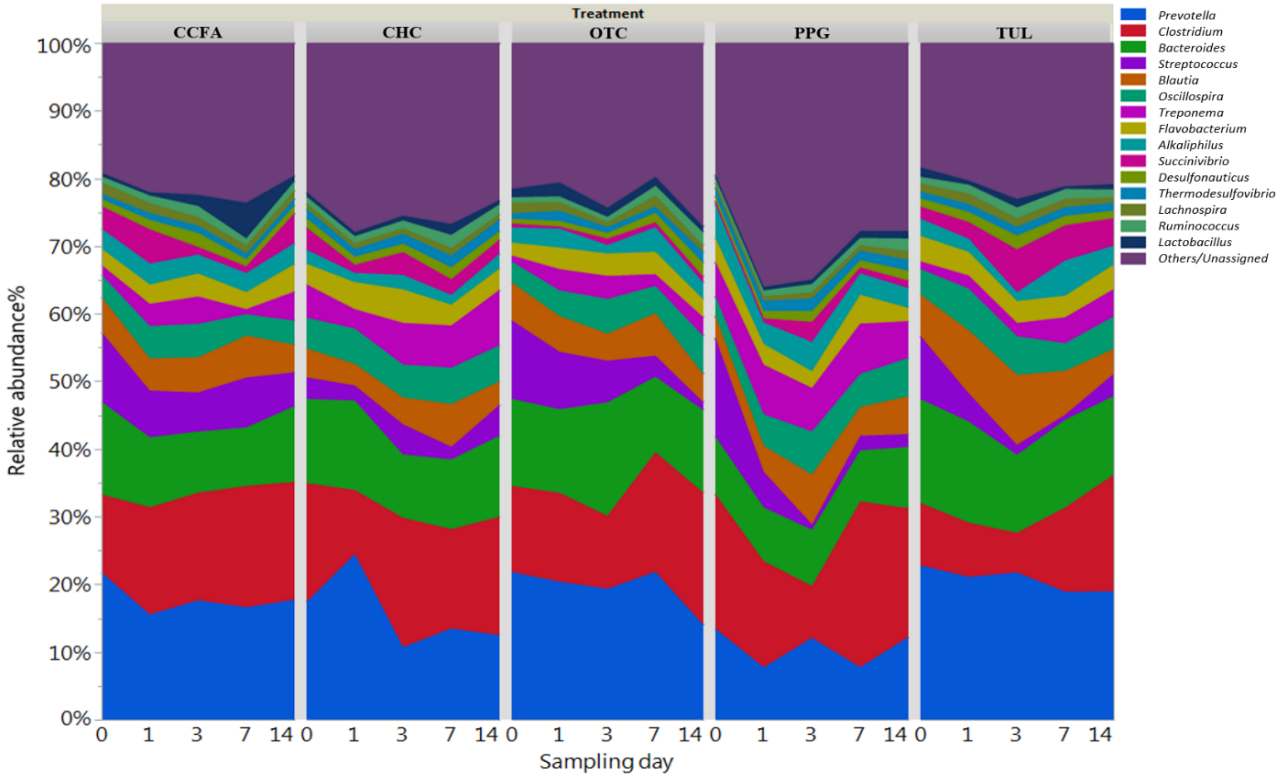


Figure 3: Bar graph illustrating the effect of various antibiotics treatment (Tulathromycin, Ceftiofur Crystalline free acid, Ceftiofur hydrochloride, Oxytetracycline, and Procaine Penicillin G) on the total *Firmicutes/Bacteroidetes* ratio at different sampling day. * $P < 0.05$.

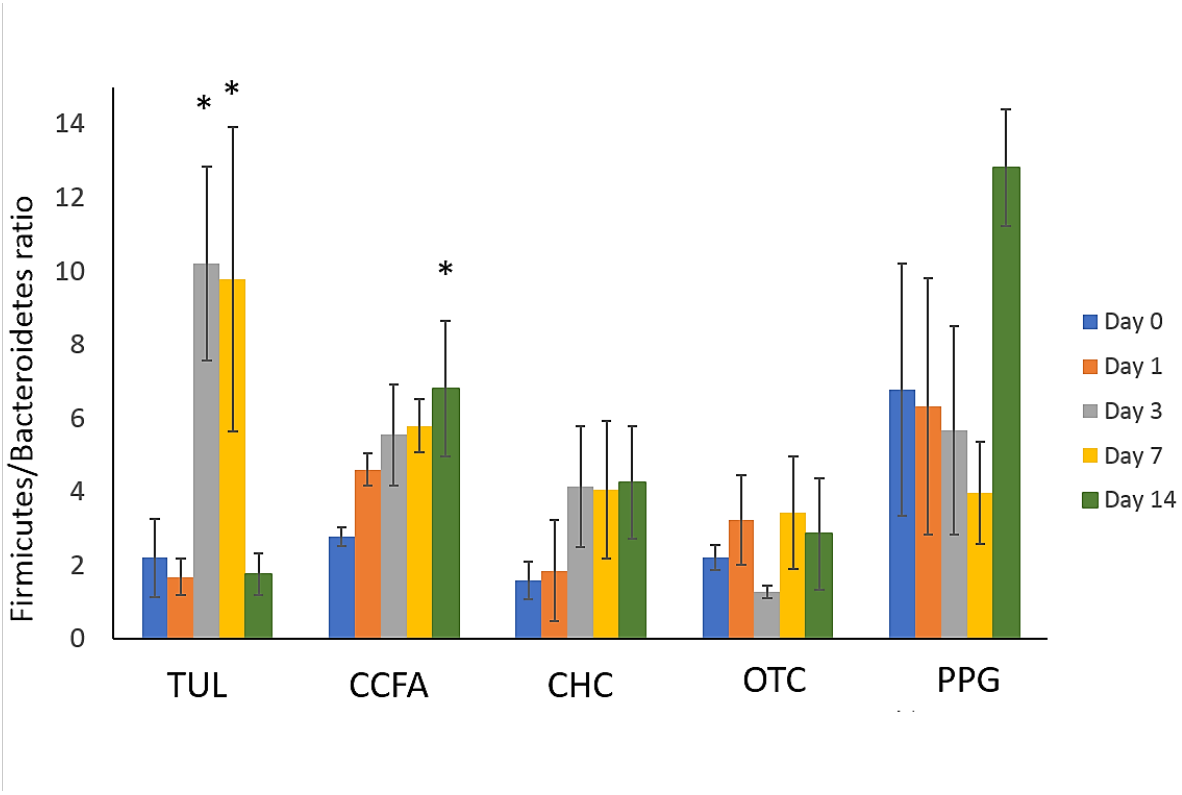


Figure 4: Bar graphs illustrating the relative abundance of top bacterial genera that averaged more than 1% observed in the fecal swabs from Tulathromycin treatment group at different sampling days. Error bars represent standard errors. * $P < 0.05$.

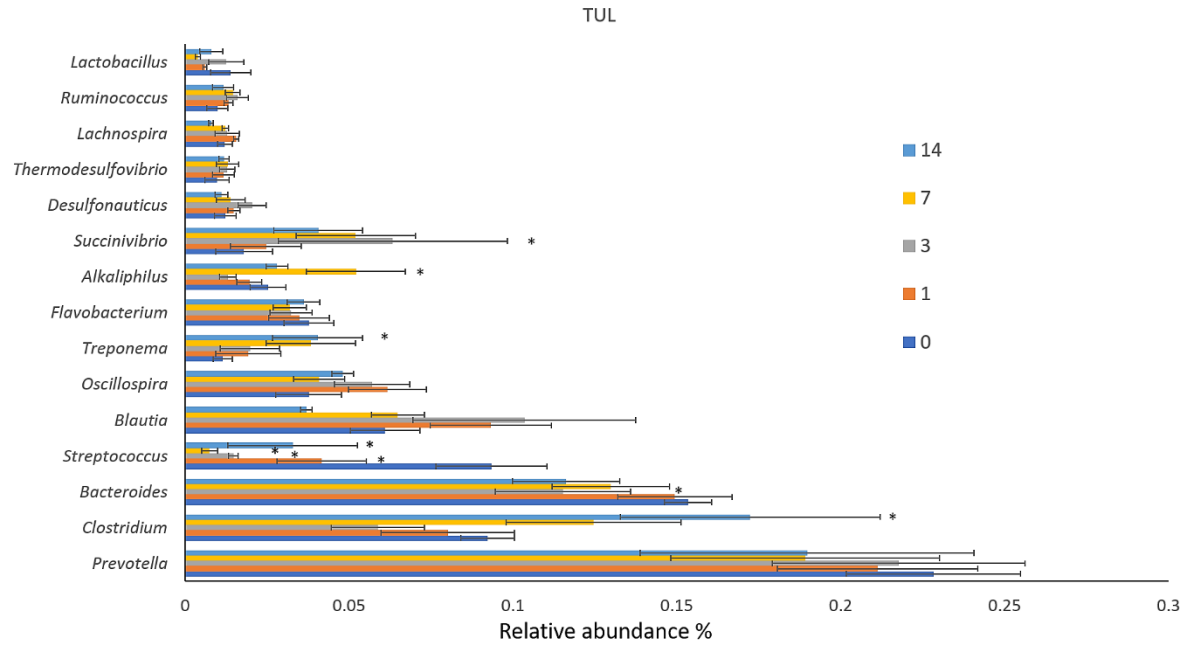


Figure 5: Bar graphs illustrating the relative abundance of top bacterial genera that averaged more than 1% observed in the fecal swabs from Oxytetracycline treatment group at different sampling days. Error bars represent standard errors. * $P < 0.05$.

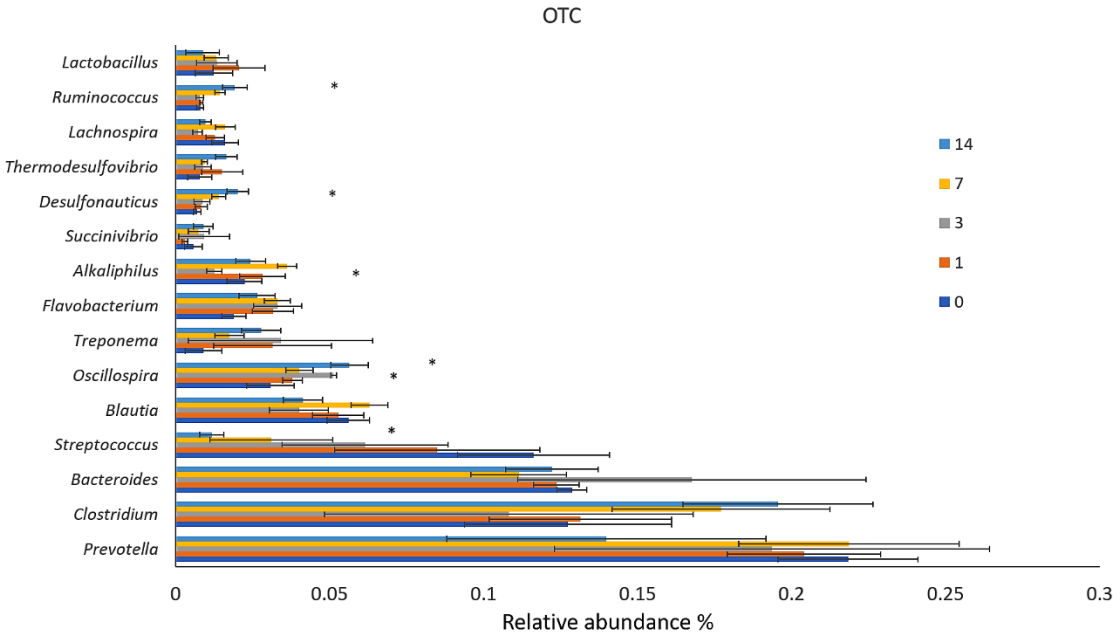


Figure 6: Bar graphs illustrating the relative abundance of top bacterial genera that averaged more than 1% observed in the fecal swabs from Procaine Penicillin G treatment group at different sampling days. Error bars represent standard errors. * $P < 0.05$.

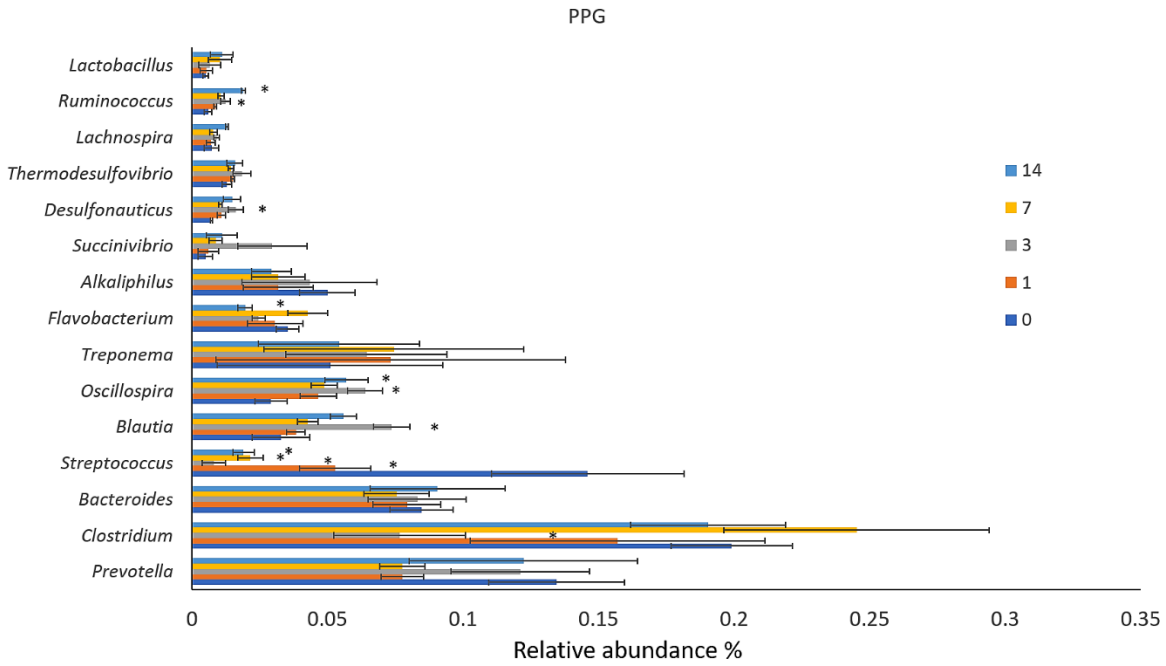


Figure 7: Bar graphs illustrating the relative abundance of top bacterial genera that averaged more than 1% observed in the fecal swabs from Ceftiofur Crystalline Free Acid treatment group at different sampling days. Error bars represent standard errors. * $P < 0.05$.

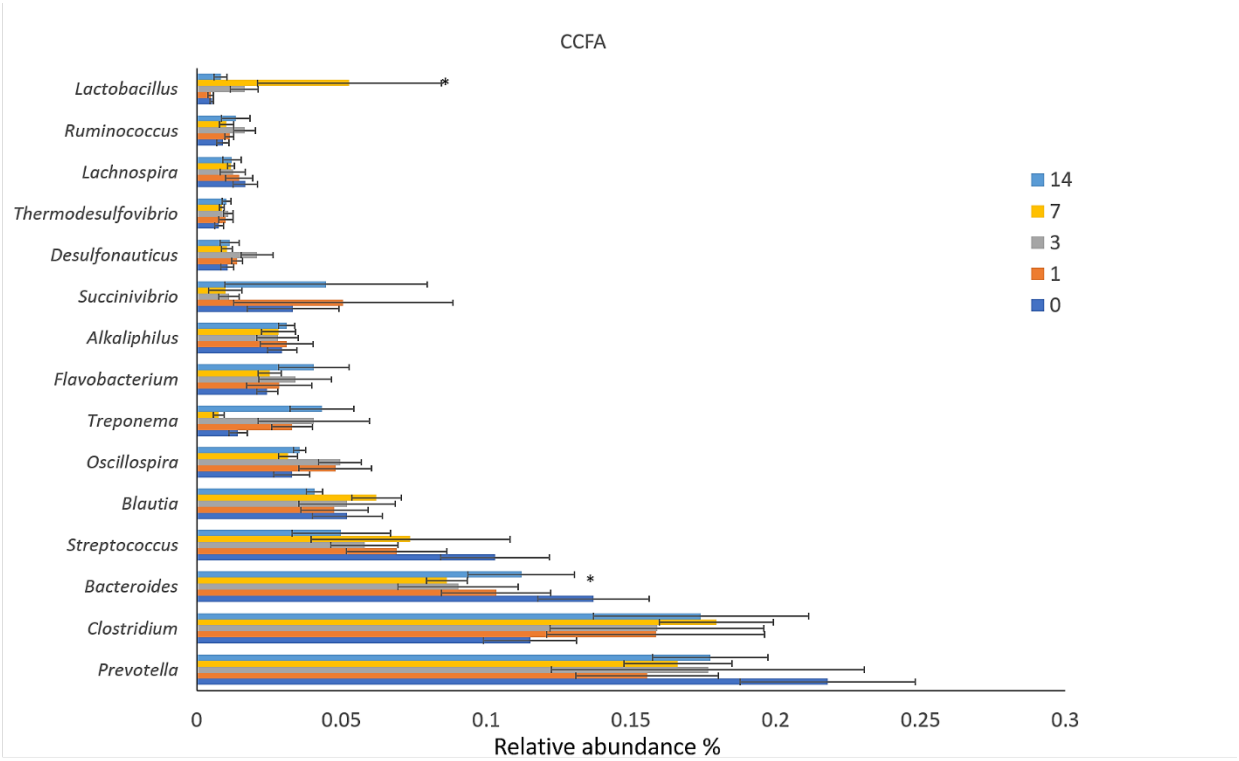


Figure 8: Bar graphs illustrating the relative abundance of top bacterial genera that averaged more than 1% observed in the fecal swabs from Ceftiofur hydrochloride treatment group at different sampling days. Error bars represent standard errors. * $P < 0.05$.

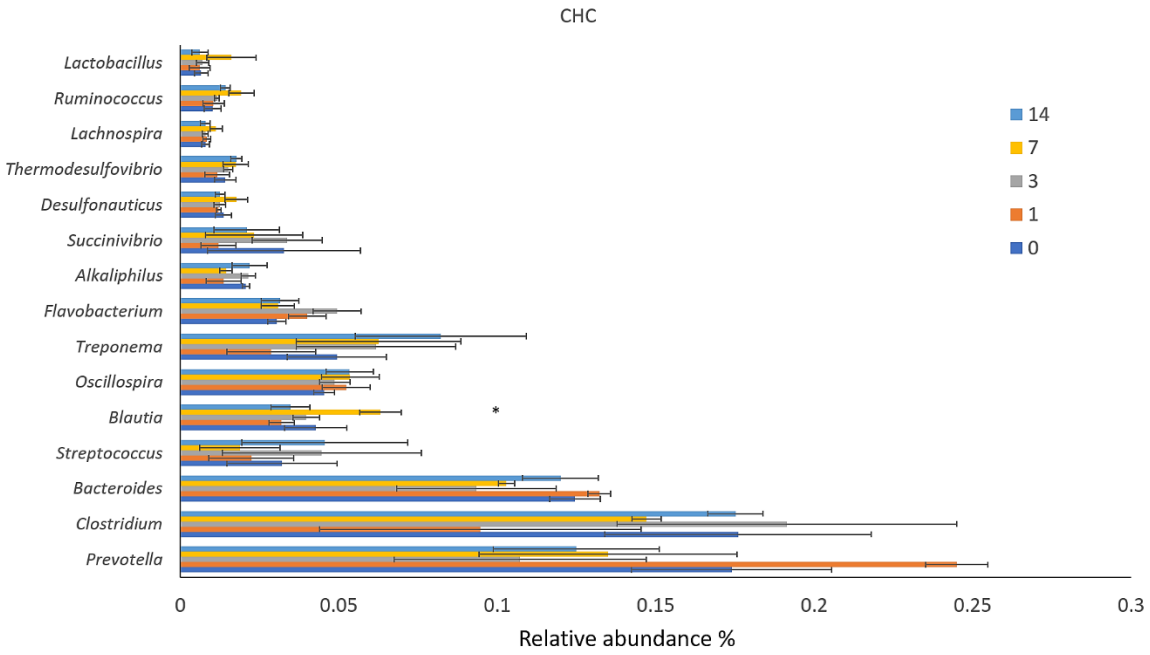


Figure 9: Bacterial diversity indices (Chao1, PD whole tree and Shannon index) measures for the fecal microbiota for different treatment groups at different sampling days. Dunnett's multiple comparisons procedure were used to compare the mean bacterial diversity indices of each sampling day (1, 3, 7 and 14) after different antibiotics administration against the day 0 before dosing. * P < 0.05.

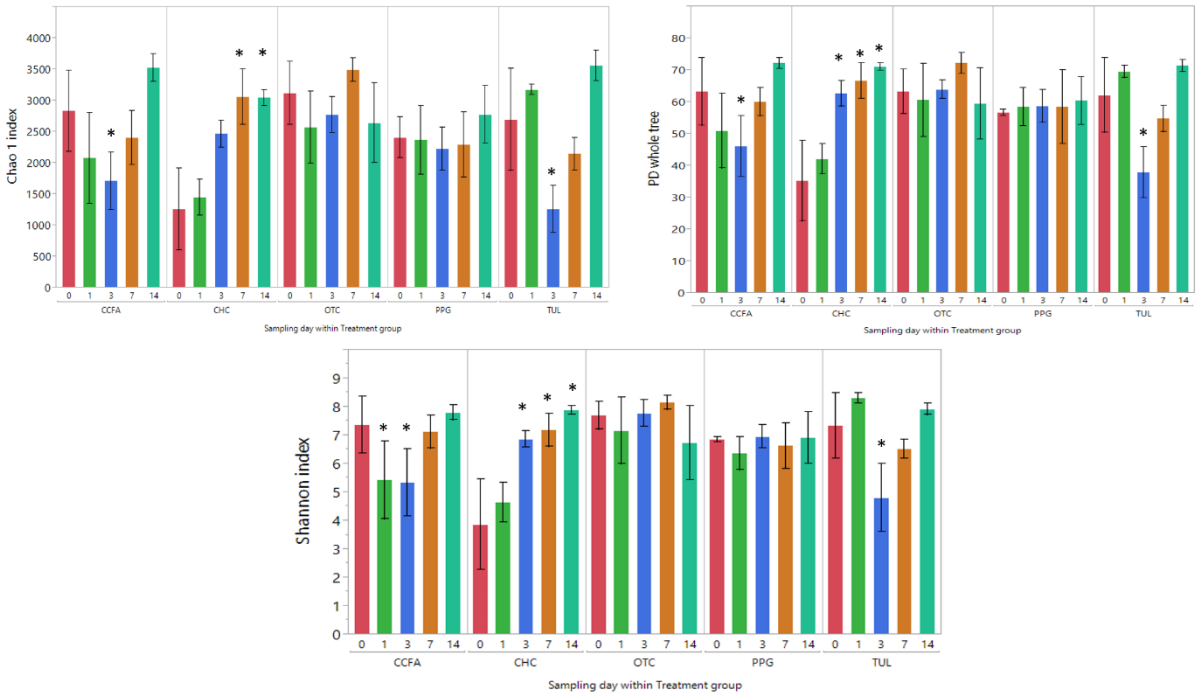


Figure 10: Linear discriminant analysis of fecal microbiota for various antibiotics adminstration at difeerent sampling days. Each dot represents an individual piglet. Linear discriminant analysis was performed using R software. Different mean relative abundances of bacterial taxa in fecal microbiota were used as covariates and sampling days for each treatment group were used as categorical variables.

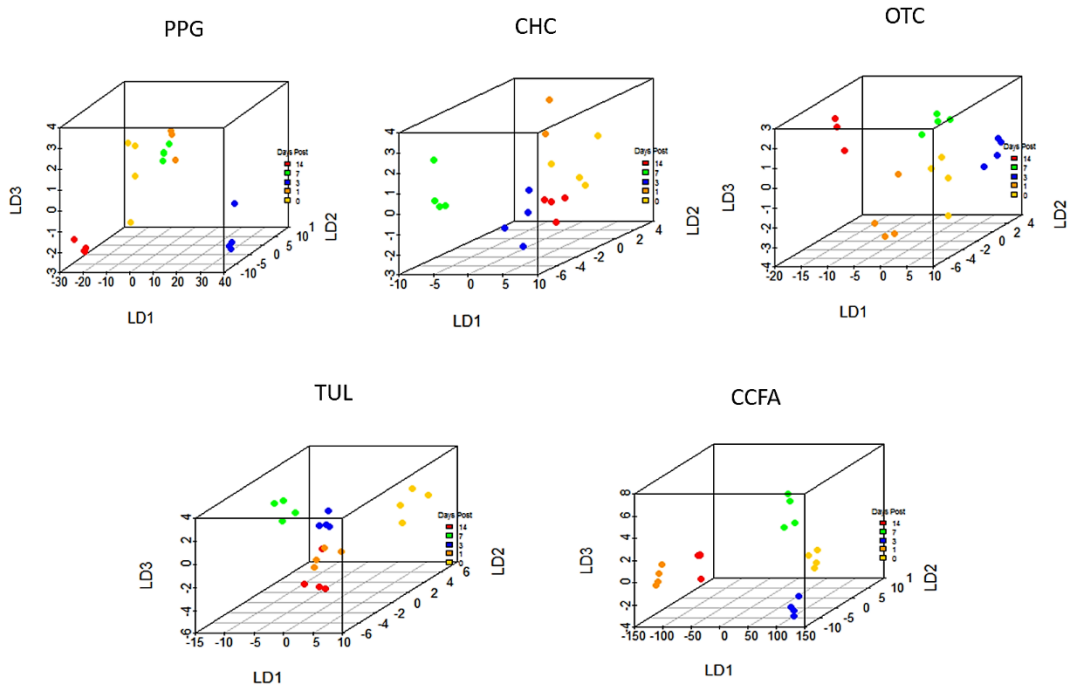


Table 1. Access Array cycling program without imaging (Fluidigm Biomark HD PCR machine) for amplifying the primer/sample combinations.

PCR Stages	**Number of Cycles**
50ºC 2 minutes	1
70ºC 20 minutes	1
95ºC 10 minutes • 95ºC 15 seconds • 55ºC 30 seconds	1
72ºC 1 minute • 95ºC 15 seconds • 80ºC 30 seconds • 60ºC 30 seconds	10
72ºC 1 minute • 95ºC 15 seconds • 55ºC 30 seconds	2
72º 1 minute • 95ºC 15 seconds • 80ºC 30 seconds • 60ºC 30 seconds	8
72ºC 1 minute • 95ºC 15 seconds • 55ºC 30 seconds	2
72ºC 1 minute • 95ºC 15 seconds • 80ºC 30 seconds • 60ºC 30 seconds	8
72ºC 1 minute	5

CHAPTER 3

EFFECT OF SINGLE DOSE OF PARENTERAL ANTIMICROBIAL ADMINISTRATION AT BIRTH ON DEVELOPMENTAL DYNAMICS OF FECAL MICROBIOTA AND PREVALENCE OF SELECTED ANTIMICROBIAL RESISTANCE GENES IN PIGLETS

3.1 ABSTRACT

Optimization of antimicrobial use in swine management systems requires full understanding of antimicrobial-induced changes on the developmental dynamics of gut microbiota and the prevalence of antimicrobial resistance genes (ARGs). The purpose of this study was to evaluate the impacts of early-life parenteral antimicrobial intervention on fecal microbiota structure, and prevalence of selected ARGs (ermB, tetO, tetW, tetC, sulI, sulII, and blaCTX-M) in neonatal piglets. A total of 48 litters were randomly allocated into one of six treatment groups soon after birth. Treatments were as follows: Control (CONT), Ceftiofur Crystalline free acid (CCFA), Ceftiofur hydrochloride (CHC), Oxytetracycline (OTC), Procaine Penicillin G (PPG) and Tulathromycin (TUL). Deep fecal swabs were collected from piglets at days 0 (prior to treatment), 5, 10, 15 and 20 after treatment. Sequencing analysis of the V3-V4 hypervariable region of 16S rRNA gene and selected ARGs were performed using Illumina Miseq platform. Our results show that, while early-life antimicrobial prophylaxis had no effect on individual weight gain, or mortality, it was associated with noticeable changes in the prevalence of selected ARGs, and minor shifts in composition of fecal microbiota during this developmental stage. Relative to CONT, only TUL treated piglets exhibited significant decline in chao1 richness index at day 20. Using Unifrac distance metrics, the microbiota composition of different treatment groups (CCFA, CHC, OTC, PPG and TUL) showed non-significant changes when compared to the CONT group. Compared to the CONT group, PPG treated piglets exhibited a significant increase in prevalence of ermB and tetW at day 20 of life. Tulathromycin intervention also resulted in a significant increase in the abundance of tetW at days 10 and 20, and ermB at day 20. Collectively, these results demonstrate that the shifts in fecal microbiota structure caused by perinatal antimicrobial intervention are modest and limited to particular groups of microbial taxa. However, early life PPG and TUL intervention could promote the selection of ARGs in herds.

3.2 INTRODUCTION

The widespread use of injectable antimicrobial in the treatment and prevention of human and animal diseases continues to rise globally (MacKie et al., 2006). Numerous concerns related to human and animal health have been raised regarding the long-term sequelae of this trend, including emergence of antibiotic-resistant bacteria, dissemination of ARGs into the environment, perturbations of the gut microbiota-ecosystem and increased risk of diseases (Chee-Sanford et al., 2001) (Hoelzer et al., 2017). Antimicrobial resistance develops when the administered antimicrobial eliminates susceptible microorganisms but leaves behind resistant strains that continue to grow and multiply in its number (Wegener, 2003). The resistant bacteria transmit their genetic resistance characteristics to their progeny through vertical evolution, or to other bacterial species through horizontal evolution (Holmes et al., 2016). Recently, several lines of evidence indicate that extensive use and misuse of existing antimicrobial increases the numbers of ARGs copies and risk of their spread among commensal bacterial population (Roca et al., 2015) (Czaplewski et al., 2016).

Traditionally, the majority of studies evaluating the effect of antimicrobial administration on emergence of antibiotic resistant bacteria and ARGs have focused on pathogenic organisms using culture-based methods (Thanner et al., 2016). While this approach has enhanced our understanding of the nature of antimicrobial resistance in a single class of organisms for certain antibiotic, it is limited in itsto ecosystem-level applications. The Aadvancements in culture independent techniques, e.g. next generation sequencing, have showed a higher diversity of microbial populations in several animal gut locations and have helped in the assessment of antimicrobial resistance determinants at the microbial ecosystem-level (Zhao et al., 2017).

Immediately after birth, the swine gastrointestinal tract is colonized by a complex microbial ecosystem that plays a crucial roles in intestinal configuration, immune system maturation, and host gene expression (Zhang et al., 2016). During this stage, the microbial ecosystem is unstable and highly susceptible to various environmental factors, including antimicrobial administration, dietary intervention and stress exposure (Schokker et al., 2014). Given the instability of microbiota at this phase, the microbial population has the potential to disseminate and transfer ARGs, which could have significant effects on development of metabolic and immune disorders (Gibson et al., 2015) (Su et al., 2017). In intensive swine management systems, newborn piglets are frequently administered antimicrobials, mainly to

prevent outbreaks of infectious diseases; however, the effects of early-life antimicrobial prophylaxis on the emergence of ARGs and its connection with the gut microbial community in piglets are poorly understood. Recently, early-life antimicrobial intervention in newborn piglets revealed long-lasting impacts on gastrointestinal microbial diversity and composition (Schokker et al., 2014). In our previous study exploring the change of fecal microbiota of eight weeks old growing piglets in response to parenteral antimicrobial administration, the fecal microbiota showed antimicrobial-specific variation in both duration and extent (Zeineldin et al., 2018). To gain further insight into the swine gut ecosystem and to find alternatives to antimicrobial, it is crucial to understand the developmental dynamics of gut microbiota and prevalence of ARGs in response to perinatal antimicrobial administration in piglets. Consequently, the aim of this study was to investigate the short-term impact of the commonly used antimicrobial during early-life on developmental dynamics of fecal microbiota, and prevalence of selected ARGs (ermB, sulI, sulII, tetC, tetO, tetW and blaCTX-M) in suckling piglets using high-throughput sequencing analysis.

3.3 MATERIALS AND METHODS

3.3.1 Ethics statement

The study procedure was conducted in compliance with the recommendations of the guidelines for the care and use of animals of University of Illinois at Urbana-Champaign. The protocol was approved by the Ethical Committee for Institutional Animal Use and Care of the University of Illinois at Urbana-Champaign.

3.3.2 Experimental design and samples collection

The experiment was conducted in a commercial swine farm in the Midwestern US with consent from the facility owner. A total of 48 sows with their newborn piglets (678 piglets in total) were used in this study based on a randomized complete block design with farrowing day and dam parity group as blocks. Approximately five days before farrowing, the pregnant sows were transferred to a farrowing pen and kept there until the end of the experiment. Sows were fed a standard lactation diet and provided ad libitum access via an automatic dry feeding system, and were given ad libitum access to water from a nipple drinker. Directly after birth, litters were randomly assigned into one of six groups; control (CONT; n= 113 pigs), ceftiofur hydrochloride

(CHC; n= 113 pigs), ceftiofur crystalline free acid (CCFA; n=115 pigs), oxytetracycline (OTC; n=114 pigs), procaine penicillin G (PGP; n=115 pigs) and tulathromycin (TUL; n= 108 pigs). Littermates were used to minimize differences arising from maternal microbiota. After farrowing, treatment was applied and all pigs were identified. All of the piglets in a litter were assigned to a single treatment group. The dosage schedule for each treatment group was; CONT (saline 1cc IM), CHC (5 mg/kg IM), CCFA (5.0 mg /kg IM), OTC (22mg/kg IM), PGP (33,000 units/kg) and TUL (2.5 mg/kg IM)]. Ceftiofur crystalline free acid and CHC are a third-generation cephalosporin with a broad-spectrum activity against both Gram-positive and Gram-negative bacteria (Chander et al., 2011). OTC is a tetracycline antibiotic that also directly targets both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001). Procaine penicillin G is one of the beta-lactam antibiotics that targets Gram-positive and Gram-negative bacteria (Ranheim et al., 2002). Tulathromycin is one of macrolides antibiotics, act by inhibit bacterial essential protein biosynthesis of both Gram-positive and Gram-negative bacteria (Schokker et al., 2014). On the basis of prosperous antimicrobial characteristics of the used antibiotics, those antimicrobial class are considered the most popular approved antibiotics used in the swine industry for the control and treatment of swine diseases (Schwarz et al., 2001).

The treated piglets were housed in a conventional farrowing pen that was approximately 1.9m x 2.6m where the sow was confined so that she could not turn around and the sidewall penning for the piglets was solid to prevent contact between litters. All farrowing pens were in a controlled environment. All pigs were allowed to suckle colostrum and piglets were not added to the birth litter (some were removed prior to treatment if there were more pigs than available mammary glands). The antimicrobial dosages and routes of administration were based on the manufacturer label instructions. The piglet's tails were not docked, and teeth were not polished. All piglets were weighed individually, and dead piglets were recorded throughout the study. Two piglets per litter were individually identified and individual deep fecal swabs (Pur-Wraps®, Puritan Medical Products, Gulford, Maine) were collected immediately prior treatment (day 0), and again on days 5, 10, 15, and 20 after dosing. All piglets were weaned at day 21 of life (a common weaning time in the pig industry). The fecal swabs were snap-frozen in sterile containers and transported to the laboratory on the same day. Samples were kept at -80° C pending further processing.

3.3.3. Extraction of genomic DNA and Illumina sequencing

Four clinically healthy piglets from each group (CONT, CCFA, CHC, OTC, PPG and TUL) at the different sampling days (0, 5, 10, 15 and 20) were selected for the microbiota analysis. In a decontaminated, sterile environment, microbial DNA was extracted from each fecal swab and from negative control sample (sterile cotton swab and extraction kit reagent) using Power Fecal DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Fecal swabs were mixed with 750 µl of Bead Solution (Power Fecal DNA Isolation kit, MO BIO Laboratories, Inc.), and bead beating was carried out in Bullet Blender 24 Gold tube holder machine (Next Advance, Inc., Averill Park, NY, USA) for 10 minute. Then the extraction process was completed according to power® Fecal DNA extraction Kit manufacturer manual. The concentration and integrity of DNA were assessed using a Nanodrop™ spectrophotometer (NanoDrop Technologies, Rockland, DE, USA), and agarose gel electrophoresis (Bio-Rad Laboratories, Inc, Hercules, CA, USA) respectively.

The hypervariable region V3-V4 of 16S rRNA gene was amplified and sequenced on the Illumina MiSeq™ sequencer (Illumina, San Diego, CA, USA) at the DNA Services lab at the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois at Urbana-Champaign, Urbana, IL). The extracted DNA was subjected to Fluidigm Access Array Amplification (Fluidigm Corporation, South San Francisco, CA, USA). All extracted DNA concentration was assessed on a Qubit (Life Technologies, Grand Island, NY, USA) using the High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA).

A total of seven primer sets targeting seven different ARGs against the most popular antibiotic classes used in the swine industry, were used (Table 2). Additionally, the primer sequences F357 -for (CCTACGGGNGGCWGCAG) and R805-rev (GACTACHVGGGTATCTAATCC) were designed with an attached eight base barcode sequence that was unique to each samples to amplify the V3-V4 hypervariable region of 16s rRNA gene. A mastermix for amplification was prepared using the Roche High Fidelity Fast Start Kit and 20x Access Array loading reagent according to Fluidigm protocols. PCR reactions consisted of DNA sample, 20X Access Array Loading Reagent, forward and reverse primer, Fluidigm Illumina linkers with unique barcode, and water to a final volume of 100 ul. PCR reactions were performed on a Fluidigm Biomark HD™ PCR machine (Fluidigm Corporation, South San Francisco, CA, USA). Amplified PCR stocks were purified on a Fragment Analyzer

(Advanced Analytics, Ames, IA) and to confirm amplicon regions sizes. The final fluidigm pools were quantitated by qPCR on a BioRad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc. CA). All pools were then combined in equimolar ratio, spiked with 15% non-indexed PhiX control library, and loaded onto the MiSeq V3 flowcell at a concentration of 8 pM for cluster formation and sequencing. The final genomic libraries were then sequenced from both ends following manufacturer's guidelines (Illumina, Inc., San Diego, CA, USA).

3.3.4 Sequence data processing and microbial community analysis

The raw sequence data were preprocessed from Illumina base call (bcl) files into compressed paired-end read fastq files (2 × 300) using bcl2fastq 1.8.4 (Illumina, San Diego, CA, USA) without demultiplexing, and then sorted by initial PCR-specific primer using a custom in-house pipeline. The generated bcl files were converted into demultiplexed compressed fastq files using bcl2fastq 1.8.4 (Illumina, CA). A secondary pipeline decompressed the fastq files, generated plots with quality scores using FastX Tool Kit. Trimmomatic was used to trim the low-quality base at the overlapping end of the raw sequence reads (Bolger et al., 2014). Barcode and sequencing primers were also trimmed from the assembled sequence. After preprocessing, the 16S rRNA gene sequences data were analyzed using the open source Quantitative Insights into Microbial Ecology (QIIME) software, version 1.9 (http://qiime.org/) (Caporaso et al., 2010). Sequence reads were quality filtered using the default parameters (Bokulich et al., 2012)**.** The de novo open-reference operational taxonomic unit (OTU) clustering process was conducted by QIIME with 97% as similarity using UCLUST clustering (Edgar, 2010), and was taxonomically assigned against the Silva reference database (Quast et al., 2013). Chimeric sequences were detected and removed using UCHIME prior to downstream analysis (Edgar et al., 2011). One sample from the TUL-treated piglets was not included in the analysis due to unsuccessful sequencing. Two OTUs detected as a contaminant in negative controls (classified as *Stenotrophomonas* and *Xanthomonas*) were removed prior to analyses. The alpha diversity (within community) were calculated within QIIME using Chao 1 richness index, Shannon diversity index. To standardize our analysis due to uneven sequencing depth, all samples were randomly rarefied to 1358 sequences per sample. To compare overall microbiota composition among samples, a beta diversity analysis was performed considering the abundance of each detected OTUs in each sample using weighted UniFrac distance and was displayed using

principal coordinate analysis (PCoA). Weighted uniFrac phylogenetic distances were used to generate the distance matrices and calculate the degree of differentiation among the samples. Finally, a Venn diagram was generated for graphical descriptions of the number of unique and shared OTUs between treatment groups.

Statistical analysis and graphing were performed using PAST version 3.13, JMP 13 (SAS Institute Inc.) and RStudio (version 1.1.383, R Studio, Inc., Boston, MA, USA). Data were logarithmically transformed or ranked when necessary to achieve normality and homogeneity of variance prior to statistical analyses. Significance difference was stated at $P < 0.05$. Statistical comparisons of weighted UniFrac distances between treatment groups at the different sampling days were determined using analysis of similarity (ANOSIM) statistical methods with 9999 permutations and Bonferroni corrected *P* values in PAST version 3.13. Due to the fact that the same piglets were sampled repeatedly over the course of the study, repeated measures ANOVA with post hoc Tukey's honestly significant difference (HSD) pairwise comparisons was performed to compare the difference in microbial relative abundance and alpha diversity indices between the treatment groups. To further identify the taxon showing significant differences between the different time points in the same groups and between the groups at the same time point, the OTUs abundance were assessed using the linear discriminant analysis (LDA) effect size (LEfSe) pipeline fitted in Galaxy (https://huttenhower.sph.harvard.edu/galaxy/) (Segata et al., 2011). We then compared the overall microbial communities between the treatment groups (CONT, CCFA, CHC, OTC, PPG and TUL) using stepwise discriminant analysis in JMP 13 (SAS Institute Inc.). For this analysis, the relative abundances of different bacterial genera in each group were used as a covariate, and the treatment groups were used as the categorical variable. The discriminant analysis was used to determine how equivalent samples, from animals in different groups, were differentiated from one another, and was illustrated using canonical loading plots.

3.3.5 Selected antimicrobial resistance genes quantification

For ARGs sequence classification, we have developed a customized version of the Antibiotic Resistance Gene Database (ARG-ANNOT) incorporated all sequences of the seven ARGs that used in this study. The customized ARG-ANNOT database was used to align the different the ARGs raw sequences reads obtained from the Illumina sequencing into different

types according to the used primers. The number of ARGs sequences depth and coverage in each ARGs have also counted. To avoid the bias, normalization of the ARGs reference sequence length by the 16S-rRNA gene sequence length was conducted. The abundance of ARGs was expressed as copy of ARG per copy of 16S-rRNA gene. The relative abundance of ARGs was calculated using the following equation (Li et al., 2015a):

$$\text{Abundance} = \sum_{1}^{n} \frac{N\ ARG - Like\ sequence \text{X}\ L\ reads\ /L\ ARG\ reference\ sequence}{N\ 16S\ sequence\ \text{X}\ L\ reads\ /L\ 16S\ sequence}$$

The difference in ARGs abundance, between the treatment groups at the different sampling days were analyzed using repeated measures ANOVA with pairwise post hoc Tukey's honestly significant difference (HSD) comparisons in PAST version 3.13. Dunnett's multiple comparisons procedure was also used to compare the mean ARGs abundance in different treatment group (CCFA, CHC, OTC, PPG and TUL) at each sampling day (0, 5, 10, 15 and 20), against the CONT group at the same time point. The difference in overall ARG abundance among treatment groups were compared using PCA fitted in STAMP software (Parks et al., 2014). Differences with a value of $P<0.05$ were considered significant.

3.3.6 Accession number

Raw paired-end Fastq sequence data obtained in this study were submitted to the Sequence Read Archive of the NCBI to make the files available for a public database with bio-project accession number PRJNA407634.

3.4 RESULTS

3.4.1 Impact of antimicrobial treatment on body weight gain and overall mortality ratio

The body weight of piglets was measured at day 0 just prior to the treatment and again at day 20 of age. There was no significant change in the average daily weight gain between day 0 and day 20 in the CCFA, CHC, OTC PPG and TUL groups compared to CONT ($P > 0.05$). The treated piglets showed non-significant changes in the overall mortality ratios (day 0 to 20) compared to control group ($P > 0.05$). However, the TUL treated piglet showed numerically increased in the mortality percent at day 15-20 (Figure 11). Our results showed that the early life antimicrobial intervention failed to reduce mortality and increase the average daily weight gain in neonatal piglets.

3.4.2 Summary of sequence data analysis

After quality filtering and removal of the low quality sequence, a total of 2,508,268 sequences were obtained from all samples. The number of sequence per sample ranged from 5307 to 48524 (mean 15201.624, SD 7324.965). Using the criterion of 97% identity cutoff, 1296 OTUs were identified among all samples, all of which were taxonomically assigned according to Silva databases classification. Collectively, most OTUs were shared among the treatment groups with only 8, 5, 18, 11, 4 and 7 OTUs were uniquely identified in piglets from the CONT, TUL, CCFA, CHC, PPG and OTC group, respectively (Figure 12).

3.4.3 Microbial taxa affected by early-life antimicrobial intervention

At the phylum level, the microbial composition in all treatment groups was markedly varied according to ages (Fig. 3). At day 0, *Proteobacteira* was the most predominant, representing 79%, 76%, 82%, 85%, 85%, and 91 % of all bacterial populations in CONT, CCFA, CHC, OTC, PPG and TUL respectively. While at day 20, *Firmicutes* was the most abundant, representing 61%, 43%, 47%, 40%, 32% and 41 % of all bacterial populations in CONT, CCFA, CHC, OTC, PPG and TUL respectively. Compared to CONT group, TUL treated piglet exhibited lower abundance of *Actinobacteria* at day 5 ($P= 0.029$). Furthermore, CONT piglets showed higher relative abundance of *Firmicutes* compared to those in PPG group at days 15 and 20 ($P= 0.031$ and 0.016) respectively (Figure 13).

At the genus level, a total of 189 genera were identified from all samples. The core fecal microbial community (defined as the genera found at a relative abundance of > 1 % in all piglets) at the baseline (day 0) was comprised of common fecal microbial genera including *Escherichia-Shigella* (41.24%), *Clostridium* (17.33%), *Fusobacterium* (4.58%), *Bacteroides* (3.39%), *Actinobacillus* (3.04%), *Streptococcus* (3.01%), and *Lactobacillus* (2.44%). A hierarchically clustered heatmap of the most predominant microbial communities at the genus level is shown in (Figure 14). Compared to CONT group, the TUL treated piglets showed decline in the abundance of *Ruminococcus* at day 15 and *Actinomyces* at days10 and 20 of life. In contrast, TUL treated piglets showed increase in the proportion of *Escherichia-Shigella* at day 5 and *Bacteroides* at day 14. In the CCFA group, the treated piglets exhibited increase in the proportion of *Campylobacter* at day 5, *Rikenellaceae RC9 gut group* at day 15 and reduction in the proportion of *Lactobacillus* at day 5, *Streptococcus* at day 5, *Prevotella* at day 15. In the CHC group, the treated piglet showed reduction in the proportion of *Streptococcus* at day 5, and increase in the proportion of *Campylobacter* at day 10. OTC treated piglet exhibited increase in the relative abundance of *Escherichia-Shigella* at day 5, *Bacteroides* at day 15, and reduction in the relative abundance of *Lactobacillus* at day 5. In the PPG group, the treated piglets showed reduction in the proportion of *Fusobacterium* at day 10 and *Clostridium* at day 20. On the other hand, the PPG pigs revealed increase in the proportion of *Olsenella* at day 15 and day 20, *Escherichia-Shigella* at day 15, *Bacteroide at* day 15 and day 20.

Alpha-diversity was computed using Chao1 richness and Shannon diversity indices (Fig. 15). Altogether, the metagenomics analysis revealed that microbial diversity and richness indices were increased with age ($P< 0.001$). Compared to CONT group, only TUL treated piglets exhibited a significant decrease in Chao 1 at day 20 ($P=0.031$) (Figure 15). Beta diversity analysis showed that the overall fecal microbiota composition at the baseline (day 0) did not differ between CONT, CCFA, CHC, OTC, PPG and TUL groups (ANOSIM, $P = 0.17$). The early life antimicrobial induced changes in the microbial community composition were not sufficient to cluster microbial populations at the different time points as shown by PCoA (ANOSIM, $P > 0.05$; Figure 16). However, there was a significant effect of sampling time on the overall microbial community composition ($P < 0.0001$, $R^2 =0.36$).

The differences in microbial relative abundance between CONT and treated piglets were additionally compared using the LEfSe algorithm (Figure 17). Compared to control group, 15, 6,

14, 8 and 9 OTUs were identified as indicator taxa in CHC, OTC, TUL, PPG and CCFA treated piglets respectively (Figure 17). Additionally, a number of potential indicator taxa that differentially represented in the treatment groups at the same age with their LDA scores are depicted in (Figure 18). Collectively, the changes in the fecal microbiota structure caused by perinatal antimicrobial intervention are limited to a particular group of microbial taxa (Figure 8).

3.4.4 Relationships among the overall microbiota composition of the six treatment groups

A multiple group similarities tree was constructed using Unifrac distance metrics to identify the similarities and differences among the antimicrobial treatment (Figure 19A). Collectively, comparison of the microbiota composition of the different treatments group (CCFA, CHC, OTC, PPG and TUL) showed no significant changes when compared to the CONT group (Table 3). However, the taxonomic composition of TUL treated piglets was separated from the compositions of the CHC and CCFA treated piglets (ANOSIM, P=0.024 and 0.015) respectively (Figure 19A & 19B). The taxonomic composition of the PPG treated piglets was closest to that in the OTC group, indicating that these two treatments resulted in more similar community structures (Figure 19A & 19B). The taxonomic compositions of the CCFA and CHC piglets were clustered together indicating that these two treatments also resulted in similar community structures (Figure 19A & 19B).

3.4.5 Effect of early life antimicrobial administration on selected ARGs

In this study, we quantified seven ARGs in relation to the bacterial 16S rRNA gene. All tested ARGs were detected, except *bla* $_{\text{CTX-M}}$ was below the limit of quantification in all samples. Across all samples, the highest level of ARGs was associated with *erm B* (33.85%), *tet W* (11.65%), and *Sul* II (9.06%). The effect of the different antimicrobial treatments on the ARGs abundance as copy of ARG per copy of 16S-rRNA gene are summarized in (Figure 20). Compared to CONT group, the early life TUL intervention resulted in significant increase in the abundance of *tet W* at days 10 and 20 (P<0.05), *erm B* at day 20 (P<0.05) (Fig. 10). PPG-treated piglets exhibited a significant increase in the prevalence of *ermB* and *tetW* at day 20 of life (P<0.05). In CCFA, CHC and OTC groups, comparisons of ARGs abundance showed no significant differences after antimicrobial administration compared to the CONT group at the same time point (P>0.05) (Figure 20). Principal component analysis of the overall ARGs

prevalence (*erm* B, *tet* O*, tet* W*, tet* C*, sul* I*,* and *sul* II) revealed that samples from CONT and TUL treated piglets were clustered into two distinct groups (ANOSIM; P= 0.015). The overall ARGs prevalence of CCFA, CHC, OTC, and PPG treated piglets showed no significance difference when compared to CONT group (ANOSIM; P>0.05**)**.

3.5 DISCUSSION

The extensive use of existing antimicrobial led to emergence of antibiotics resistant bacteria and various ARGs in the environment, which is thought to pose an imminent threat to animal and human health (Berendonk et al., 2015). Several research studies have revealed microbial shifts in the swine gastrointestinal microbiota after antimicrobial administration (Kim et al., 2012) (Zhao et al., 2018) (Zeineldin et al., 2018). In order to overcome these conflicts, production systems must adapt to reduce the overall demand for antimicrobial. A key step in the process is to understand the mechanism and magnitude by which antibiotic administration affects the microbial mucosal integrity, emergence of ARGs, and overall host health. The ability to link the changes in the emergence and dynamics of ARGs and resident microbiota would allow the exploration of complex interactions that have not been possible in the past. Multiple lines of evidence indicate that the gastrointestinal tract of swine is a complex and diverse microbial ecosystem, where extensive communication between host, mucosal communities, and the surrounding environment, occur concurrently (Li et al., 2017a).

Parenteral antimicrobial are extensively utilized therapeutically in swine for control or prevention of infectious disease (Pyörälä et al., 2014). Sound scientific evidence shows that antimicrobial intervention can display either a detrimental or a beneficial effect on gut health (Phillips et al., 2004), but this has not been widely studied in neonates. This study used 16S rRNA genes sequencing approach to quantify the impacts of a single dose of early life antimicrobial on fecal microbiota structure, and prevalence of selected ARGs (*erm*B, *tet*W, *tet* O, *tet*C, *sul*I, *sul*II, and *bla* $_{\text{CTX-M}}$) in neonatal piglets. In line with other studies, our results demonstrated that the fecal microbial communities in all treatment groups were dominated by *Firmicutes, Proteobacteria* and *Bacteroidetes* at the phylum level (Maradiaga et al., 2018), and by *Escherichia-Shigella, Bacteroides, Lactobacillus, Clostridium*, and *Streptococcus* at the genus level (Maradiaga et al., 2018). In terms of temporal changes, time-dependent dynamics of the

piglet fecal microbiota were observed. The neonatal piglets at day 0 had a significantly greater proportion of *Escherichia-Shigella*, *Fusobacterium, Clostridium*, and *Actinobacillus.* The piglet fecal microbial communities composition observed in this study at day 0 soon after birth was similar to that published by (Kubasova et al., 2017). Although *Escherichia* and *clostridium* are the first genera colonize the gastrointestinal tract in different species (Rodríguez et al., 2015) (Slifierz et al., 2015), the existence of *Fusobacterium* member in gut microbiota in day 0 old piglets is of concern since *Fusobacterium* promotes the development of swine dysentery (Durmic et al., 1998). In 20-day-old piglets, *Lactobacillus, Bacteroides, Ruminococcus,* and *Ruminococcaceae UCG-005* were the most abundant microbiota member, which similar to a previous report (Slifierz et al., 2015) (Kubasova et al., 2017). While our study revealed that aging is the most significant driver of development of the fecal microbiotas, understanding the early colonization pattern of gut microbiota will open the door to new perspectives on the utility of early life antimicrobial administration to healthy neonates in swine management systems.

Contrary to the common disruption of swine gut microbiota that can result from infeed antibiotic exposure (Looft et al., 2012) (Kim et al., 2012) (Kim et al., 2016) (Zhao et al., 2018), the changes in microbiota composition that were observed with early life antimicrobial intervention were modest and restricted to a particular group of taxa. The observed changes in the developmental dynamics of fecal microbial populations showed antimicrobial-specific variations in both duration and extent. Our findings are generally in line with a previous study that evaluated the impact of antimicrobial treatment on the microbiota composition and resistance gene reservoir (Choo et al., 2018). Using 16S rRNA gene sequencing, Choo et al. domenstared that the disruption in microbiota composition was restricted to relatively small group of *Actinomyces* species (Choo et al., 2018). Additionally, the noticed reduction in the relative abundance of *Actinomyces* population in response to TUL treatment are in agreement with other study (Smith et al., 2005). Species of *Actinomyces* genus, facultative gram-positive anaerobes, are characteristically considered lactate utilizing commensal bacteria and can be habitually detected in the female genital tract, and gastrointestinal tract of healthy individuals (Takahashi, N., 1999)(Smith et al., 2005). Whether these decreases in the abundance of *Actinomyces* are of clinical consequence, however, is not currently definite. Of particular interest in our results is the decrease in proportion of *Ruminococcus* in the CCFA, CHC and TUL treated piglets compared with CONT group. Member of these genera are commonly associated with gut

health (Yu et al., 2018). Additionally, TUL, PPG and OTC treated piglets showed increase in the proportion of *Escherichia-Shigella* and *Bacteroides*. *Escherichia spp*. are commonly found in the farm environment and are considered indigenous to the piglets gut microbiota (Yang et al., 2004) (Bischoff et al., 2002) (Chapman et al., 2006). Similarly, increase the abundance of *Bacteriodes* spp. during early life is considered disease predisposing condition (Korpela et al., 2016) (Tran et al., 2018).

PPG treated piglets also exhibited increase in the proportion of *Olsenella* at day 15 and day 20. Members of genus *Olsenella* are Gram positive rods and consider skatole-producing bacterium, which is responsible for boar taint and off-flavor taint (Dewhirst et al., 2001) (Gupta et al., 2013)(Li et al., 2015b). In CCFA and CHC group, the treated piglets exhibited increase in the proportion of *Campylobacter* at day 5 and day 10 respectively. *Campylobacter spp.,* particularly *C. jejuni* are considered one of the most common cause of human enteritis (Dicksved et al., 2014) and swine dysentery (Yang et al., 2017). Taken together, our result suggests that early life antimicrobial intervention may make the gastrointestinal tract more susceptible to potential pathogenic bacteria (Schokker et al., 2015).

Bacterial diversity within community are often used as a crucial measure of functional resilience and homeostasis of gastrointestinal microbial ecosystem (Lozupone et al., 2012). We used Chao1, and Shannon indices to compare the alpha diversity among different treatment groups. Bacterial diversity indices suggest that the piglet's fecal microbiotas were rich and diverse, and undergo intricate development during the first 20 days of life. Similarly, gastrointestinal tract of piglets during early life, showed an age-dependent manner of microbial population evolution and acquisition (Bian et al., 2016). In the present study, Only TUL intervention decreased Chao1 richness index only at day 20. In line with other study, the early life antimicrobial induced changes in the microbial community composition were not sufficient to cluster microbial populations at the different time points as measured by beta diversity analysis (Zhang et al., 2016) (Li et al., 2017b). In contrast (Looft et al., 2014) observed a significant changes in diversity indices after early life of carbadox administration of in 6-weeks-old piglets. The discrepancies between the present study and the previous research might be resulted from the use of different type of antibiotics, dosage and route of administration, and different environmental conditions or the ages of study populations (Li et al., 2017b).

To investigate the indicator taxa that significantly discriminated between CONT and other treatment groups, we used a well-established approach, LEfSe (Segata et al., 2011). In this study, LEfSe revealed 15, 6, 14, 8 and 9 OTUs as indicator taxa in CHC, OTC, TUL, PPG and CCFA treated piglets respectively. These results further support the concept that the shifts in the fecal microbiota structure caused by perinatal antimicrobial intervention are modest, and are limited to a particular group of microbial taxa.

As we mentioned previously, the use of antimicrobial has been compromised due to the emergence of resistant bacteria and increase ARGs abundance and diversity that can endure beyond the antibiotics cessation. We assessed carriage of seven different ARGs genes (*Tet W, Tet O, Tet C, Sul I, erm B, Sul II,* and *bla CTX-M*) in relation to the bacterial 16S rRNA gene, based on their identification in previous research. The tested ARGs belongs to the most abundant type of ARGs conferring resistance to macrolides, beta lactams, sulfonamide and tetracycline, and can be carried by common members of the gut microbiota (Li et al., 2016). Our results demonstrate that the ARGs are developed in the gut microbiota from the first day of life. Compared to CONT group, TUL and PPG treated piglets exhibited significant increase in abundance of macrolide resistance *ermB* gene. This finding is in line with the increased carriage of *erm*B after long-term administration of antimicrobial in healthy individuals (Choo et al., 2018). Macrolides *erm* B resistance genes can horizontally transfer between microbial species via transformation or conjugation, permitting commensal microbiota to serve as resistance reservoirs (Roberts et al., 2011). Additionally, the TUL and PPG treated piglets exhibited significant increase in abundance of *tetW* resistance genes, encoding for efflux pump proteins. The enrichment of these efflux pump proteins resistance genes in the absence of tetracycline antimicrobial administration results in negative selection of bacteria carrying these genes from the whole community (Gerzova et al., 2015). Interestingly, the change in the proportion of bacterial population carrying the resistance genes *ermB* and *tetW* had a similar temporal pattern. This might indicate that these genes can be loaded together on the same genetic transferable element (transposon) or the resistant population showed multiple drug resistance bacteria in these piglets (Rubio-Cosials et al., 2018). These findings suggest that single dosages of TUL and PPG antimicrobial can increase ARGs abundance even for antimicrobial class not administered to the animals. Moreover, increases the levels of transmissible ARGs within the developing fecal

microbiota highlight microbial ecosystem potentiality to act as a resistance reservoir (Looft et al., 2012).

Our study had a number of experimental limitations that should be considered. The metagenomics analysis was conducted on a relatively small number of piglets, though similar to other published metagenomics studies (Yu et al., 2018). Furthermore, our analysis focused on the short-term impact of antimicrobial administration on fecal microbiota. Finally, our study focused on identification of selected transmissible antibiotic resistance genes, and we did not evaluate the change in the antimicrobial resistome that carried by the whole microbial ecosystem. Despite these experimental limitations, our study results provide preliminary insight into an area of investigation that could be of great relevance to swine gut health. Understanding of the factors that influence the developmental dynamics of gut microbiota would be important in establishing which management approaches can be used to promotes and maintain a hemostatic microbial ecosystem during this important phase of production.

3.6 CONCLUSION

This study demonstrated that antimicrobial intervention had relatively minor effects on the gut microbiota development during early life in comparison to control but alterations were noticeable in particular taxa. However, early life TUL and PPG intervention could promote selection of ARGs in herds. These changes may help us to understand the impacts of early antibiotics exposure on gut microbial composition and development of ARGs in swine management system. Understanding when and how and gut microbial communities changes in response to antimicrobial administration will aid in the development of new antibiotics alternatives.

3.7 FIGURES AND TABLES

Figure 11: (A) Bar graph illustrating the body weight (kg) at day 0 and day 20, and the average weight gain from day 0 to day 20 of age in different treatment groups. (B) Bar graph illustrating the Mortality percent of piglets from day 0 to day 5 (Day 0-5), from day 5 to day 10 (day 5-10), from day 10 to day 15 (day 10-15), and from day 15 to day 20 (day 15-20) in different treatment group. There was no significant change in the average daily weight gain and overall mortality ratio ($P > 0.05$).

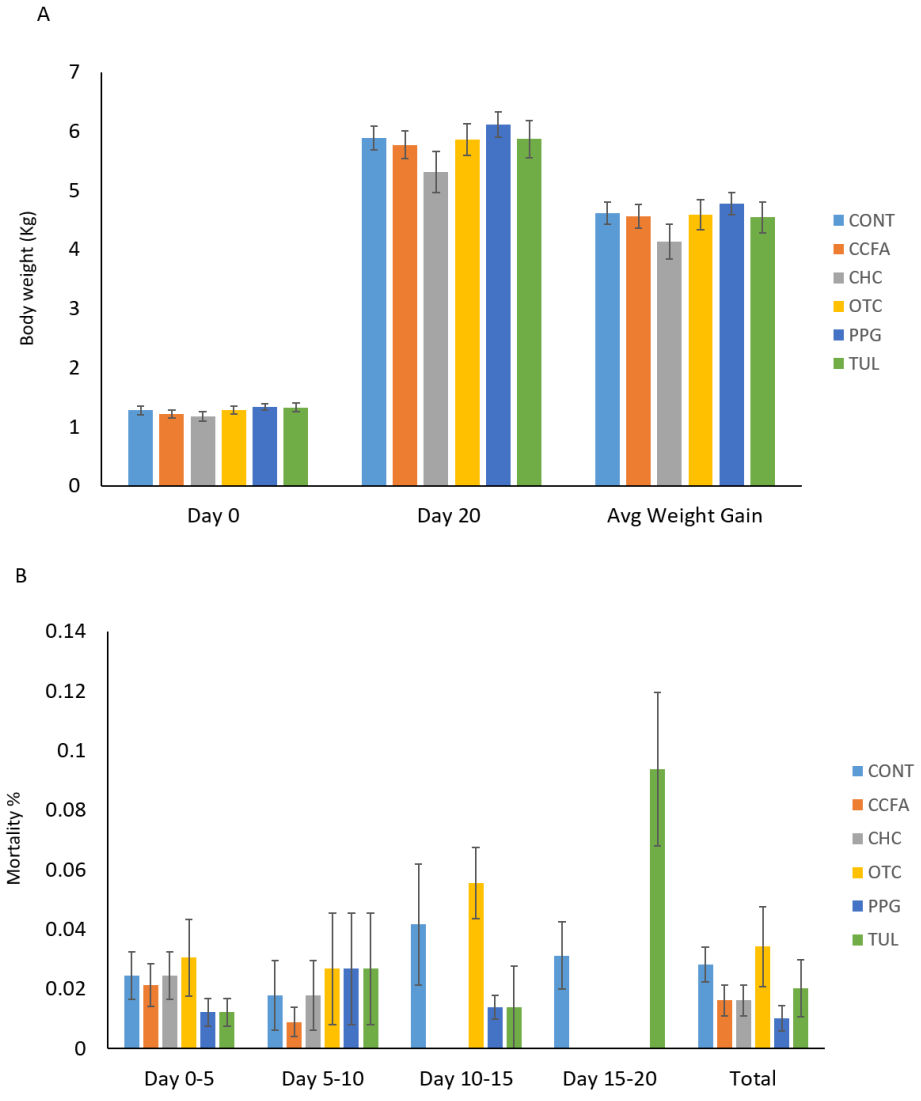


Figure 12: Venn diagram depicting the common and unique OTUs among the different treatments groups (CONT, CCFA, CHC, OTC, PPG, and TUL). A total of 842 OTUs were represented core microbiota and shared between all treatments groups.

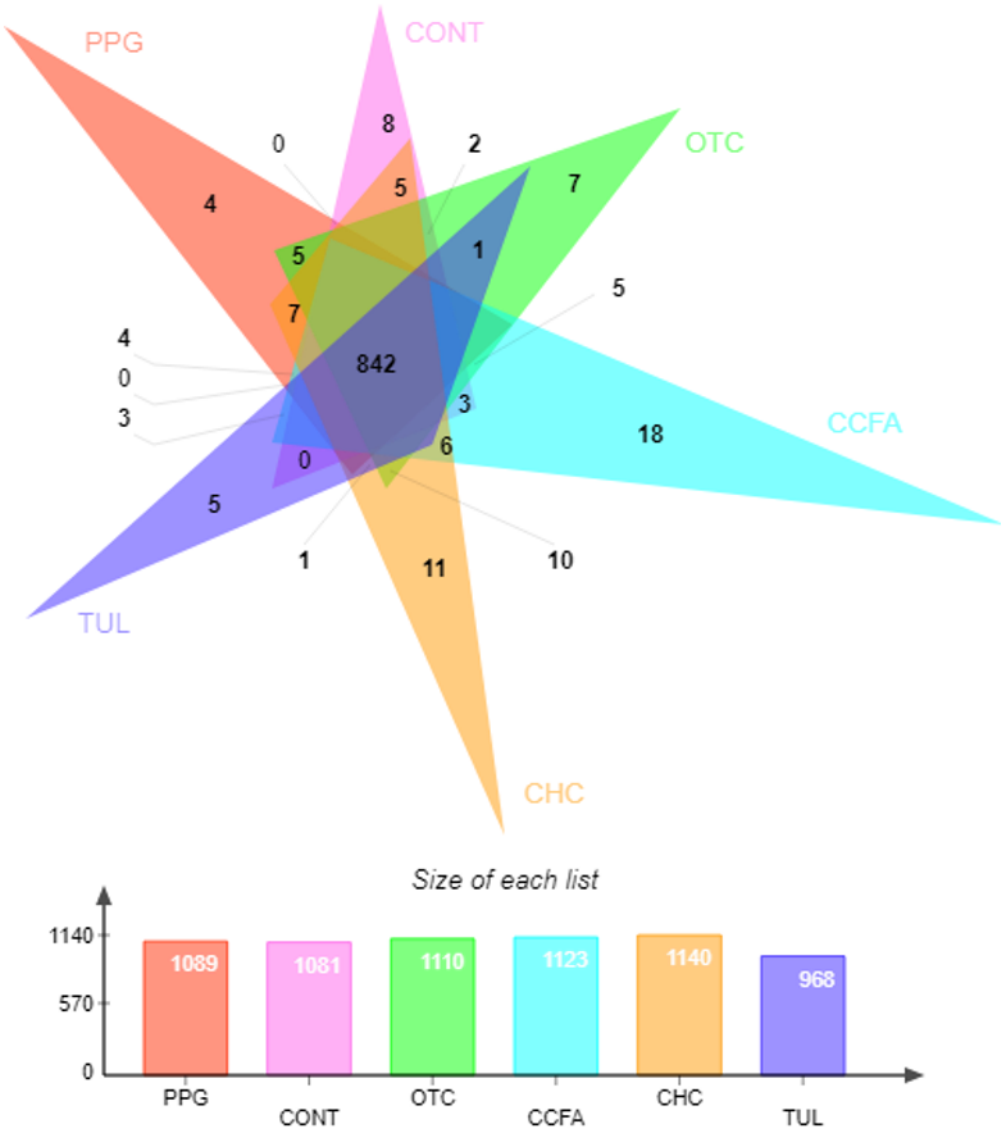


Figure 13: Taxonomic classification of 16S rRNA gene sequences at the phylum level for CONT, CCFA, CHC, OTC, PPG and TUL treated piglets at each sampling time points. Only those bacterial phyla that averaged more than 1% of the relative abundance across all samples when sequencing V3-V4 hypervariable regions are displayed.

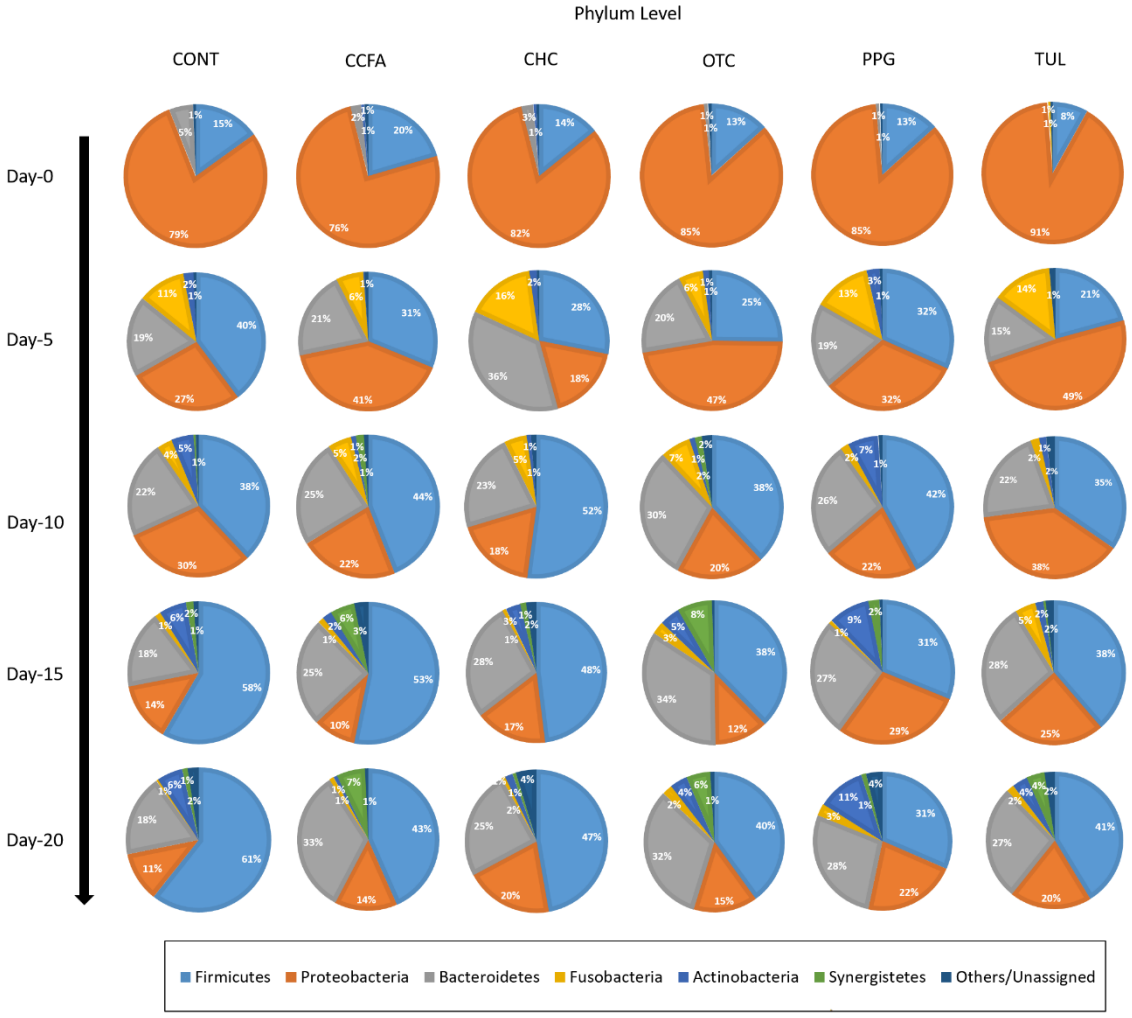


Figure 14: Heatmap cluster analysis of the most predominant microbial communities at the genus level for CONT, CCFA, CHC, OTC, PPG and TUL treated piglets at each sampling time points. Only those bacterial genera that averaged more than 1% of the relative abundance across all samples when sequencing V3-V4 hypervariable regions are displayed.

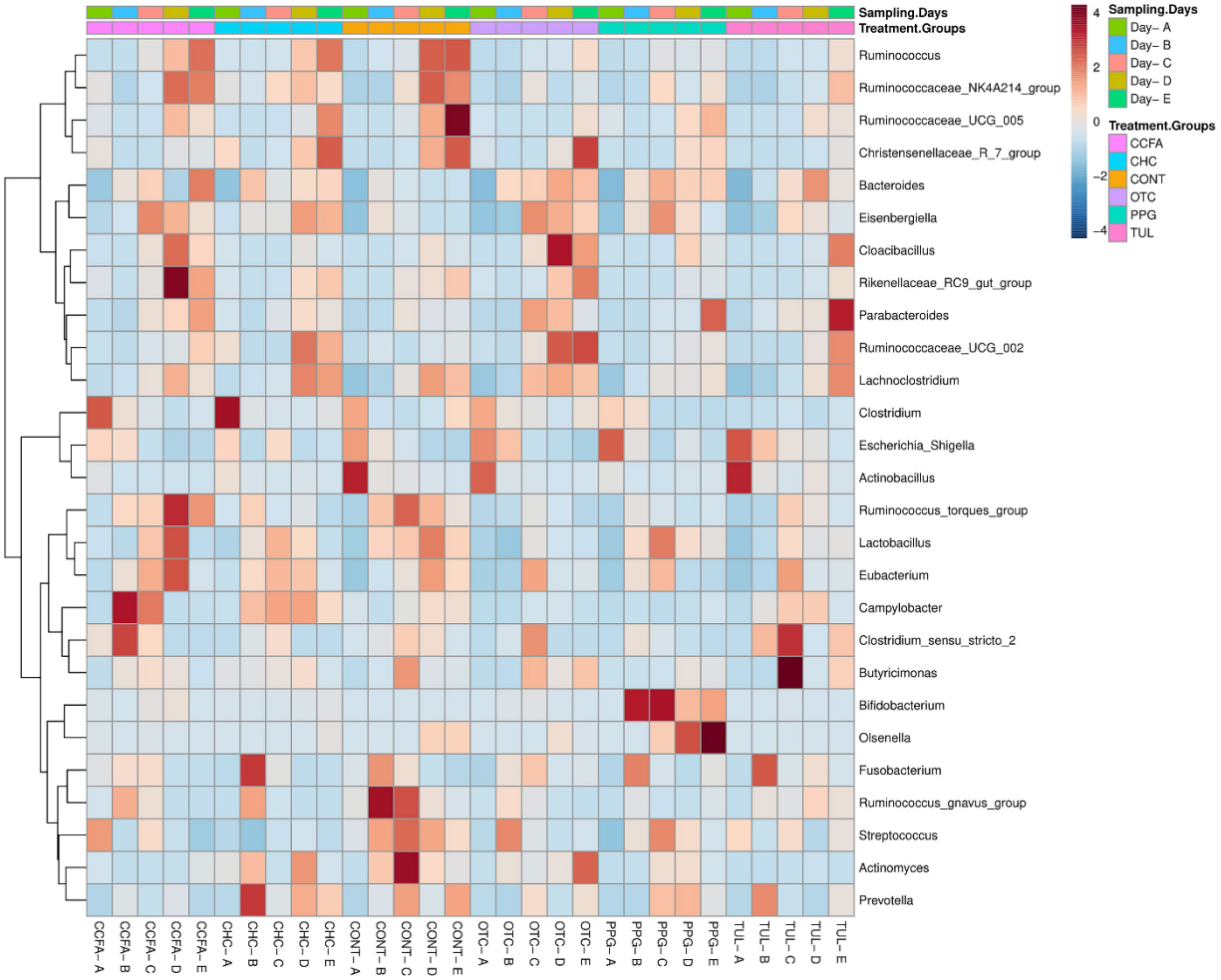


Figure 15: The difference in bacterial diversity indices (Chao1 and Shannon) between the different treatment groups (CONT, CCFA, CHC, OTC, PPG and TUL) at different sampling days (Day 0, 5, 10, 15, and 20).

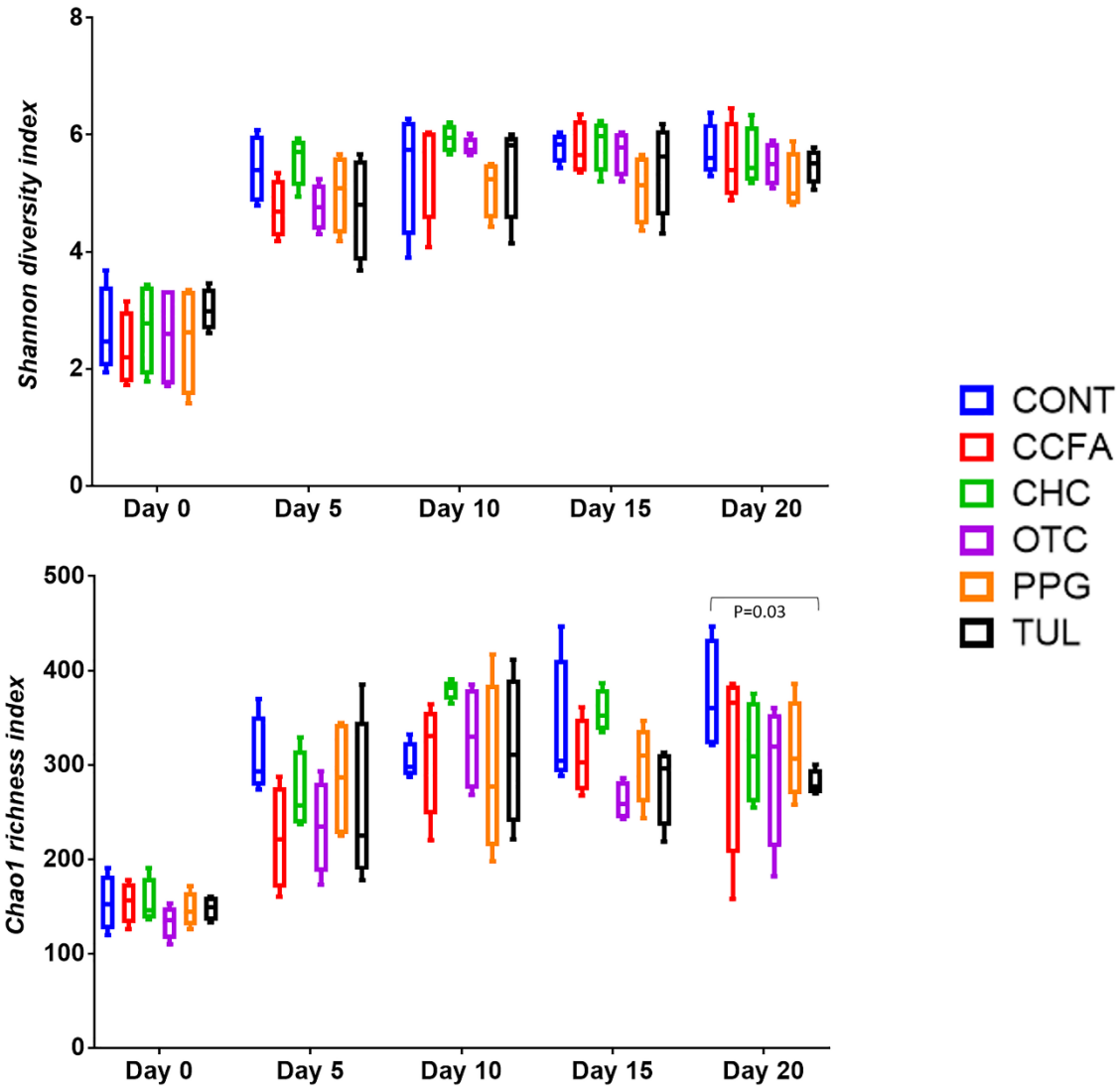


Figure 16: Principal Coordinate analysis (PCoA) for different treatment groups (CONT, CCFA, CHC, OTC, PPG and TUL) at different sampling days. The percent variation explained by each coordinate is indicated on the axes. The individual data points, which represent total fecal microbiota compositions of each piglet, are depicted. Significance between groups was analyzed using analysis of similarity (ANOISM) with 9999 permutations and Bonferroni corrected P values.

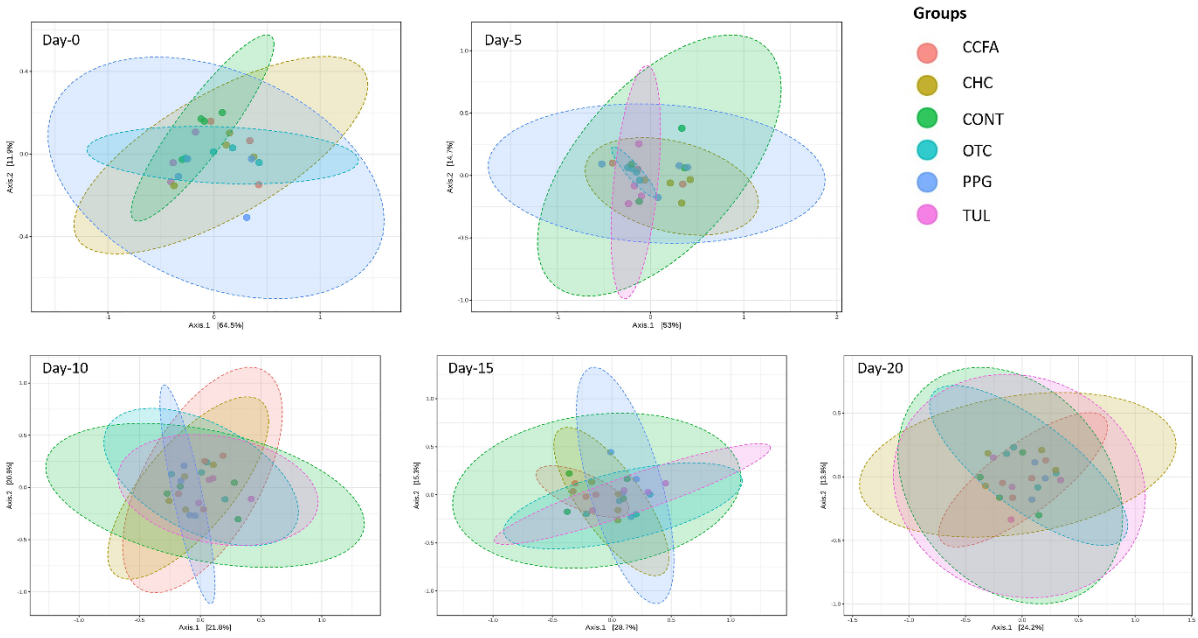


Figure 17: LDA Effect Size (LEfSe) analysis of fecal microbiota depicting the top OTUs with the highest linear discriminant analysis LDA score log10 ≥ 2.0 that discriminate between the CONT group and CCFA, CHC, OTC, PPG and TUL treated piglets. Each color refers to each group and its corresponding indicator taxa.

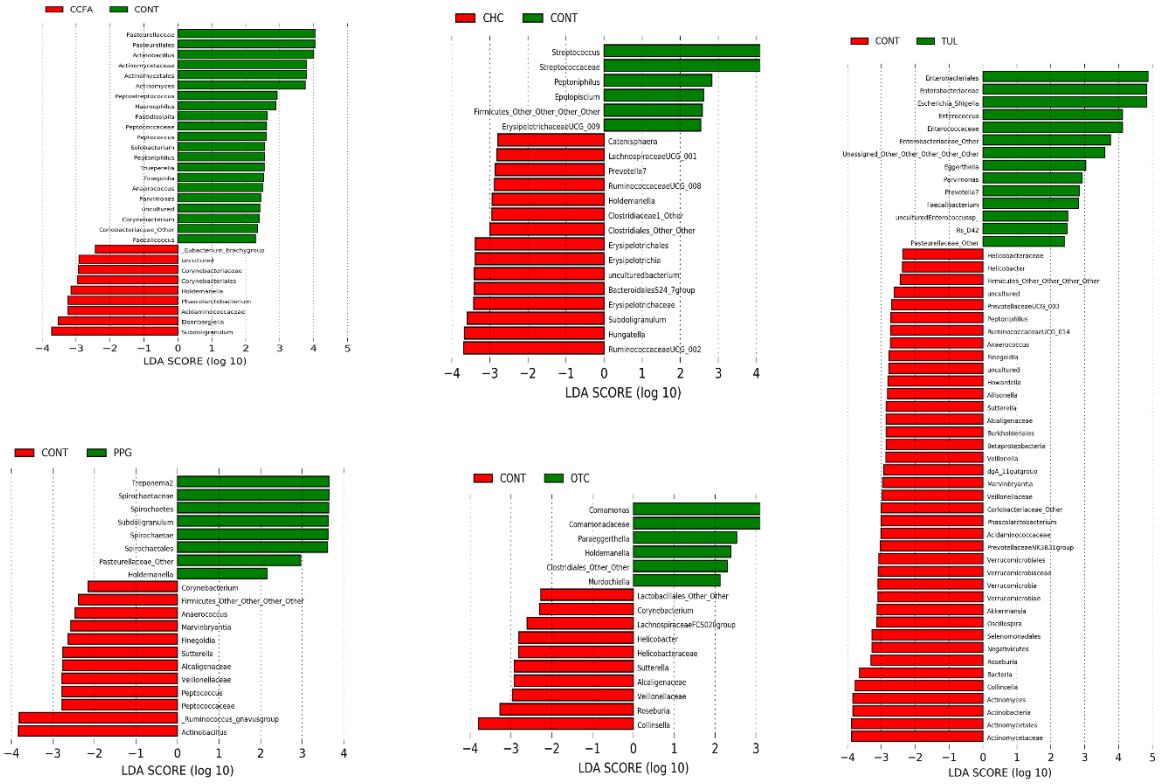


Figure 18: Identification of indicator bacterial taxa associated with statistically significant differential abundance between the different treatments groups (CONT, CCFA, CHC, OTC, PPG and TUL) at the different sampling days. The top OTUs with the highest LDA score log10 ≥ 2.0 that discriminate between the CONT group and CCFA, CHC, OTC, PPG and TUL treated piglets at each time point are depicted. Each color refers to each group and its corresponding indicator taxa.

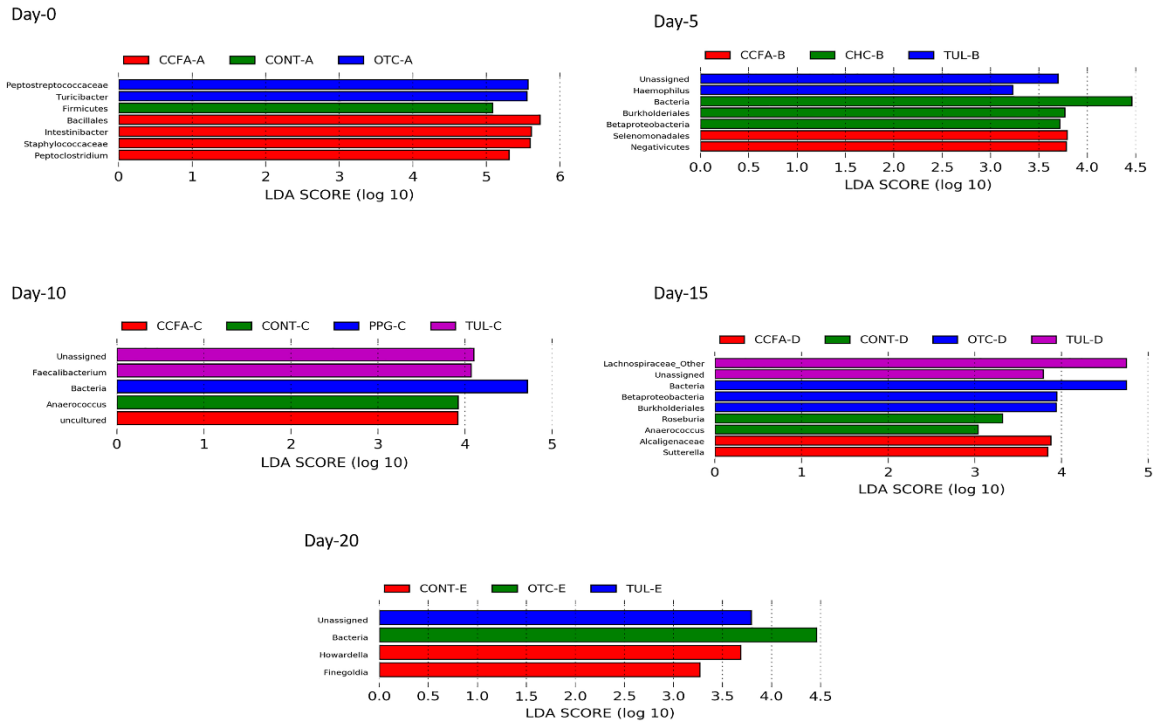


Figure 19: (A) Multiple group similarities tree was constructed using Unifrac distance metrics to identify the similarities and differences among the antimicrobial treatment. (B) Discriminant analysis of fecal microbiota in the different treatments groups (CONT, CCFA, CHC, OTC, PPG and TUL). Different mean relative abundances of bacterial genera in fecal microbiota were used as covariates, and sampling groups were used as categorical variables. Differences in the fecal microbial profiles of the different groups are illustrated by canonical 1 and 2.

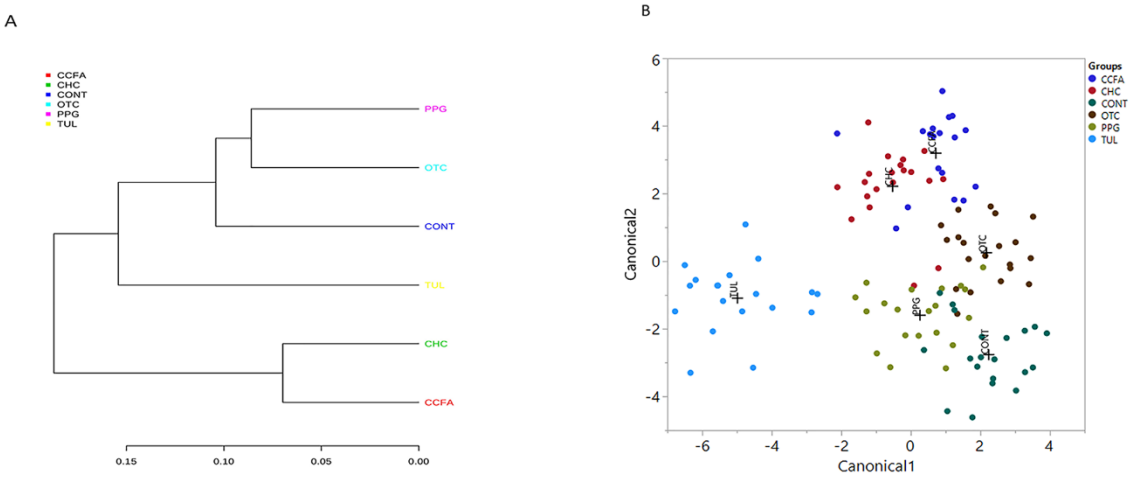


Figure 20: Line graphs illustrating the difference in abundance of antibiotics resistance gene (ermB, SulI, SulII, TetC, TetO, and TetW) between the CONT group and CCFA, CHC, OTC, PPG and TUL treated piglets at different sampling days.

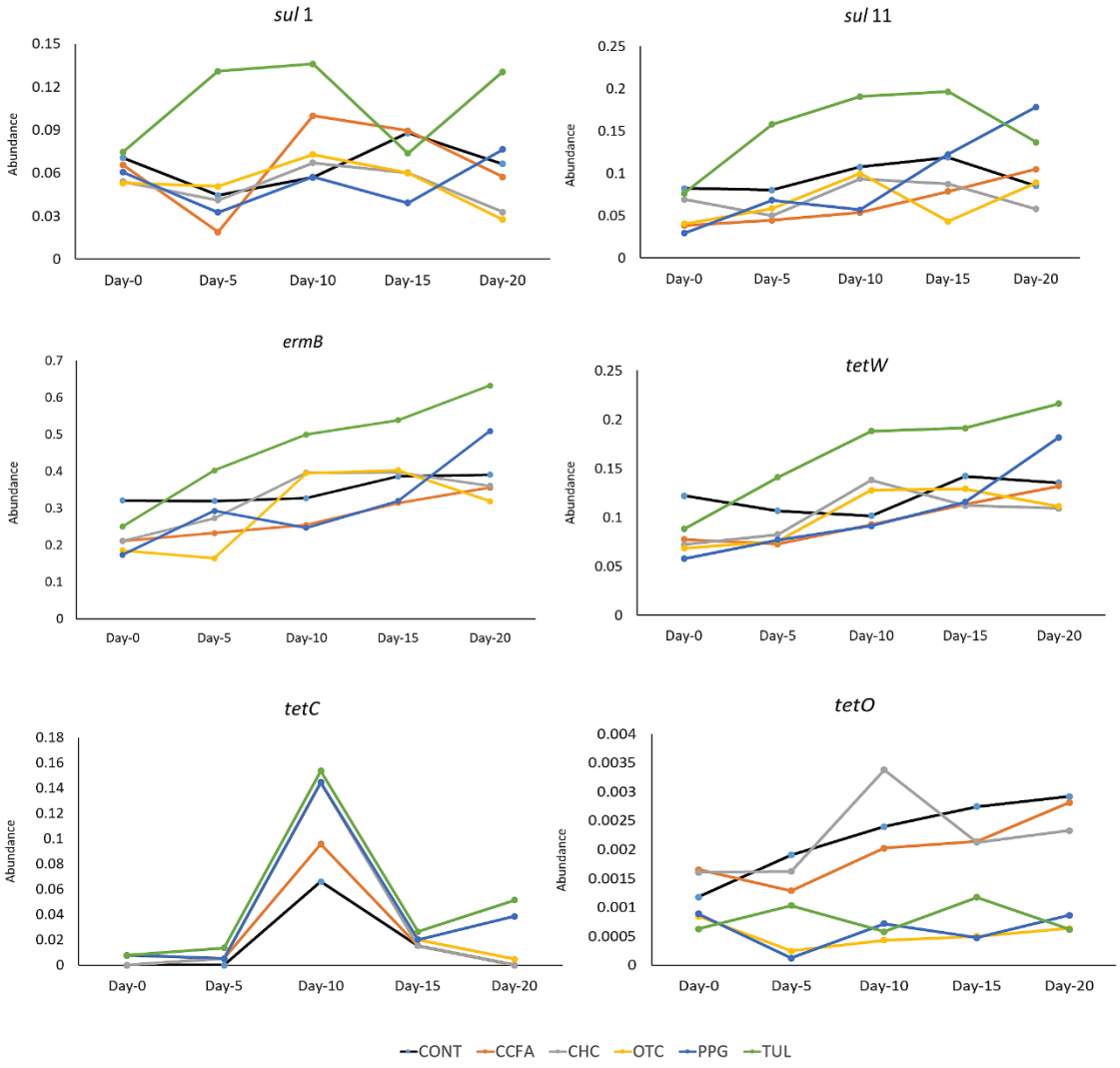


Table 2: Primers targeting selected antibiotic resistance genes used in this study.

Target gene	Primer	Sequence 5'-3'	Amplicon size (bp)
tet O	tetO_F	ACGGARAGTTTATTGTATACC	1920
	tetO_R	TGGCGTATCTATAATGTTGAC	
tet W	tetW_F	GAGAGCCTGCTATATGCCAGC	1920
	tetW_R	GGGCGTATCCACAATGTTAAC	
tet C	tetC_F	GCGGGATATCGTCCATTCCG	1191
	tetC_R	GCGTAGAGGATCCACAGGACG	
sul I	sul I_F	CGCACCGGAAACATCGCTGCAC	840
	sul I_R	TGAAGTTCCGCCGCAAGGCTCG	
sul II	sul II_F	TCCGGTGGAGGCCGGTATCTGG	816
	sul II_R	CGGGAATGCCATCTGCCTTGAG	
erm B	erm B_F	GGTTGCTCTTGCACACTCAAG	738
	ermB_R	CAGTTGACGATATTCTCGATTG	
bla $_{CTX-M}$	CTX-M_F	CTATGGCACCACCAACGATA	876
	CTX-M_R	ACGGCTTTCTGCCTTAGGTT	

Table 3: The result of the nonparametric ANOSIM test (analysis of similarities) with 9999 Monte Carlo permutations to evaluate the UniFrac distances significance between the treatment groups.

	CCFA	**CHC**	**CONT**	**TUL**	**OTC**	**PPG**
CCFA		0.8935	0.4816	**0.0243**	0.4357	0.3249
CHC			0.3806	**0.015**	0.3179	0.2128
CONT				0.196	0.6121	0.1837
TUL					0.1124	0.0759
OTC						0.2548
PPG						

CHAPTER 4

NEGLIGIBLE IMPACT OF PERINATAL TULATHROMYCIN METAPHYLAXIS ON THE DEVELOPMENTAL DYNAMICS OF FECAL MICROBIOTA AND THEIR ACCOMPANYING ANTIMICROBIAL RESISTOME IN PIGLETS

4.1 ABSTRACT

While the antibiotic resistance profiles of cultured pathogens have been characterized in swine, the fluctuations in antimicrobial resistome associated with the developing gastrointestinal microbiota have not been elucidated. The objective of this study was to assess the impact of perinatal tulathromycin (TUL) metaphylaxis on developmental dynamics of fecal microbiota and their accompanying antimicrobial resistome in piglets. Sixteen litters were given one of two treatments (CONT; saline 1cc IM and TUL; 2.5 mg/kg IM) directly after birth. Deep fecal swabs were collected at day 0 (prior to treatment), and again at days 5 and 20 after treatment. Whole genome, metagenomics sequencing approach was performed on the extracted DNA, and fecal microbiota structure and antimicrobial resistome were assessed. Collectively, the swine fecal microbiota and their accompanying antimicrobial resistome were diverse and established soon after birth. Across all samples, a total of 127 antimicrobial resistome related to 19 different classes of antibiotics were identified. The majority of identified antimicrobial resistome were observed in both experimental groups and at all-time points. The magnitude and extent of differences in microbial composition, and antimicrobial resistome, between the TUL and CONT groups were statistically insignificant. However, both the fecal microbiota composition and antimicrobial resistome were changed significantly between the sampling days. In combination, these results indicate that the perinatal TUL metaphylaxis has no measurable benefit, or detriment impacts on either the overall fecal microbial community or their antimicrobial resistome of these young piglets.

4.2 INTRODUCTION

The widespread use of antimicrobial in treatment and prevention of human and animal diseases continues to rise globally (MacKie et al., 2006). The over use of existing antimicrobial, results in perturbations of the gut microbiota, promotes the selection of antibiotic-resistant microorganisms, and the emergence of various antimicrobial resistomes (Czaplewski et al., 2016) (Hoelzer et al., 2017). There is widespread concern regarding the contribution of antimicrobial use in livestock to the development of antimicrobial resistance in people (Founou et al., 2016) (Connelly et al., 2018). To overcome the resistance problem, livestock production systems must optimize the use of antimicrobial treatment (Maron et al., 2013). The key step in this process is to understand the mechanism and extent by which antimicrobial treatment affects the resident microbiota, and their accompanying antimicrobial resistome (Allen et al., 2014). The ability to link the changes in the developmental dynamics of antimicrobial resistome and resident microbiota is crucial in managing and preventing this global health threat.

Most of studies evaluating the effect of antimicrobial intervention on the emergence of antibiotic resistant bacteria and antimicrobial resistome have frequently focused on phenotypic resistance using culture methods (Thanner et al., 2016). While this approach has enhanced our understanding of the nature of antimicrobial resistance in a single class of organisms for certain antibiotics, they are limited to ecosystem-level applications (McEwen and Fedorka-Cray, 2002). The advancements in high-throughput sequencing techniques, have demonstrated that highly diverse bacterial populations are present in animal gut ecosystem and have helped researchers to quantitatively assess the dissemination of antimicrobial resistome in different environments (Zhao et al., 2017). During neonatal phase, newborn piglets are frequently exposed to antimicrobial metaphylaxis to control infectious disease (Yun et al., 2017). Disruption of the establishment of the gut microbiota and their accompanying antimicrobial resistome as a result of antimicrobial administration during this critical phase of production may produce important implications for swine health later on life (Kelly et al., 2017).

Like other macrolides, TUL is bacteriostatic antimicrobial, which act by inhibiting the biosynthesis of essential bacterial proteins and stimulates the disassociation of ribosomal peptidyl-tRNA during translocation process (Schokker et al., 2014). On the basis of it favorable antimicrobial characteristics, TUL is utilized therapeutically in swine for control or prevention of infectious disease at a single dosage of 2.5 mg/kg.BW (Pyörälä et al., 2014). Recently, early-life

TUL intervention in day 4 old piglets revealed long-lasting impacts on gastrointestinal microbial diversity and composition (day 176) but had limited short term effect (Schokker et al., 2014). In our previous study exploring the change of fecal microbiota of 8 week old growing piglets in response to TUL administration, the dysbiotic fecal microbiota after TUL intervention revealed a rapid recovery towards the baseline (pre-treatment) community composition (Zeineldin et al., 2018). To gain further insight into swine gut ecosystem and find alternatives to antimicrobial, it is important to understand the developmental dynamics of gastrointestinal microbiota and prevalence of antimicrobial resistome in response to perinatal antimicrobial metaphylaxis. Consequently, the aim of this study was to investigate the short-term impact of perinatal TUL metaphylaxis on the developmental dynamics of fecal microbiota and their accompanying antimicrobial resistome in neonatal piglets.

4.3 MATERIALS AND METHODS

4.3.1 Ethics statement, animals and samples collection

The present study was conducted in a commercial swine farm in the Midwestern US with consent from the facility owner. All procedures were carried out in agreement with principles and guidelines of the Institutional Animal Care and Use Committee of University of Illinois at Urbana-Champaign. The protocol was evaluated and approved by the Ethical Committee for Institutional Animal Use and Care of the University of Illinois at Urbana-Champaign. A total of 16 sows with their newborn piglets (221 piglets in total) were used in this study. Approximately five days before farrowing, the pregnant sows were transferred to the farrowing pen and kept there until the end of the experiment. Sows were given ad libitum water and fed a standard lactation diet via an automatic dry feeding system. No antimicrobial were administered to the sows before or after farrowing. Farrowing followed normal procedures for the farm except that no piglets were transferred into the experimental litters. If more pigs were present than the dam had milk glands, piglets were removed. All litters contained 12 to 14 pigs after this procedure. Directly after birth (<6 hours), litters were randomly assigned to one of two groups; CONT (n=8 litters) and TUL group (n=8 litters). In TUL group, a total of 108 piglets were treated with 2.5 mg TUL/kg IM (Draxxin®, Zoetis US, Chicago Heights, IL, USA). In CONT group, a total of 113 piglets were treated with saline 1cc/IM. The piglet's tails were docked, and 200 mg of Iron dextran was administered at three days of age. Males were surgically castrated at the same time

according to farm protocols. Daily physical examination was performed individually to evaluate the attitude and appetite of all pigs and their dams by farm staff. Piglets were individually identified with in liter. Weights of all piglets were recorded on study days 0 and at day 20 and two pigs per litter were selected and weighed at days 5, 10, 15 in addition to their weights at days 0 and 20. All piglets were weaned at day 21 along with all other litters in the farm that were not enrolled in the study. The two piglets that were selected in each litter and individual deep fecal swabs (Pur-Wraps®, Puritan Medical Products, Gulford, Maine) were collected immediately prior treatment (day 0), and again on days 5, and 20 after treatment. The fecal swabs were kept in dry ice-chilled boxes, transported to the laboratory on the same day and stored at -80° C until further processing.

4.3.2 Fecal DNA extraction and whole-genome sequencing

Genomic DNA was extracted from subgroups of fecal swabs (CONT; n=12 and TUL; n=12) and from negative control sample (sterile cotton swab and extraction kit reagent) using Power Fecal DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer's standard protocol (Zeineldin et al., 2017a) (Zeineldin et al., 2017b). The fecal swabs were randomly selected from the piglets that remained healthy throughout the sucking period. For each sample, total DNA concentration and integrity was evaluated by optical density using a Nanodrop™ spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at wavelengths of 260 and 280 nm, and agarose gel electrophoresis (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Extracted DNA were immediately stored at −20 °C pending sequencing and then shipped on dry ice for sequencing at the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois at Urbana-Champaign, Urbana, IL, USA).

DNA libraries were constructed using the Nextera DNA Flex Library Preparation Kit (Illumina, Inc., San Diego, CA). Briefly, 100ng of DNA were tagmented, cleaned up with magnetic beads and amplified for 5 cycles of PCR using Illumina Enhanced PCR Mix and Nextera FS dual indexed primers. Amplified DNAs were cleaned, and size selected for fragments 250bp to 750bp in length, using a double-sided bead purification procedure. The final libraries were quantitated using Qubit High-Sensitivity DNA (Life Technologies, Grand Island, NY) and the average size was determined on the AATI Fragment Analyzer (Advanced Analytics, Ames, IA). Libraries were pooled evenly, and the pool was cleaned using a 1:1 ratio with

AxyPrep Mag PCR Cleanup beads (Axygen, Inc. Union City, CA), then evaluated again on AATI Fragment Analyzer (Advanced Analytics, Ames, IA). The final pool was diluted to 5 nM concentration and further quantitated by qPCR (Bio-Rad Laboratories, Inc. CA). The pool was then denatured and spiked with 4% non-indexed PhiX control library and loaded onto the MiSeq V3 flowcell at a concentration of 10 pM for cluster formation and sequencing. Finally, the libraries were sequenced from both ends of the molecules to a total read length of 250nt from each end following manufacturer's guidelines (Illumina, Inc., San Diego, CA).

4.3.3 Sequence data processing and microbial community analysis

Raw sequence data files were de-multiplexed and converted to fastq files using Casava v.1.8.2 (Illumina, Inc. San Diego, CA). Sequence reads quality were assessed using FastQC software (Andrews, 2010). Adaptor sequence and low-quality reads were trimmed from the raw sequence data using Trimmomatic software (Bolger et al., 2014). Fastq sequence files were then uploaded to the Metagenome Rapid Annotation Using Subsystems Technology (MG-RAST) webserver to determine the taxonomic composition of fecal microbiota at the phylum, genus, and species levels (Glass et al., 2010). MG-RAST utilizes a high-performance data-mining algorithm along with curated genome databases that rapidly disambiguates millions of short reads of a metagenomics sequence into discrete microorganisms engendering the identified sequences. In MG-RAST, sequences reads were subjected to quality control, including dereplication (removal of sequences produced by sequencing artifacts), removal of host-specific species sequences, length filtering (removal of sequences with a length >2 standard deviations from the mean), and ambiguous base filtering (removal of sequences with >5 ambiguous base pairs). Normalization was performed using a log2-based transformation [log2 (x + 1)], followed by standardization within each sample and linear scaling across all samples (Gaeta et al., 2017). We used a nonredundant multisource protein annotation database (M5NR) as the annotation source for microbial classification. Microbiota abundance was analyzed using a best-hit classification approach with a maximum e value of 1×10^{-5}, a minimum identity cutoff of 60%, and a minimum alignment length cutoff of 15. We used the SEED subsystem as the annotation source for predicted metabolic functional gene profiles. To be publicly available, we deposited whole genome-sequencing data in MG-RAST under the following accession numbers: from mgm4779141.3 to mgm4779164.3.

The microbiota alpha diversity was calculated within PAST version 3.13 using Chao 1, Shannon, Simpson and Evenness indices. Beta diversity was computed using principal component analysis (PCA) based on non-phylogenetic Bray–Curtis distance metrics implemented in MicrobiomeAnalyst (Dhariwal et al., 2017). The difference in overall microbial composition between the CONT and TUL group was determined using non-parametric multivariate analysis of variance (PERMANOVA) with 9999 permutations and Bonferroni corrected *P* values in PAST version 3.13. The difference in microbial relative abundance and alpha diversity metrics between the two groups (CONT and TUL) at each time point (Day 0, 5, and 20) were analyzed using Mann–Whitney pairwise comparison test with sequential Bonferroni significance in PAST version 3.13. Significance difference was stated at $P < 0.05$. To further quantify the overall microbial composition similarities between the two groups at each time point, the relative abundance values of microbiota at genus level were assessed using the linear discriminant analysis (LDA) effect size (LEfSe) pipeline using Galaxy (https://huttenhower.sph.harvard.edu/galaxy/) (Segata et al., 2011). The difference in overall predictive function gene profiles between CONT and TUL groups were compared through the software STAMP (Statistical Analysis of Metagenomic Profiles) (Parks et al., 2014). Principal component analysis and heatmap diagram were also performed using STAMP software.

4.3.4 Antimicrobial Resistome Identification

To assess and quantify the abundance of the antibiotic resistome in our data, we used SRST2 pipeline (Michael et al., 2014). SRST2 is based on Bowtie2 and is used to map raw reads and cluster sequence that shares a high similarity directly against a database of preference, using CD-hit with an identity threshold of 80% (Clausen et al., 2016). For antimicrobial resistome sequence classification, we used antibiotic resistance gene database (ARG-ANNOT database) that incorporated all sequences of known antibiotics resistance genes. The ARG-ANNOT database was used to align the different the antimicrobial resistome raw sequence reads obtained from the Illumina sequencing. Antimicrobial resistome alpha diversity metrics were computed using the Shannon index, Simpson's index, Chao1 richness estimate and Pielou's evenness index. The difference in antibiotic resistome abundance and diversity, between the CONT and TUL groups at the different sampling days were analyzed using Mann–Whitney pairwise comparison test with sequential Bonferroni significance in PAST version 3.13. Additionally, the

two-sided Welch's t-test and Benjamini–Hochberg FDR correction were used to compare the overall difference in antimicrobial resistome between CONT and TUL groups using STAMP software (Parks et al., 2014). Differences were considered significant at $P < 0.05$. Principal component analysis and heatmap diagram were also performed using STAMP software. The difference in overall antimicrobial resistome between the CONT and TUL group was determined using PERMANOVA with 9999 permutations and Bonferroni corrected P values in PAST version 3.13.

4.4 RESULTS

4.4.1 Impact of TUL metaphylaxis on the body weight gain and overall mortality ratio

Piglet's body weight was measured at day 0 just prior to the treatment and again at day 20 of age. There was no significant change in the average daily weight gain between day 0 and day 20 in the TUL group compared to CONT (means ± SE; 4.61± 0.18 vs. 4.54 ± 0.26, P > 0.05, Figure 21A). The TUL treated piglets showed non-significant changes in the overall mortality ratios (day 0 to 20) compared to control group (means ± SE; 0.028± 0.005 vs. 0.021 ± 0.009, P > 0.05, Figure 21B). Our results showed that the early life TUL intervention failed to reduce mortality and increase the average daily weight gain in neonatal piglets.

4.4.2 Whole genome, metagenomics sequencing summary

Across all 24 fecal samples, shotgun metagenomic sequencing generated a total of 19,236,952 raw sequence reads (mean number of sequences per sample: 400,742.88; median: 394,675; range: 358,524– 464,985). The average Phred quality score of raw sequence reads across all samples was 33.7 and only 1.01 % of all reads were removed due to low quality. Using the criterion of MG-RAST taxonomic classification, 3,833,882 taxonomic hits were identified among all samples, all of which were taxonomically assigned according to RefSeq classification. Collectively, a total of 2,010,187 and 1,829,585 hits were identified in piglets from the CONT and TUL group, respectively.

4.4.3 Taxonomical classification of the fecal microbiota

Across all samples, around 29 bacterial phyla, 586 genera and 1468 species were detected using MG-RAST webserver. Collectively, the microbial composition at both phylum and genus

level in CONT and TUL varied greatly according to ages (Figure 22A). At the phylum level, *Proteobacteira* was the most predominant at day 0, representing 62 % and 70 % of all bacterial populations in CONT and TUL respectively. While at day 20, *Firmicutes* was the most abundant, representing 52 % and 60 % of all bacterial populations in CONT and TUL respectively. When selectively comparing changes between CONT and TUL, there was no significant change in bacterial phyla that averaged more than 1% of the relative abundance. However, TUL treated piglets, exhibited significant increase in some very rare phyla like *Chloroflexi* (0.24 % vs 0.11 % in CONT piglets, p = 0.03) and *Chrysiogenetes* (0.0057 % vs 0.0008 % in CONT piglets, p = 0.014) at day 20.

At the genus level, the predominant bacterial genera that averaged more than 1% of the relative abundance across all samples at the baseline (day 0) was comprised of common fecal microbial genera including *Escherichia* (50.72%), *Bacteroides* (7.73%), *Clostridium* (7.03%), *Shigella* (5.61%), *Streptococcus* (2.18%), *Fusobacterium* (1.74%) and *Salmonella* (1.31%) *Lactobacillus* (1.01%). Distribution of most abundant bacterial genera in both CONT and TUL groups at different sampling days are depicted in (Figure 22B). Even though there was no significant change detected in bacterial genera that averaged more than 1% between the two groups, in-depth analysis at genera-level suggested that treatment with TUL was associated with moderate changes in the microbial profile in the feces of these young piglets. Compared to CONT piglets, the TUL treated piglets revealed significant increase in abundance of *Eubacterium* (3.67 % vs 1.87 % in CONT piglets, p= 0.042), *Butyrivibrio* (0.73 % vs 0.25 % in CONT piglets, p= 0.041), *Bacillus* (0.88 % vs. 0.37 % in CONT piglets, p= 0.031), *Ethanoligenens* (0.89 % vs. 0. 52 % in CONT piglets, p= 0.039) at day 20. The TUL treated piglets showed also a significant decline in the abundance of *Actinobacillus* (0.015 % vs 0.19 % in CONT piglets, p= 0.038) at day 5 when compared to CONT group at the same time point. At the species level, the microbial composition in CONT and TUL varied greatly according to ages (Figure 23A). Additionally, some bacterial species showed significant difference when compared CONT and TUL treated piglets at days 5 and 20 (Figure 23B &C).

To further characterize the difference of fecal microbiota between the two groups and determine indicator taxa in each group, LEfSe algorithm was performed to measure the contribution of microbial taxa to the identified differences between the two groups. Based on LEfSe algorithm, the changes in the fecal microbiota structure caused by perinatal TUL

intervention are limited to a particular group of microbial taxa (Figure 4). Compared to CONT group, 3, 3 and 8 OTUs were identified as indicator taxa in TUL treated piglets at days 0, 5 and 20 respectively (Figure 24). At day 5, the TUL treated piglets exhibited a high contribution of the taxa *unclassified Erysipelotrichaceae unclassified Bacteroidetes* and *Mucilaginibacter.* While at day 20*, Ruminococcus, Ethanoligenens, Butrivibrio, unclassified Lachnospiraceae, Dehalococcoides, Thermoanaerobacterium, Abiotrophia* and *Cellulosilticum* were enriched in TUL group.

We next investigate the effects of early life TUL metaphylaxis on the fecal microbiota diversity. Alpha-diversity was computed using several metrics (Chao1, Shannon, Simpson and Evenness indices). Alpha diversity metrics showed non-significant changes between the CONT and TUL groups (Figure 25). However, the metagenomics analysis in both experimental groups revealed that microbial diversity and richness indices were increased with age (Fig 5). Beta diversity analysis also showed that the TUL induced changes in the microbial community composition were not sufficient to cluster microbial populations at days 0, 5 and 20 as shown by PCA of Bray–Curtis distance (PERMANOVA, $P > 0.05$; Figure 26).

4.4.4 Effect of TUL metaphylaxis on microbial functional profiles

In addition to a taxonomic profile, whole genome, metagenomics sequencing provides information about metagenome predicted functional profile. The relative abundance of the functional profiles at level 2 is depicted in (Figure 27A). There was no significant difference in the overall metabolic functional capability at level 2 pathway, between the CONT and TUL group (Figure 27B and C). However, the overall predicted functional profiles in both CONT and TUL varied greatly according to ages (Figure 28A). Furthermore, the extended bar plot revealed some significant difference in the relative abundance of some metabolic and resistance to antibiotics functional potential between the CONT and TUL treated piglets (Figure 28B and C).

4.4.5 Effect of TUL metaphylaxis on antimicrobial resistome

To assess the consequence of early life TUL metaphylaxis on development of antimicrobial resistome in the gut of newborn piglets, whole genome sequence reads data were analyzed for the presence of antibiotics resistome in the fecal microbiomes using SRST2 software. We determined the presence and abundance of antibiotic resistome by mapping the sequence reads from all samples to ARG ANNOT database that have all known antibiotic resistance genes. Across all

samples, a total of 127 antimicrobial resistance genes related to 19 different classes of antibiotics were identified. The detected ARGs included genes conferring resistance to antibiotics including lipopeptide, aminocoumarin, tetracycline, fluoroquinolone, beta-lactam, aminoglycoside, streptogramin, macrolide, lincosamide, lipopeptide, rifamycin, phenicol, peptide, glycopeptide, nucleoside, sulfonamide, fluoroquinolones, coumarin, rifampin, and diaminopyrimidine. A heatmap of identified antibiotic resistome in the fecal microbiota at class level of each piglets in both CONT and TUL group was depicted in (Figure 29). The identified antimicrobial resistome were observed in both CONT and TUL groups and at all-time points. The highest level of antibiotics resistome across all samples were associated with *tetQ* (10.22%), *tetO* (7.21%), and *tetW* (6.24%), *PmrC* (4.65%), and *APH(3')-IIIa* (3.77%). The magnitude and extent of differences in the 50-predominant antimicrobial resistome, between the TUL and CONT groups were statistically insignificant (Figure 30). The distribution of antimicrobial resistome in both experimental groups to the universal presence of antibiotic resistance genes in the resident fecal microbiota rather than resistance induced by TUL intervention.

To gain further insight, we calculated several alpha-diversity indexes for antimicrobial resistome in both CONT and TUL groups using several metrics (Chao1, Shannon, Simpson and Pielou's evenness indices) (Figure 31). Alpha diversity metrics showed non-significant changes between the CONT and TUL groups ($P > 0.05$, Figure 31). However, the metagenomics analysis revealed that antimicrobial resistome diversity and richness indices were increased with age (Figure 11). Principal component analysis also revealed that the overall fecal antimicrobial resistome did not differ significantly between the TUL and CONT groups (PERMANOVA, $P = 0.353$; Figure 32A). However, the antimicrobial resistome at gene level across all samples varied greatly according to ages (PERMANOVA, $P < 0.001$; Figure 32B).

4.5 DISCUSSION

In this study, we used whole genome metagenomics sequencing to assess the significance of early life developmental dynamics of the fecal microbiota and their accompanying antimicrobial resistome in newborn piglets in response to TUL metaphylaxis soon after birth. This study is very crucial because of the critical public health concerns regarding the use of antimicrobial in livestock. Additionally, this study was performed in a commercial swine farm to improve the practical relevance of our results. The findings of this study revealed that the single dose of TUL prophylaxis in newborn piglets failed to reduce mortality and increase the average daily weight gain in neonatal piglets. In these piglets, the early life microbial composition after birth was predominantly comprised of *Escherichia*, *Bacteroides*, *Clostridium*, *Shigella*, *Fusobacterium,* and *Streptococcus*. These taxa create an anaerobic environment important for establishing other health beneficial strict anaerobes (Pantoja-Feliciano et al., 2013). The piglets fecal microbial communities composition observed in this study soon after birth was similar to that published by (Kubasova et al., 2017). Although *Escherichia* and *clostridium* are the first genera colonize the gastrointestinal tract in different species (Rodríguez et al., 2015) (Slifierz et al., 2015), the existence of *Fusobacterium* member in gut microbiota of day 0 old piglets is of concern since *Fusobacterium* promotes the development of swine dysentery (Durmic et al., 1998). In 20-day-old piglets, *Clostridium, Bacteroides, Escherichia, Lactobacillus,* and *Prevotella* were the most abundant microbiota member, which similar to a previous report (Slifierz et al., 2015) (Kubasova et al., 2017). While our study revealed that the age is the most significant contributor in fecal microbiota development, understanding the early colonization pattern of gut microbiota will open the door to new perspectives on the utility of early life antimicrobial administration to healthy neonates in swine management systems.

TUL is a bacteriostatic macrolide with therapeutic properties that are desirable in swine for treatment, control or prevention of infectious disease (Pyörälä et al., 2014). Macrolides inhibit bacterial protein biosynthesis as well as inhibit ribosomal translation by preventing peptidyl transferase ribosomal translocation (Mazzei et al., 1993). While, there have been contradictory reports in the published studies regarding the impact of macrolide antibiotic intervention on the microbiota structure, our results are broadly consistent with a previous study that assessed the effects of TUL intervention on the fecal microbiota and their accompanying resistome of commercial feedlot calves (Doster et al., 2018). Using Shotgun sequencing, Doster

et al. reported that fecal microbiome and antimicrobial resistome were not significantly different between the two treated and control groups (Doster et al., 2018). Similarly, our results revealed that the perinatal TUL metaphylaxis has no measurable benefit, or detriment impacts, on either the overall microbial community or the antimicrobial resistome present in the feces of these young piglets. The TUL-piglet fecal microbiota is relatively similar to CONT piglets with only minor alteration in non-abundant microbial taxa. For instance, the relative abundance of *Eubacterium*, *Butyrivibrio* and *Bacillus*, *Ethanoligenens* were significantly increased in TUL treated piglets. While the abundance of *Actinobacillus* was decreased in TUL treated piglets at day 5. In line with other studies, the disruption in microbiota composition profound after macrolides prophylaxis was restricted to relatively small group of taxa (Choo et al., 2018). The high relative abundance of *Eubacterium*, *Butyrivibrio* in TUL treated piglets was interesting finding. Member of these genera comprises some of the most prevalent communities in the neonates gut and are commonly associated with gastrointestinal health, through production of short-chain fatty acid, especially butyrate (Cresci et al., 1999) (Jandhyala et al., 2015) (Choo et al., 2018). Using our extensive whole genome data, we additionally identified taxa at the species level, which offering us the opportunity to study the microbiota diversity at a much higher resolution. The use of TUL metaphylaxis was associated with non-significant changes in microbial diversity. This suggests that TUL had no effect on the fecal microbiota diversity in sucking piglets. A primary reason could be that TUL is actively absorbed from injection site, distributed rapidly to tissue particularly the respiratory tract, with exceptionally long elimination half-life in lung tissue (6 days in pigs) (Benchaoui et al., 2004). Further, TUL excretion is somewhat slower (about 70% within 23 days) with the excreted dose being divided between urine (40%) and feces (32%). Similar to our findings, macrolides administration in cattle did not change the microbiota composition and diversity in lower gastrointestinal tract (Thomas et al., 2017). The modest changes in fecal microbiota composition following early life TUL metaphylaxis are likely to reflect a combination between the resident microbiota resistance mechanism and the relatively weak gastrointestinal selective pressure of single-dose from TUL treatment (Choo et al., 2018).

In this study, we used MG-RAST functional analysis to predict the metagenomics potential functional gene features and identified some functional profiles that showed significant different between CONT and TUL group at level 2 and level 3 Hierarchical KEGG pathways

classification. The most abundant gene functions across all samples were related to carbohydrates metabolism, amino acids and derivatives, protein metabolism, DNA metabolism, RNA metabolism, cell wall and capsule, membrane transport, and virulence, disease and defense. These functions are essential for the survival and development of the microbial community. Similar to the highly diverse and developed fecal microbiota compositions, the overall predicted functional profiles in both CONT and TUL varied greatly according to ages. Similarly, this age variability in microbiota predicted functional potential has been also detected in RNA and DNA - based surveys (Phillips et al., 2004) (Qin et al., 2010). While these are only statistical presumptions in functional features of the taxonomically assigned microbial population in our study, similar changes have been declared after different antimicrobial therapy in human (Pérez-Cobas et al., 2013). The changes in some functional genes profiles between the CONT and TUL treated piglets implied that these functional features might play crucial role under stress conditions, as is the case of antimicrobial administration (Wang and Quinn, 2010). Additionally, the observed increase in cephalosporin β-lactam antibiotics resistance genes in TUL treated piglet's means that horizontal gene transfer is a major mechanism of concern for the spread of antimicrobial resistance genes (Chambers et al., 2015). Further investigations into the functional profiles associated with microbial community changes (i.e. which community members have the same functional features and could alternate for one another), either by direct metabolites measurement or by transcriptome analysis, will be an essential next step to better understand the effect of early life TUL interventions on microbiota function in piglets.

One important consequence of overuse of antimicrobial in livestock production is the spread of ARGs, which is a serious public health problem (MacKie et al., 2006). The resident microbial communities could act as a potential reservoir for antimicrobial resistome for the pathogens (Marshall et al., 2009). Recently, the use of next generation sequencing provides a significant tools for estimation of antimicrobial resistome abundnace in gastrointestinal microbial community (Thomas et al., 2017). In this study, we were able to detect a total of 127 antimicrobial resistance genes related to 19 different classes of antibiotics across all samples using a metagenomics whole genome, sequencing strategy. Similar to the previous report, the identified antimicrobial resistome were observed in both CONT and TUL groups and at all-time points (Yassour et al., 2016) (Connelly et al., 2017). Additionally, the antimicrobial resistome

were detected even in the absence of antimicrobial exposure (Tsukayama et al., 2018). In our study, the CONT piglets not exposed to antimicrobial displayed a high frequency of ARGs.

One of the major findings of our study is that the change in the proportion antimicrobial resistome in both TUL and CONT piglets had a similar temporal pattern. This apparent lack of TUL effect is likely explained by the dominance of sequences coding for resistance to tetracyclines (*tetQ*, *tetO*, and *tetW)* across all samples (Chambers et al., 2015).

The whole genome metagenomics approach also enabled us to identify several genes encoding resistance to the macrolides, which were of special interest given that TUL belongs to this class. Interestingly, the magnitude and extent of differences in the proportion of macrolides resistance genes sequence between the TUL and CONT groups were statistically insignificant ($P > 0.10$; Fig 8). Similarly, macrolide treated population did not result in a significant increase in the macrolide resistance genes (erm(A), erm(B), erm(C), erm(F), mef(A/E), and msrA (Choo et al., 2018). In combination, there was no measureable effect of TUL treatment on antimicrobial resistome in this group of piglets. Since we used only single dose of TUL and the total study duration in this study was only 20 days, the antimicrobial resistome profile we determined here may not be representation of longer term effect of such antimicrobial metaphylaxis (Thomas et al., 2017).

While the results of this study were important and could open new avenues in understanding the role of antimicrobial administration in the neonatal stages, our study had a number of experimental limitations that should be considered. The major limitation in this study were the small sample size, though similar to other published metagenomics studies (Thomas et al., 2017) (Yu et al., 2018). In addition, the higher cost associated with shotgun metagenome sequencing restricts using a larger sample size. Finally, our analysis focused on the short-term impact of TUL administration on fecal microbiota (20 days). Despite these experimental limitations, our study results provide preliminary insight into an area of investigation that could be of great relevance to swine gut health. Therefore, further long-term studies across larger population and with different class of antimicrobial should be conducted to determine the beneficial and or the detriment effects of early life antimicrobial prophylaxis on gut microbial community structure and antibiotics resistome in our livestock management system.

4.6 CONCLUSION

This study demonstrated that TUL metaphylaxis at birth had relatively minor effects on the developmental dynamics of gut microbiota and their accompanying antimicrobial resistome in suckling piglets. The current study provides an extended discussion not only help us to understand the interplay between antibiotics and the developmental changes piglet gut microbiota and ARGs, but also outline important future directions for exploring the potential long-term impact of these changes on pig health and productivity. Additionally, this study suggesting that the TUL treatment may be administered without incurring sever changes to the fecal microbiota and their accompanying antimicrobial resistome during the sucking periods in swine. Understanding when and how and gut microbial communities' changes in response to antimicrobial administration will open the door to new perspectives on the utility of early life antimicrobial administration to healthy neonates in our livestock management systems.

4.7 FIGURES

Figure 21: (A) Bar graph illustrating the body weight (kg) at day 0 and day 20, and the average weight gain from day 0 to day 20 of age. (B) Bar graph illustrating the Mortality percent of piglets from day 0 to day 5 (Day 0-5), from day 5 to day 10 (day 5-10), from day 10 to day 15 (day 10-15), and from day 15 to day 20 (day 15-20). The piglets were treated with a Single dose of TUL at day 0 soon after birth (TUL, N = 108), or treated with saline (CONT, N = 113). There was no significant change in the average daily weight gain and overall mortality ratio ($P > 0.05$).

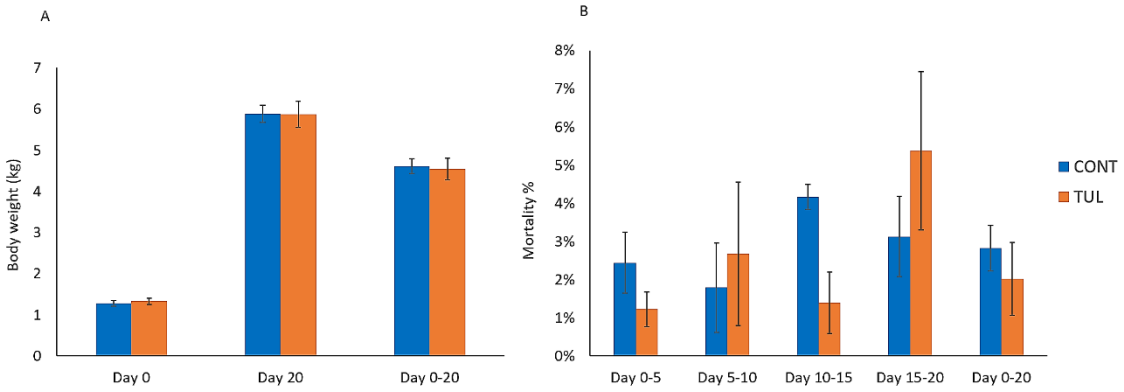


Figure 22: Taxonomic classification of whole genome metagenomic sequences at the (A) phylum level and genus level (B) for the control (CONT) and tulathromycin (TUL) treated piglets at each sampling time days (0, 5 and 20). Only those bacterial phyla and genera that averaged more than 1% of the relative abundance across all samples are displayed.

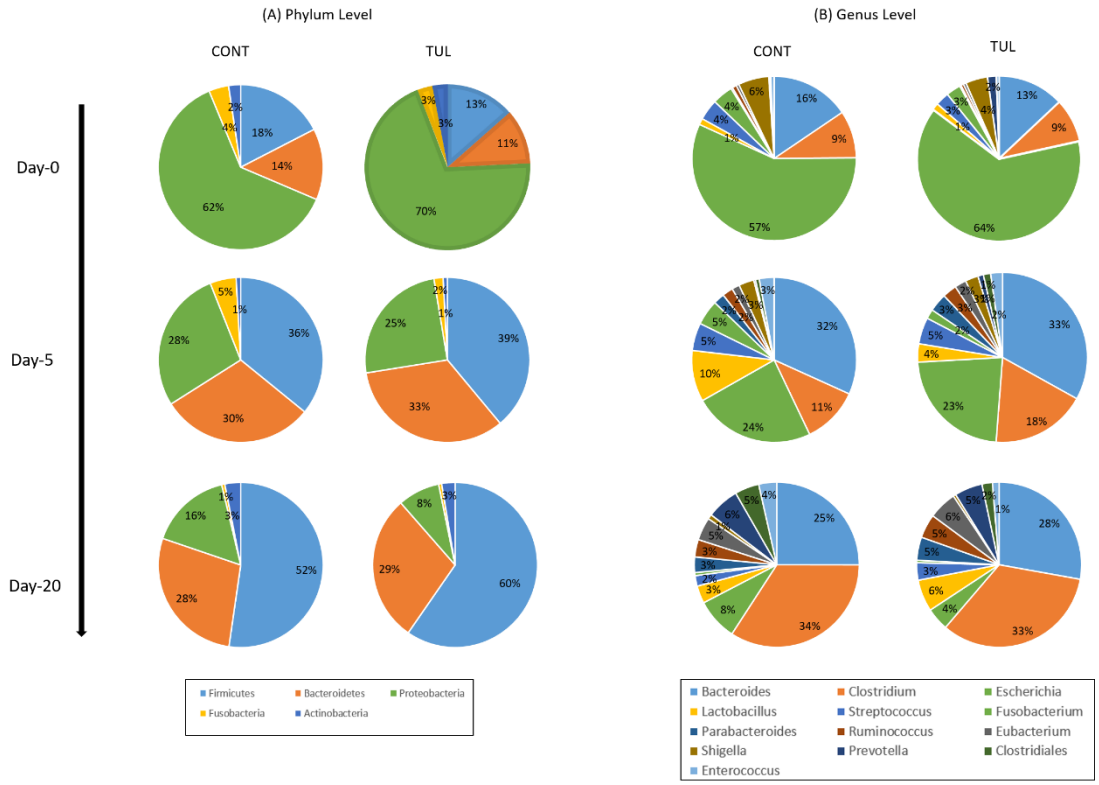


Figure 23: (A) Principal component analysis (PCA) for the microbial composition at species level across all samples at different sampling days (0, 5 and 20). The percent variation explained by each component is indicated on the axes. The individual data points, which represent total fecal microbiota compositions of each piglet, are depicted. Significance between groups was analyzed using PERMANOVA with 9999 permutations and Bonferroni corrected P values. (B) Bacterial species that showed significant difference when compared CONT and TUL treated piglets at days 5. (C) Bacterial species that showed significant difference when compared CONT and TUL treated piglets at days 20.

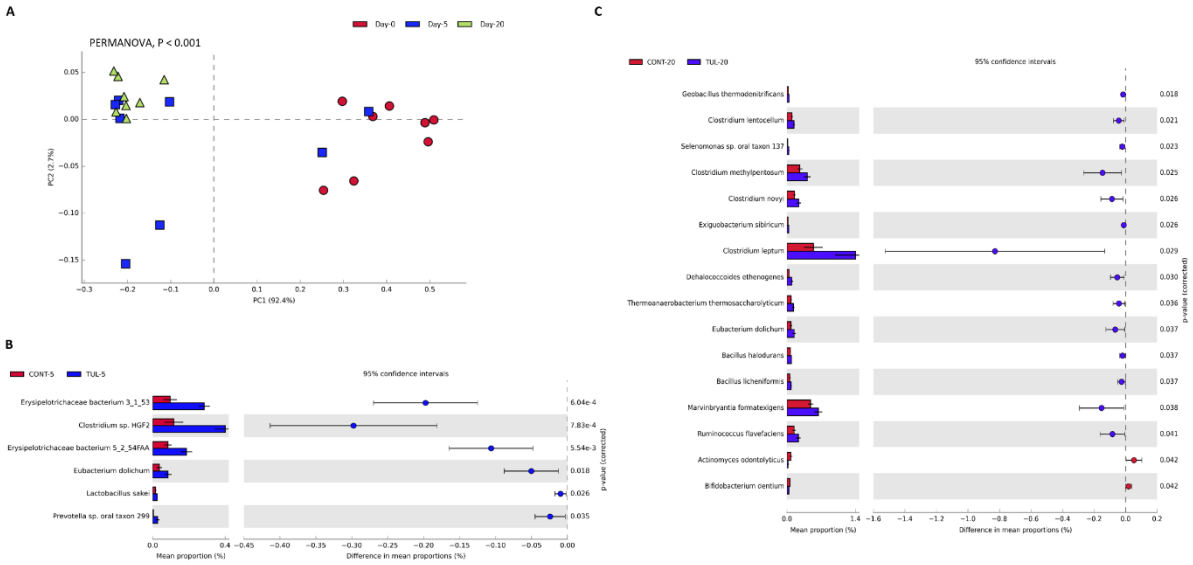


Figure 24: Linear discriminant analysis effect size measuring the contribution of microbial taxa to the identified differences between the control and tulathromycin treated piglets and determining indicator taxa in each group.

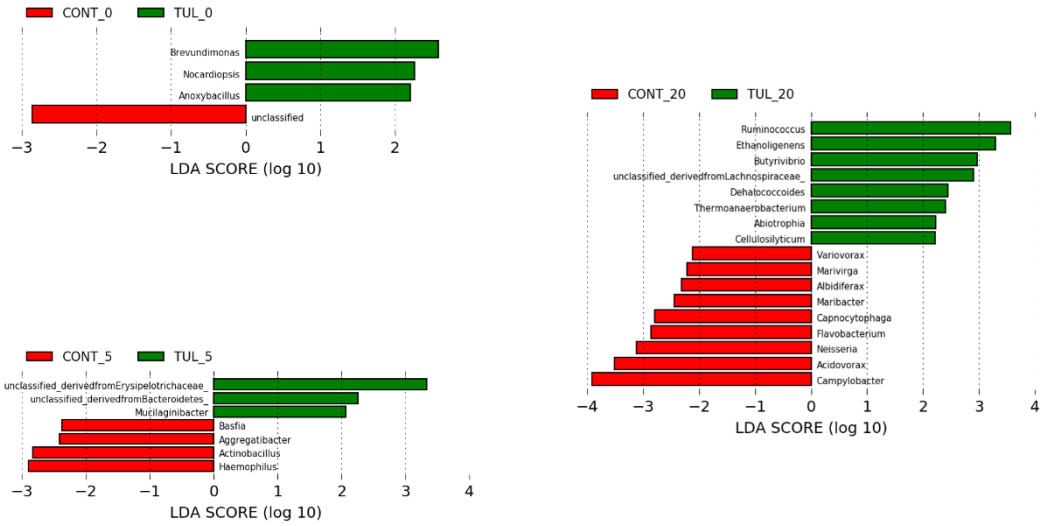


Figure 25: The difference in bacterial diversity indices (Chao 1, Shannon, Simpson and Evenness) measures between the control (CONT) and tulathromycin (TUL) groups at different sampling days (0, 5 and 20). The individual data points, which represent bacterial diversity for each piglet, are depicted. Error bars represent the standard errors.

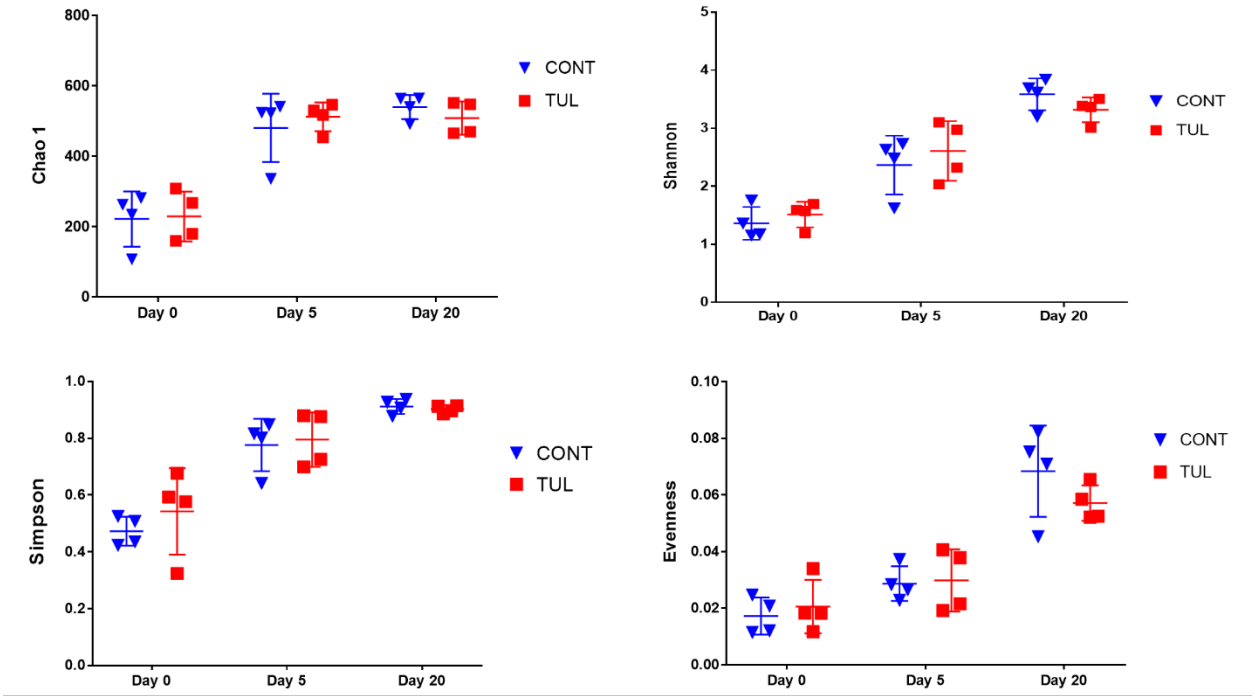


Figure 26: Principal component analysis (PCA) based on non-phylogenetic Bray–Curtis distance metrics for control (CONT) and tulathromycin (TUL) treated piglets at different sampling days (0, 5 and 20). The percent variation explained by each principal component is indicated on the axes. The individual data points from control (red circle) and TUL (green circle) which represent total fecal microbiota compositions of each piglet are also depicted. Significance between groups was analyzed using PERMANOVA with 9999 permutations and Bonferroni corrected P values.

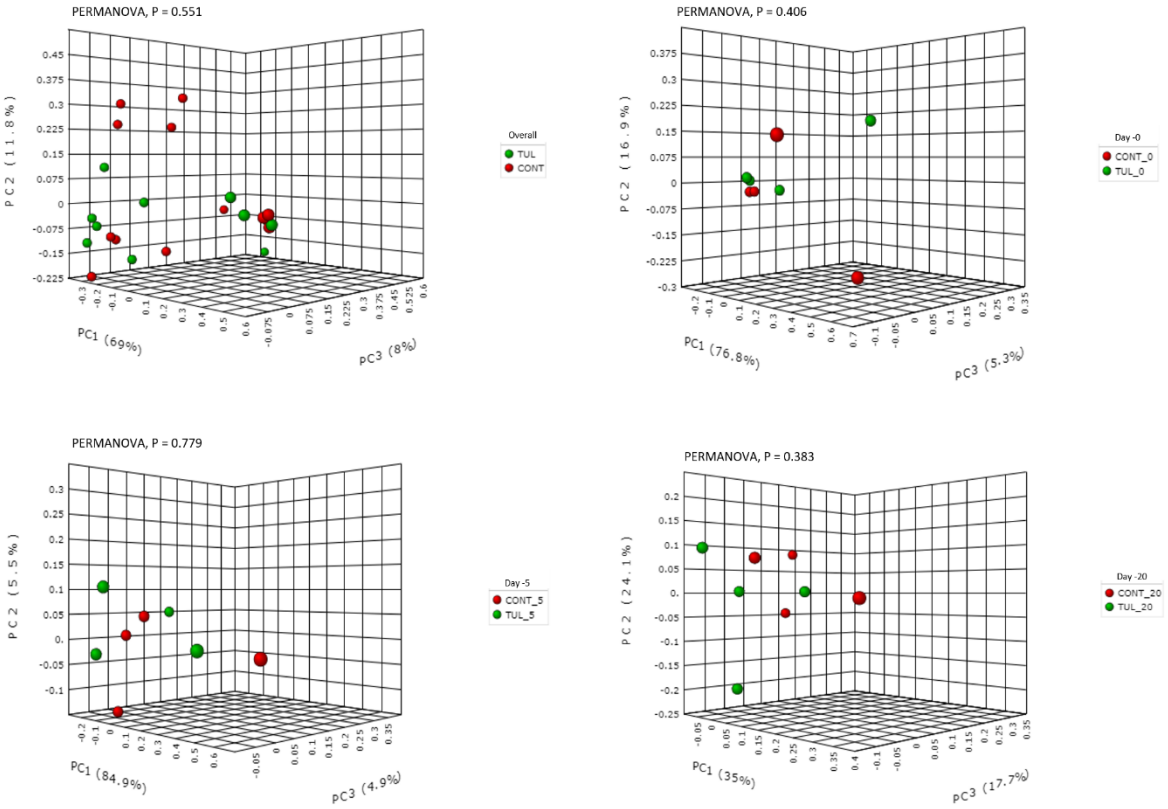


Figure 27: Inferred predictive functional features of piglet's fecal microbiota. **(**A) Heatmap cluster analysis of metagenomics functional capability at level 2 KEGG pathway based on differentially abundant functional features between the control (CONT) and tulathromycing (TUL) groups, and at different sampling days (0, 5 and 20). (B) The relative abundance of the functional profiles at level 2 in the TUL treated piglets compared to the CONT group (P-value > 0.05). (C) Principal component analysis (PCA) based on non-phylogenetic Bray–Curtis distance metrics for the overall functional gene profiles between CONT and TUL treated piglets. The percent variation explained by each principal component is indicated on the axes. The individual data points from CONT (red circle) and TUL (blue square) are also depicted.

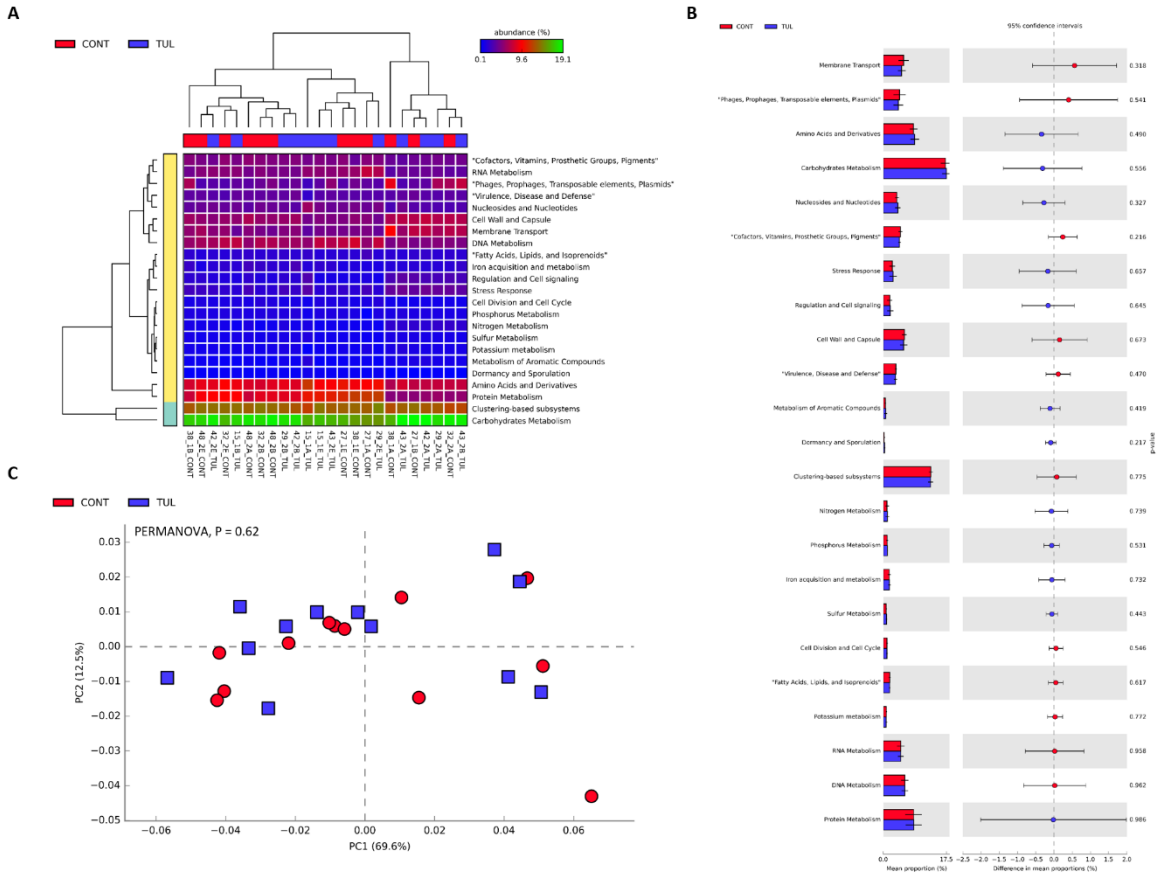


Figure 28: (A) Principal component analysis (PCA) for the predicted functional profiles at level 3 across all samples at different sampling days (0, 5 and 20). The percent variation explained by each component is indicated on the axes. The individual data points, which represent total fecal microbiota compositions of each piglet, are depicted. Significance between groups was analyzed using PERMANOVA with 9999 permutations and Bonferroni corrected P values. (B) Extended bar plot showed the statistically significant difference in functional gene features in the TUL treated piglets compared to the control group P-value < 0.05 was considered significant. (C) Extended bar plot showed the statistically significant difference in resistance to antibiotics functional potential in the TUL treated piglets compared to the CONT group P-value < 0.05 was considered significant.

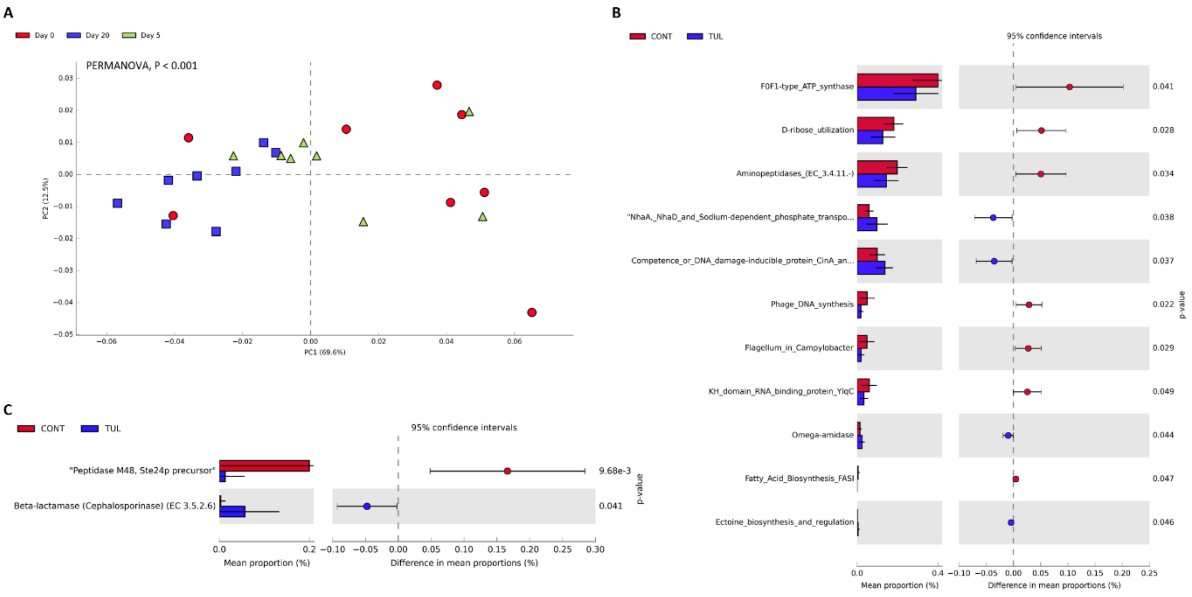


Figure 29: Heatmap of identified antibiotics resistome in the fecal microbiota at class level of each piglets in both control (CONT) and tulathromycin (TUL) groups.

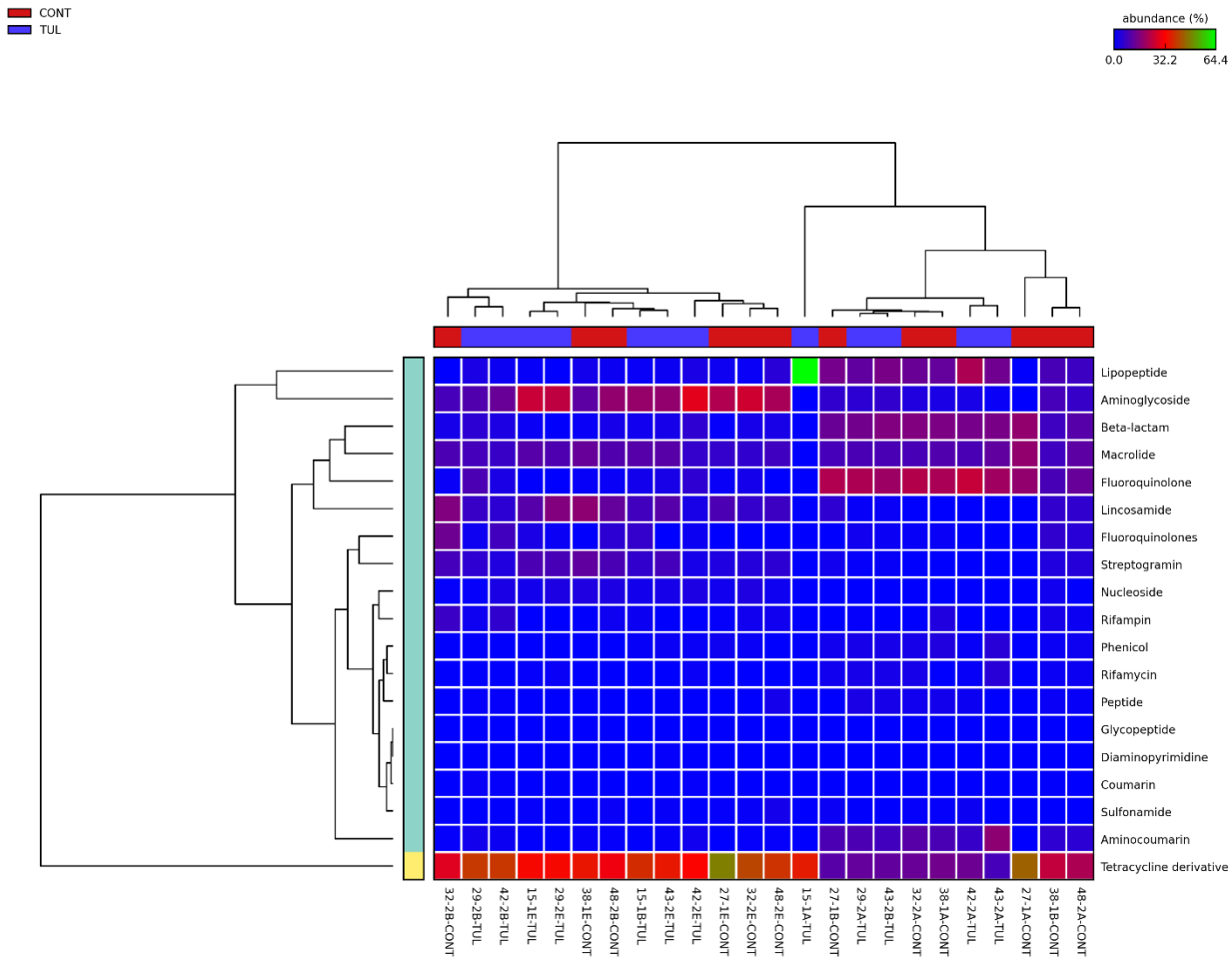


Figure 30: Extended bar plot showed the 50-predominant antimicrobial resistome, between the control (CONT) and tulathromycin (TUL) groups. P-value < 0.05 was considered significant.

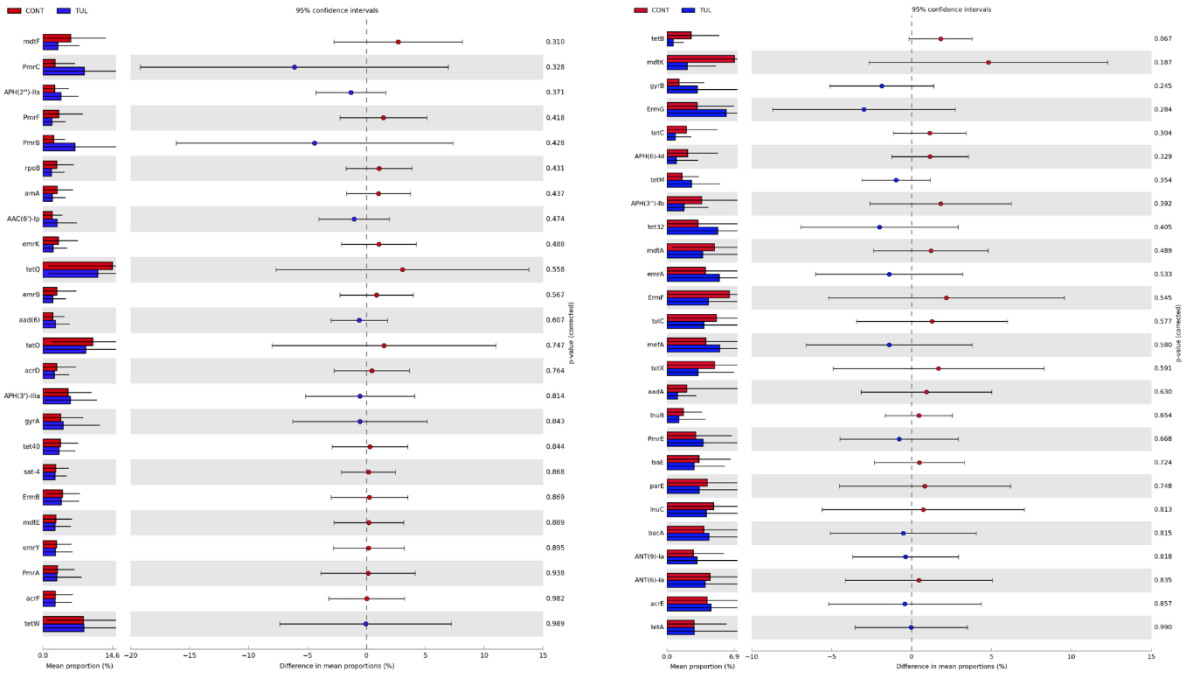


Figure 31: The difference in bacterial diversity indices (Chao 1, Shannon, Simpson and Pielou's evenness indices) measures between the two groups control (CONT) and tulathromycin (TUL) at different sampling days (0, 5 and 20). The individual data points, which represent bacterial diversity for each piglet, are depicted. Error bars represent the standard errors.

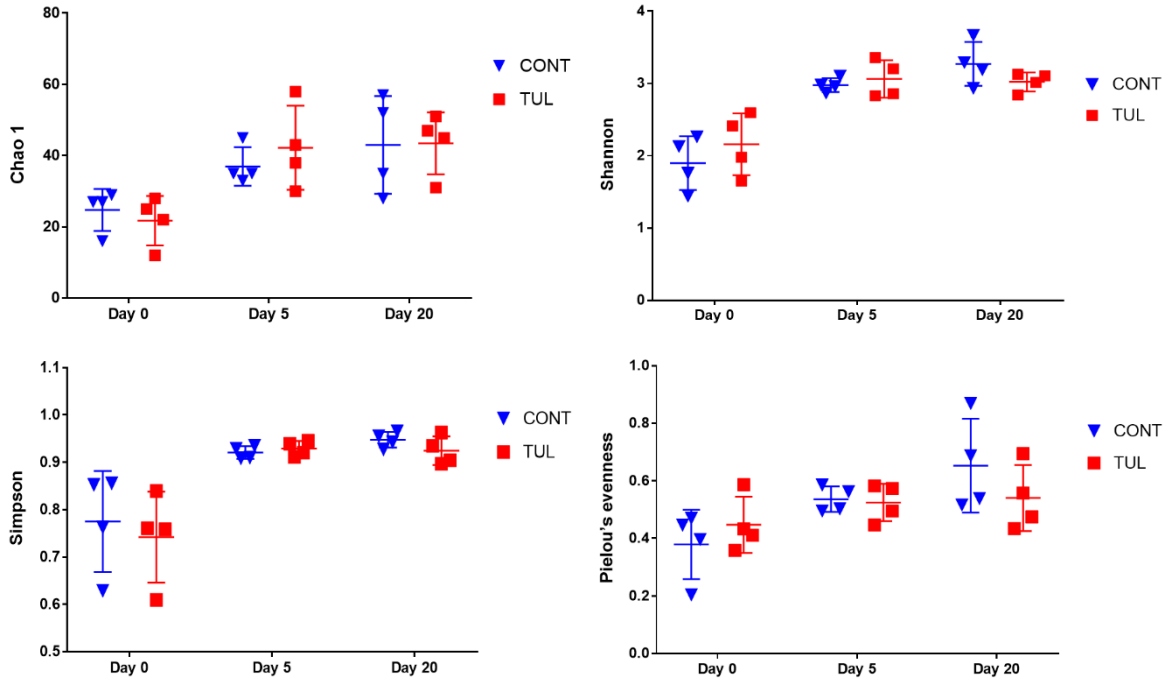
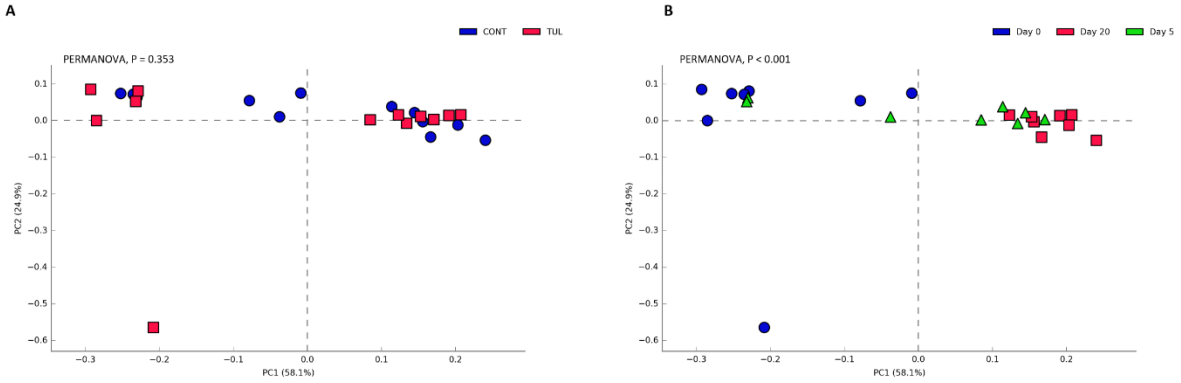


Figure 32: (A) Principal component analysis (PCA) based on non-phylogenetic Bray–Curtis distance metrics for the overall fecal antimicrobial resistome profiles between control and tulathromycin treated piglets. (B) Principal component analysis (PCA) for the overall fecal antimicrobial resistome across all samples at different sampling days (0, 5 and 20). The percent variation explained by each component is indicated on the axes. The individual data points, which represent total fecal microbiota compositions of each piglet, are depicted. Significance between groups was analyzed using PERMANOVA with 9999 permutations and Bonferroni corrected P values.



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