The Effects of Zinc Therapy on the Co-selection of Methicillin-resistance in Livestock-associated *Staphylococcus aureus* and the Bacterial Ecology of the Porcine Microbiota

by

Mackenzie Jonathan Slifierz

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Doctor of Philosophy
in
Pathobiology

Guelph, Ontario, Canada

ABSTRACT

THE EFFECTS OF ZINC THERAPY ON THE CO-SELECTION OF METHICILLIN RESISTANCE IN LIVESTOCK-ASSOCIATED *STAPHYLOCOCCUS AUREUS* AND THE BACTERIAL ECOLOGY OF THE PORCINE MICROBIOTA

Mackenzie J. Slifierz
University of Guelph, 2016

Advisor:
Dr. J. Scott Weese

With increasing pressure to reduce the use of conventional antibiotics in livestock production systems, high-dose zinc therapy has become a popular alternative, but there is insufficient research assessing its risk to the health of animals, humans, and the environment. The objectives of this investigation were: (i) to determine whether zinc could inadvertently co-select for antibiotic-resistance given the co-location of the zinc-resistance gene (*czrC*) within a bacterial resistance island, and (ii) to better understand the early-life porcine microbiota and to determine whether zinc impacts the bacterial ecology of the porcine nasal microbiota.

First, epidemiological and experimental studies were conducted to determine the effects of zinc on the co-selection of antibiotic-resistance genes in methicillin-resistant *Staphylococcus aureus* (MRSA) which is commonly carried by pigs and is a potential risk to public health. These studies revealed that exposure to high doses of in-feed zinc can affect the prevalence and persistence within a nursery pig herd and that the presence of MRSA on farms is strongly associated with the use of zinc therapy in nursery diets.

Second, the porcine nasal and fecal microbiotas were studied using next-generation sequencing. Under normal conditions, pigs have a very rich and diverse microbiota which undergoes a rapid evolution during the first several weeks of life. When given zinc therapy, the nasal microbiota demonstrated an increased richness of bacteria, greater relative abundance of uncommon bacterial taxa, and a less established core microbiota comprised of only a highly zinc-resistant bacterial genus. These findings suggest that zinc therapy may increase susceptibility to additional bacteria beyond what is normally observed among the nasal microbiota; although, it cannot be said whether this has positive or negative implications for host health.

The use of a therapeutic zinc diet in swine production systems appears to be associated with the co-selection of livestock-associated MRSA. Evidence also suggests that zinc therapy may increase the susceptibility of the nasal microbiota to colonization with zinc-resistant bacterial species, such as livestock-associated MRSA. In conclusion, zinc therapy appears to be at least one factor that is driving the emergence and persistence of MRSA in commercial swine herds.

Acknowledgements

Everyone who was involved with this research has my complete gratitude. This great milestone could not have been accomplished without the support of my mentors, colleagues, friends, and family, who are oftentimes considered as one and the same.

I would like to thank my exceptional advisor, Dr. Scott Weese, for giving me the opportunity to take on this challenge and for mentoring me throughout the process. I would also like to express my deepest appreciation to the other outstanding members of my advisory committee: Dr. Bob Friendship and Dr. Jan MacInnes. Your advice and guidance has been invaluable, and for that I am truly thankful. I also wish to recognize Joyce Rousseau who was a mentor in the laboratory and provided essential support throughout this endeavor.

I wish to acknowledge and thank those who made direct and indirect contributions towards this research project: Bryan Bloomfield, Karen Richardson, Dr. Glen Cassar, Dr. Terri O'Sullivan, Amanda Perri, Amy Sturgeon, Mohammad Jalali, Rebecca Flancman, Diego Gomez, Jeffrey Gross, and the rest of the staff at the Advanced Analysis Centre's Genomics Facility at the University of Guelph. Their helpfulness and support throughout this research project is very much appreciated.

Last but not least, I could not have achieved this without my wife, Melissa Cummings, whose encouragement and dedication has been beyond measure.

Table of Contents

List of Tables

List of Figures

CHAPTER 1: Introduction and Review of the Literature

1.1 − Introduction

High levels of in-feed zinc are commonly used in swine production as an antimicrobial-alternative therapy for prevention of post-weaning diarrhea. They are also applied non-therapeutically as for growth promotion. Despite its widespread use, the mechanisms of this therapy are unclear and potential consequences of using this therapy are not well understood, including its effects on the bacterial communities that drive the metabolism and health of the host. The overarching objective of this thesis is to investigate the effects of zinc therapy on the microbial populations within pigs, including the short-term impacts on the microbial ecology and the persistence of undesirable phenotypic traits such as antimicrobial resistance.

The first part of this thesis will explore the hypothesis that zinc therapy is a driver of antimicrobial resistance. The recently discovered zinc resistance gene (*czrC*) in staphylococci of porcine origin has been found to co-locate with other antimicrobial resistance genes within a mobile genetic element known as the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (Cavaco et al., 2010). Due to the co-location of *czrC* and other antimicrobial resistance genes, it is suspected that exposure to high doses of zinc may cause co-selection and co-retention of antimicrobial-resistance genes in the absence of antimicrobials (Cavaco et al., 2010). This hypothesis will be tested through both experimental and epidemiological methods.

The second part of this thesis will describe the evolution of the developing bacterial communities (microbiota) that populate the gut and nasal cavity of swine, and

determine whether zinc therapy impacts the porcine nasal microbiota. High-throughput next-generation sequencing of the 16S rRNA gene (V4 region) will be used to characterize the developing microbiota under normal conditions and after exposure to zinc therapy. It is anticipated that this will further the understanding of the developing porcine microbiota as well as determine the effects of therapeutic zinc on the bacterial ecology of the porcine nasal microbiota.

1.2 – Zinc therapy in swine production

Pigs require zinc in their diet and a ration supplemented with approximately 50-125 ppm zinc is nutritionally suitable for normal growth and development (Jacela et al., 2010). However, higher concentration of zinc are also used therapeutically in swine production as a means to control *Escherichia coli* diarrhea (colibacillosis), particularly during the early nursery phase when pigs are just weaned (Jacela et al., 2010). Typically, therapeutic or pharmacological levels of zinc are added to the first-phase nursery ration at 2,000 – 3,000 ppm, which pigs will receive for 2-3 weeks (Jacela et al., 2010). Although pigs have a demonstrable resilience to high levels of in-feed zinc, it is generally not recommended to use >3,000 ppm zinc or to prolong exposure as this can lead to zinc toxicosis which is characterized by depressed feed in-take and growth, arthritis, gastritis, and hemorrhaging of the brain, lymph nodes, spleen, and axilla (Brink et al., 1959).

The most common source of zinc used in swine production is zinc oxide (ZnO), but other sources such as zinc sulfate and zinc methionine are also used, although less frequently due to mixed reports of their effectiveness (Jacela et al., 2010). Zinc oxide is approximately 74% zinc (Jacela et al., 2010) and is a relatively inexpensive (~$7.50 CND

per metric ton of feed). Furthermore, there is research which has demonstrated that high levels of ZnO can also increase growth performance in healthy nursery pigs, although the effectiveness varies by farm and the mechanism responsible is still unclear (Hill et al., 2000). Given its therapeutic and cost effectiveness, in addition to its growth-promoting abilities, it is unsurprising that concentrated ZnO has been rated as one of the top alternatives for replacing conventional in-feed antimicrobials (Pluske et al., 2013).

Nevertheless, the use of zinc therapy in swine production has generated some concern. It is unclear how high doses of zinc affect the microorganisms that populate the gastrointestinal and respiratory tracts of pigs and, although it may provide protection against some diseases, it is unknown whether this therapy may cause susceptibility to other porcine or zoonotic pathogens. Zinc therapy may also play a role in co-selection of resistance genes in bacteria due to the co-location of zinc-resistance and antimicrobial-resistance genes within a shared plasmid or mobile genetic element (Cavaco et al., 2010). Lastly, there is also concern that concentrated zinc excreted in the manure may have a detrimental effect on the environment when the manure is used to enrich the soil with nutrients. As there is increasing pressure to reduce the use of conventional antimicrobials in livestock production systems, zinc is becoming a popular alternative, but there is insufficient research assessing its risk to the health of animals, humans, and the environment.

1.3 – *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium that is a common commensal of humans and animals. It is estimated that 20% of the general human population is

persistently colonized with *S. aureus* and an estimated 60% of the population carries this bacterium intermittently (Kluytmans et al., 1997). Although many people and animals carry *S. aureus* without incident, colonization with this bacterium does increase the risk for opportunistic infections in certain situations (Kluytmans et al., 1997). *S. aureus* remains one of the leading causes of infections in many developed nations; it is estimated to cause over 500,000 hospitalized infections and 12,000 deaths each year in North America which costs in excess of $6 billion USD in healthcare expenditures (Diekema et al., 2001; Klein et al., 2007).

The primary niche of *S. aureus* in humans and animals is the nares (Williams, 1963), although it can colonize other body sites including the skin, oropharyngeal cavity, respiratory tract, gastrointestinal tract, and urogenital area (Madigan et al., 2008). In some situations, *S. aureus* colonization at inappropriate or compromised sites (wounds) within the host can lead to infection. Opportunistic infections commonly caused by *S. aureus* include skin and soft tissue infections, osteomyelitis, arthritis, pneumonia, meningitis, bacteremia, and toxic shock syndrome, in addition to its ability to cause toxin-mediated food poisoning (Madigan et al., 2008).

Antimicrobial therapy has routinely been used to treat many types of staphylococcal infections; however, the acquisition of resistance to common antimicrobial agents has made treatment more difficult and costly (Klein et al., 2007). Methicillin-resistant *S. aureus* (MRSA) has emerged as a serious nosocomial disease in healthcare settings worldwide (Stevens et al., 2010). Many strains of MRSA have acquired tolerance to a multitude of antimicrobials including beta-lactams, fluoroquinolones, macrolides, licosamides, streptogramins, tetracyclines,

aminoglycosides, and glycopeptides. Some clinical isolates of MRSA present resistance to over a dozen antimicrobial agents (Maple et al., 1989). This situation has become unsettling as MRSA continues to develop resistance to antimicrobials and is outpacing the list of effective drugs. To combat the threat of MRSA, intensive global surveillance programs have been developed (Diekema et al., 2001; Zhanel et al., 2013), and strict "search-and-destroy" policies have been implemented with noticeable success (Vos et al., 2005). There is increasing pressure on pharmaceutical companies to develop novel antimicrobials, and there is greater advocacy for antimicrobial stewardship in both human and animal medicine.

1.3.1 – Infections in humans and animals

Staphylococcus aureus is the leading cause of skin and soft tissue infections, bacteremia, and pneumonia in humans in almost all regions of Canada, the United States, Europe, Latin America, and the West Pacific (Diekema et al., 2001). In the United States, there were 478,000 estimated cases of *S. aureus*-related hospitalizations in 2005 (Klein et al., 2005). The U.S. Centers for Disease Control and Prevention also estimates that staphylococcal food poisoning accounts for 185,000 illnesses and 1,700 hospitalizations in the US each year (Mead et al., 1999) which costs billions of dollars in lost productivity and healthcare resources (Buzby and Roberts, 2007). Furthermore, patients hospitalized with an *S. aureus* infection have longer hospital stays, incur more hospital charges, and are 5-times more likely to die when compared to other patients (Noskin et al., 2005). Similar reports in Canada demonstrate the prevalent and burdensome nature of *S. aureus* infections on population health; it is estimated that invasive *S. aureus* infections

(hospital- and community-associated) occur at a rate of 28.4 cases per 100,000 Canadians (Laupland et al., 2003). When compared to other rates of diseases that are reportable in the Canadian province of Ontario, invasive *S. aureus* infections are among the most frequently occurring infectious disease (see Figure 1.1). Overall, infections arising from *S. aureus* have a tremendous impact on human health and create a considerable burden for healthcare systems worldwide.

Infections arising from antimicrobial-resistant strains of *S. aureus*, namely methicillin-resistant *S. aureus* (MRSA), are more difficult and costly to treat when compared to methicillin-sensitive *S. aureus* (MSSA) infections (Klein et al., 2007). MRSA infections can be broadly categorized as hospital-associated (HA-MRSA), community-associated (CA-MRSA), or livestock-associated (LA-MRSA), in addition to some further categorizations such as hospital-acquired/community-onset or laboratory-acquired MRSA infections. Typically, the primary types (HA-, CA-, and LA-MRSA) have distinguishable traits and antimicrobial resistance patterns, although these patterns are changing due to the migration of MRSA between environments (Mediavilla et al., 2012). Furthermore, there is also a significant transformation in the epidemiology of MRSA in Canada; HA-MRSA cases are decreasing while CA-MRSA cases are increasing (Nichol et al., 2013).

Staphylococcus aureus has also been reported to colonize and infect various animal species including dogs, cats, horses, cattle, pigs, poultry, and some marine mammals, although infections in most of these species is uncommon with the exception of cattle (Weese, 2010). Wound and postoperative infections, urinary tract infections, otitis, and pyoderma are typical *S. aureus*-related infections seen in dogs and cats

(Weese, 2010). In cows, *S. aureus* is a major etiological agent of bovine mastitis which occurs at a rate of 23 cases per 100 cow-years in Canada, representing a significant problem and expense to dairy production systems (Olde Riekerink et al., 2008). *S. aureus* infections are less relevant in other livestock species, including pigs which rarely experience *S. aureus*-related infections although it has been implicated as an etiological agent in ear necrosis (Park et al., 2013). The more immediate concern with colonization among household pets and livestock species is that this may represent a risk to humans and that antimicrobial use in livestock production may be contributing to the emergence of antimicrobial-resistance in *S. aureus*.

1.3.2 – Resistance to conventional antimicrobials

Since the widespread introduction of therapeutic antimicrobials in the early 1940s, *Staphylococcus aureus* has developed resistance to several commonly used antimicrobial agents. It first developed resistance to penicillin in the 1950s (Stevens et al., 2010), and in 1961 the first case of methicillin resistance was reported in the literature (Jevons, 1961). Methicillin-resistant *S. aureus* (MRSA) carries the *mecA* gene which codes for penicillin-binding protein 2a (PBP2a); a peptidoglycan-synthesizing protein with a low affinity for beta-lactam antimicrobials (e.g., methicillin and oxacillin). Recently, a novel methicillin-resistance gene (*mecC*) has been described in the literature, although its relevance is not clear apart from its potential to go undetected by diagnostic laboratories screening for methicillin-resistance (Paterson et al., 2014).

The *mecA* gene is located within the Staphylococcal Cassette Chromosome *mec* (SCC*mec*); a mobile genetic element located within the chromosomal genome of

staphylococci. The SCC*mec* has been described as a "resistance island" due to the tendency for multiple antimicrobial-resistance genes to accumulate within this region (Ito et al., 1999). The SCC*mec* also contains one or more recombinase genes (*ccr*) which code for enzymes responsible for site-specific integration and excision of the SCC*mec* element (Katayama et al., 2000).

Current evidence suggests that the SCC*mec* can transfer horizontally between staphylococci species, although the mode of transfer is still unknown (Berglund et al., 2008; Smyth et al. 2010; Vanderhaeghen et al., 2012) and this is probably an uncommon event given the size of the cassette. One study has demonstrated that the rare mobile genetic element *sasX*, which increases virulence and pathogenicity, was able to spread between different MRSA sequence types, strongly supporting the theory of horizontal gene transfer (Li et al., 2012). Many non-*S. aureus* staphylococci species act as a reservoir for the SCC*mec* and it is speculated that these reservoirs may contribute to the development of new (*de novo*) MRSA strains by horizontal gene transfer (Smyth et al., 2010; Vanderhaeghen et al., 2012; Tulinski et al., 2012). The ability for multiple antimicrobial-resistance genes to accumulate within a mobile genetic element that is transferable between staphylococci species is concerning and further investigation into the mechanism of horizontal gene transfer between staphylococci is warranted.

In addition to methicillin-resistance, MRSA is also resistant to a multitude of antimicrobial agents (see Table 1.1), including, and most commonly, fluoroquinolones, macrolides, lincosamides, streptogramins, tretracyclines, and aminoglycosides. Resistance to glycopeptides, oxazolidonones, lipoglycopeptides, and glycylcyclines has also been reported (Richter et al., 2011; Zhanel et al., 2013; Nimmo et al., 2011; ECDC,

2013; Sader et al., 2007; Jones et al., 2013; Mendes et al., 2012; Jones et al., 2014; Rubin and Chirino-Trejo, 2011). However, resistance to these antimicrobials is rare. The following section will review some of the most common and relevant antimicrobial-resistance mechanisms in *S. aureus*.

One of the most common resistance phenotypes found in *S. aureus* is resistance to fluoroquinolones, particularly among human hospital-associated strains (Pantosti et al., 2007). Fluoroquinolone antimicrobials act by interfering with topoisomerases, the class of enzymes responsible for unwinding DNA. This essentially inhibits duplication of bacterial DNA (Hooper, 2002). Point mutations in the quinolone-resistance-determining-region (QRDR) of topoisomerase IV and DNA gyrase lead to fluoroquinolone resistance in *S. aureus* (Hooper, 2002). Resistance may be enhanced in some strains by the presence of a fluoroquinolone efflux pump encoded by *norA* (Kaatz et al., 1993).

Staphylococcus aureus has also acquired broad-spectrum resistance to macrolide (e.g. erythromycin), lincosamide (e.g. clindamycin), and streptogramin B antimicrobials – collectively termed MLS_B resistance (Leclercq, 2002). Methylation of the antimicrobial target site of the ribosome leads to this broad cross-resistance and is conferred by an array of *erm* (erythromycin ribosome methylase) genes (Leclercq, 2002). The transposon-bound *erm*(A) is frequent amongst MRSA strains whereas the plasmid-bound *erm*(C) is more closely associated with MSSA strains (Leclercq, 2002). A novel multi-resistance plasmid carrying *erm*(T) has recently been reported in MRSA ST398 of porcine origin (Gómez-Sanz et al., 2013). Resistance to macrolide and streptogramin B antimicrobials can also be acquired by an efflux pump encoded by the plasmid-bound *msr*(A) gene

(Ross et al., 1996). Additionally, procurement of *lnu*(A) confers resistance to only lincosamindes, although this mechanism is uncommon in *S. aureus* (Leclercq, 2002).

Another class of antimicrobials that *S. aureus* has acquired widespread tolerance to is the tetracyclines. Tetracycline antimicrobials inhibit protein synthesis in bacteria by binding to the 30S ribosomal subunit during translation thereby blocking aminoacyl-tRNA from attaching to the A-site of the ribosome (Madigan et al., 2008). Resistance to tetracyclines is present in some strains of *S. aureus* and is particularly prevalent among MRSA associated with livestock, which is likely a consequence of widespread tetracycline use in livestock feed (Kreausukon et al., 2012; Stegger et al., 2013). Resistance to tetracyclines can occur through two mechanisms; protection of the ribosome from tetracyclines as conferred by the *tetO* and *tetM* genes (plasmid-bound) or active efflux of tetracyclines as conferred by the *tetK* and *tetL* genes (chromosomal- or transposon-bound) (Pantosti et al., 2007).

Aminoglycoside antimicrobials (e.g. gentamicin, amikacin, kanamycin, neomycin, and streptomycin) are still used to treat staphylococcal infections as these agents exert a synergistic effect when used in combination with other antimicrobials such as vancomycin and penicillin (Shlaes et al., 1991). Aminoglycosides act by disrupting protein synthesis during translation, and although different aminoglycosides have distinct functions, the result is often a defective or truncated protein. Resistance to aminoglycosides usually results from the acquisition of plasmid-bound genes that encode aminoglycoside-inactivating enzymes; however, other mechanisms exist including ribosomal modification and changes in cell permeability (Shaw et al., 1993). The clinical effectiveness of aminoglycoside therapy is limited as resistance has become widespread

amongst clinical isolates of *S. aureus*, especially in MRSA (Maple et al., 1989; Schmitz et al., 1999).

One of the most important antimicrobials used to treat MRSA infections are the glycopeptide antimicrobials. Glycopeptides bind to the acyl-D-alanyl-D-alanine of peptidoglycan thereby blocking the synthesis of the cell wall. The treatment of multidrug-resistant staphylococcal infections has depended on the continued effectiveness of glycopeptide antimicrobials (vancomycin and teicoplanin) for over 40 years (Sakoulas and Moellering, 2008). However, in 1997, the first clinical isolate of intermediate vancomycin-resistant *S. aureus* (VISA) was reported (Hiramatsu et al., 1997). This was subsequently followed by the identification of highly vancomycin-resistant *S. aureus* (VRSA) in the United States in 2002 (CDC, 2002). At present, infections caused by VISA and VRSA are rare and have only been reported sporadically with no secondary cases (Moellering, 2012). These strains are still susceptible to several antimicrobial agents (ceftaroline, daptomycin, linezolid, minocycline, trimethoprim-sulfamethoxazole, rifampin, and tigecycline) (Saravolatz et al., 2012), and their pathogenicity is attenuated as the production of virulence factors appears to be inhibited by the mechanisms responsible for vancomycin resistance (Peleg et al., 2009). However, an alarming report by researchers in Brazil shows that the most common strain of CA-MRSA (ST8 / USA-300) has recently acquired vancomycin resistance with no loss in pathogenicity (Rossi et al., 2014). Intermediate vancomycin-resistance is conferred by a thickened cell wall whereas complete resistance to vancomycin is conferred by the *vanA* operon (Pantosti et al., 2007). Research by Weigel et al. (2003) demonstrates that VRSA likely acquired a

plasmid carrying transposable *vanA* operon from vancomycin-resistant e*nterococci* (VRE) through horizontal gene transfer.

Despite resistance to vancomycin, there are still a number of old and new antimicrobials that are effective for treating staphylococcal infections, although the toxic side-effects and costliness of these alternative antimicrobials are unwelcoming (Rivera and Boucher, 2011). Some of the effective antimicrobials used to treat multidrug-resistant staphylococcal infections include rifampicin, daptomycin, linezolid, tigecycline, trimethoprim-sulfamethoxazole, and quinupristin-dalfopristin (Goldberg et al., 2010; Rivera and Boucher, 2011; Zhanel et al., 2013; Oksuz and Gurler, 2013). Additionally, the US Food and Drug Administration (FDA) recently approved telavancin (lipoglycopeptide) in 2009 (Goldberg et al., 2010), ceftaroline (cephalosporin) in 2010, and dalbavancin (lipoglycopeptide) in 2014 for use in treating *S. aureus* infections, including MRSA (Rivera and Boucher, 2011). Treatment of MRSA infections often employs a synergistic combination of these antimicrobials, but certain combinations can reduce therapeutic effectiveness (Moellering, 2012). However, MRSA has demonstrated resistance or reduced susceptibility to all of the previously described antimicrobial therapies, usually rarely and sporadically (see Table 1). Several new anti-staphylococcal agents are also being explored (Moellering, 2011), including tedizolid (an oxazolidinone antimicrobial) which has recently been approved by an FDA advisory committee for treating MRSA infections. Nevertheless, further research and development of new antimicrobial therapies for MRSA infections is warranted.

1.3.3 − Resistance to heavy metals

Staphylococcus aureus is able to acquire resistance to many heavy metal compounds including arsenic, cadmium, copper, zinc, nickel, mercury, lead, chromium, silver, tin, lithium, aluminum, barium, and strontium (Ug and Ceylan, 2003; Li et al., 2011; Yilmaz et al., 2013; Gómez-Sanz et al., 2013). Unfortunately, heavy metal resistance has become associated with antimicrobial resistance due to co-location of resistance genes within plasmids and transposable elements (Li et al., 2011; Gómez-Sanz et al., 2013). Exposure to heavy metals can lead to co-selection of plasmids or transposable elements that contain both heavy metal and antimicrobial resistance genes (Baker-Austin et al., 2006). Strong evidence supports the phenomenon of co-selection of antimicrobial resistance genes through heavy metal exposure in several bacterial species (Calomiris et al., 1984; Hasman and Aarestrup, 2002; Stepanauskas et al., 2006; Medardus et al., 2014). Hence, exposure to heavy metals may contribute to the selection and persistence of antimicrobial resistance even in the absence of antimicrobial exposure.

Resistance to heavy metals is particularly common in MRSA isolated from livestock. Studies have identified several heavy metal resistance genes in LA-MRSA including *czrC* (cadmium and zinc resistance), *cadD*/*cadX* (cadmium resistance), *mco*/*copA*/*copB* (copper resistance), and *arsRBC*/*arsDARBC* (arsenic resistance operons) (Cavaco et al., 2010; Li et al., 2011; Gómez-Sanz et al., 2013). The mechanisms and characteristics of resistance for these heavy metals are summarized in Table 1.2. Of particular interest is the zinc and cadmium resistance gene *czrC* which is commonly carried by MRSA of porcine origin (Cavaco et al., 2010). The *czrC* gene is located within the SCC*mec* alongside *mecA* which presents a potential opportunity for co-selection

(Cavaco et al., 2011). This concern is compounded by the fact that pigs are commonly exposed to high doses of in-feed zinc oxide (≥2,000 mg/kg) as a prophylactic therapy to control post-weaning *E. coli* diarrhea (colibacillosis) and it is often used as an alternative to conventional antimicrobials (Puske et al., 2013). The widespread use of zinc oxide combined with the opportunity for zinc-mediated co-selection of resistance to β-lactam antimicrobials may provide an explanation for the persistence of MRSA on "antimicrobial-free" pig farms (Weese et al., 2011; Osadebe et al., 2013; van de Vijver et al., 2013; Park et al., 2013). Further investigation is needed to determine whether therapeutic doses of in-feed zinc oxide affect the prevalence and persistence of MRSA within and between swine herds. Additionally, zinc was also widely used in hospital antiseptic solutions and in over-the-counter products such as nasal sprays, lozenges, topical skin ointments, and cosmetics.

1.3.4 – Molecular epidemiology and bacterial typing techniques

Molecular typing methods are useful epidemiological tools that allow for the surveillance of bacterial dissemination and evolution. For *S. aureus*, there are 4 main molecular typing methods; *spa* typing, multilocus sequence typing (MLST), pulse-field gel electrophoresis (PFGE) macrorestriction analysis, and multilocus variable-number tandem repeat analysis (MLVA) (Stefani et al., 2012). The use of these methods varies by laboratory, profession, and country. However, spa typing and MLST typing have become the preferred methods in most regions as there are practical limitations with using PFGE and MLVA. Although PFGE is considered the gold standard due to its considerable discriminatory power and high reproducibility, it is very labour intensive, has low

throughput, and in some circumstances many isolates may be labelled as non-typeable due to restriction enzyme resistance (Bannerman et al., 1995; Argudín et al., 2010). On the other hand, MLVA overcomes many of the limitations of PGFE, being rapid and having high throughput, but there is a lack of standardized methodology and nomenclature (Stefani et al., 2012). Development and widespread use of MLST and *spa* typing is due to the many advantages of these sequence-based methods, including: medium to high throughput, high discriminatory power, unambiguous results, standardized nomenclature, and highly reproducible data that are easily shared through online databases (Stefani et al., 2012). Given the preference for these typing methods, the following section will briefly review the use of *spa* typing and MLST for *S. aureus* in addition to SCC*mec* typing that is used for classification of MRSA.

One of the most attractive typing methods for *S. aureus* is *spa* typing. This method uses sequence data for the variable polymorphic X region of the surface protein A (*spa*) gene of *S. aureus* (Stefani et al., 2012). The variable-number tandem repeats (VNTR) in this region and can form many arrangements which have generated over 13,000 unique *spa* types (Mediavilla et al., 2012). There are two standardized nomenclatures used for *spa* typing; Ridom StaphType and the Kreiswirth index (Harmsen et al., 2003; Koreen et al., 2004). Types can easily be assigned via an online database (ww.SeqNet.org), which currently boasts the largest and most comprehensive typing database for *S. aureus*, containing almost 300,000 isolates with submissions from more than 100 countries. The rapid and easy dissemination of results has made *spa* typing a very important epidemiological tool. It has proven useful for local, regional, and national epidemiological studies, and has been used to investigate local outbreaks, although it is

less useful in settings that have a single or few endemic clones, such as small hospitals (Stefani et al., 2012). Nevertheless, *spa* typing is rapid, cost efficient, has high-throughput, excellent reproducibility, good discrimination, and a standardized nomenclature.

Another widely used typing technique for *S. aureus* is MLST. Sequencing of specific fragments of 7 housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpiA, yqiL*) allows for the identification of alleles of each gene. The alleles are assigned an identification number and when these numbers are joined they form a unique allelic profile that corresponds to a specific sequence type (ST) (Mediavilla et al., 2012). Closely related STs, which may differ by only a few single nucleotide polymorphisms but share at least 5 identical alleles, can be grouped into clonal complexes (CC) (Mediavilla et al., 2012; Stefani et al., 2012). The standardized protocol and nomenclature for MLST can be easily accessed through an online database (http://saureus.mlst.net). Although MLST is standardized, reproducible, unambiguous, and has medium-throughput, it does have several limitations including high cost, more prone to errors, and intermediate discrimination, such that its usefulness is limited to national and regional epidemiology (Stefani et al., 2012).

There is an additional molecular typing method, known as SCC*mec* typing, that is used widely and exclusively for MRSA classification. Typing of the SCC*mec*, a mobile genetic element that is the defining feature of MRSA, is completed by identifying two important characteristics of the SCC*mec*: the type of the cassette chromosome recombinase (*ccr*) gene complex, and the class of the *mec* gene complex. The type of *ccr* gene complex is determined by the *ccr* gene(s) present; there are multiple *ccr* genes

(*ccrA, ccrB, ccrC*) and multiple allotypes for some of the *ccr* genes (*ccrA1*, *ccrA2*…) (IWG-SCC, 2009). For the *mec* gene complex, combinations of the *mecA* gene and its regulatory factors (*mecR1*, *mecI*…) determine the class of that complex (IWG-SCC, 2009). Together, the type of the *ccr* gene complex (Types 1−8) and the class of the *mec* gene complex (Class A, B, C1, C2, D, E) determines the SCC*mec* type, of which there are currently 11 defined types (SCC*mec* types I−XI) and multiple subtypes (IWG-SCC, 2009). By itself this typing method has low discriminatory power but it can be used in combination with other typing methods to improve discrimination; such is the case when differentiating the North American (SCC*mec* type IVa) and Latin American (SCC*mec* type IVc) clones of ST8 (Mediavilla et al., 2012).

1.4 − Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA)

In 2004, methicillin-resistant *Staphylococcus aureus* (MRSA) was identified in pigs and a pig farmer in the Netherlands (Voss et al., 2005). Since then, a worldwide emergence of MRSA in swine production has been reported (Cuny et al., 2010). Specific sequence types (ST) of MRSA, namely ST398 and ST9, are strongly associated with pigs (Cuny et al., 2010). Current research suggests that methicillin-susceptible *S. aureus* (MSSA) ST398 originating from humans colonized pigs and acquired resistance, presumably through exposure to antimicrobial agents routinely used in swine production (Price et al., 2012), with subsequent evolution so that genetic markers of pig- or human-adaptation are now identifiable (Stegger et al., 2013). MRSA has also been identified in other livestock species including cattle and poultry (Weese, 2010). These strains of MRSA that are frequently isolated from livestock species and contain livestock-specific

genetic markers are collectively referred to as livestock-associated MRSA (LA-MRSA). The identification of MRSA in livestock production systems has generated concern about the use of antimicrobials in agriculture and whether LA-MRSA poses a risk to human health.

1.4.1 − Molecular and genetic characteristics of LA-MRSA

The predominant sequence types of MRSA that are associated with pigs are ST398 (Europe and North America) and ST9 (Asia) (Cuny et al., 2010). Further, ST398 contains several *spa* types that show distinct geographical distributions, including t034 and t011 which are predominant in North American pigs and European pigs, respectively (see Table 1.3). However, there are also some strains of MRSA ST398 that are non-typeable using the standard *spa* typing method (Shopsin et al., 1999; Votintseva et al., 2014). This is due to a deletion of the IgG-binding region of the *spa* gene, and the increased frequency of this deletion among ST398 may be a pig-specific immunological adaptation (Votintseva et al., 2014). Nevertheless, an improved technique now allows for the determination of the *spa* type of these previously non-typeable strains (Votintseva et al., 2014).

There is also commonality in SCC*mec* types that are identified in LA-MRSA. The predominant SCC*mec* types found in LA-MRSA strains are types IV and V (van Duijkeren et al., 2008; Park et al., 2013). Both SCC*mec* types IV and V have been identified in ST398, and previous research has identified ST398 isolates with different SCC*mec* types (IV and V) circulating within the same herd (van Duijkeren et al., 2008). Variability in the SCC*mec* is frequent among LA-MRSA strains to such an extent that 3

novel SCC*mec* types were discovered in MRSA of animal origin and there are many other composite SCC*mec* types carried by LA-MRSA, many of which are non-typeable (Vandendriessche et al., 2013).

In addition to common strains and SCC*mec* types that are associated with pigs, there are particular features distinguishable in LA-MRSA including genetic markers, resistance profiles, and virulence factors. One of the most distinct traits of MRSA associated with pigs is its resistance to antimicrobials that are commonly used in swine production systems, namely tetracyclines. Screening a reference library of human- and livestock-associated MRSA ST398 isolates revealed that *tetM* was absent in all human-associated MRSA ST398 but was present in 99% of porcine-associated MRSA ST398 (Stegger et al., 2013). The cadmium and zinc resistance gene *czrC* is also commonly found in MRSA of porcine origin which is unsurprising as high doses of in-feed zinc are used therapeutically in swine production (Cavaco et al., 2011).

Several novel antimicrobial-resistance genes have also been discovered in MRSA of animal origin. Three novel plasmid-bound resistance genes were identified in isolates of porcine MRSA ST398; *dfrK*, a trimethoprim resistance gene (Kadlec et al., 2009a), *erm*(T), which confers resistance to macrolides, lincosamides, and streptogramin B antimicrobials (Kadlec et al., 2009b), and *vga*(C), which confers resistance to lincosamindes, pleuromutilin, and streptogramin A antimicrobials (Kadlec et al., 2009c). Additionally, *vga*(E), which also confers resistance to lincosamides, pleuromutilin, and streptogramin A antimicrobials, was another variant identified in MRSA ST398 of porcine origin (Schwendener and Perreten, 2011). A novel resistance gene (*ampA*) to apramycin, an antimicrobial used exlusively in veterinary medicine, was also discovered

in porcine and bovine isolates of MRSA ST398 (Feßler et al., 2011). Furthermore, a novel *mecA* homologue, *mecC*, was recently identified in MRSA isolates from people and cattle (Garcia-Alvarez et al., 2011), although the frequency of *mecC* among livestock-associated MRSA appears low (Vandendriessche et al., 2013).

Virulence factors found in *S. aureus* that are relevant to human infections include enterotoxins, exfoliative toxins, toxic shock syndrome toxin (*tst-1*), Panton-Valentine leukocidin (PVL), immune evasion proteins, nasal and skin colonization factors (*sasX*), biofilm/adhesive molecules (*hbl*), and the argenine catabolic mobile element (ACME), among others (Fluit, 2012). Among LA-MRSA, the presence of enterotoxin genes, *tst-1*, PVL, and ACME is relatively rare (Fluit, 2012). Some colonization and adhesion factors are common amongst LA-MRSA (Fluit, 2012), however the frequency of hypervirulent determinants such as *sasX* have yet to be investigated. Overall, most strains of LA-MRSA have significantly attentuated pathogenicity due to the loss of many virulence factors and this has likely contributed to its low rate of infections among humans, although the risk of dissemination of virluence factors among LA-MRSA warrants continuing survellience.

1.4.2 – Epidemiology of LA-MRSA

Methicillin-resistant *Staphylococcus aureus* has been isolated from pigs throughout the world with an observed herd-level prevalence of 9−83% among some of the top pork producing countries (see Table 3). The prevalence of MRSA is particularly high among nursery (weaner) pigs. A longitudinal study demonstrated that the prevalence of MRSA dramatically increases following weaning (Weese et al., 2011a). This is the

time when pigs are mixed with naïve pen-mates and exposed to nursery rations containing antimicrobial compounds, although no antimicrobials were used in the study by Weese et al. (2011a). However, after peaking at 2-3 weeks post-weaning, the prevalence declines considerably until the end of the nursery phase (Weese et al., 2011a). The decline in MRSA shedding continues into the grower-finisher phase and until market-weight, at which point less than 5% of pigs are still shedding MRSA (Weese et al., 2011b).

An intermediate prevalence of MRSA among sows suggests that this population may act as a reservoir of MRSA. Shedding of MRSA by sows is a significant risk factor for MRSA carriage among suckling pigs (Weese et al., 2011a). Attempts to reduce MRSA shedding by sows, including washing sows with livestock shampoo or disinfectants, has been unsuccessful (Verhegghe et al., 2013). Current research is exploring targeted decolonization of MRSA in livestock using naturally occurring bacteriophages (Kraushaar et al., 2013).

Additional risk factors for MRSA colonization among swine herds have been identified or suggested in the literature. Since the identification of MRSA in swine production there has been escalating concern that conventional antimicrobials are the primary drivers of resistance in these bacteria. However, a large study of 202 swine herds in Germany identified only 2 significant risk factors, including herd size and time, and was unable to find an association between MRSA and antimicrobial usage (Broens et al., 2011). Additionally, several studies have documented the commonness and long-term persistence of MRSA in pigs raised without exposure to antimicrobials (Weese et al., 2011; Osadebe et al., 2013; Park et al., 2013) and in organic husbandry (Vijver et al.,

2013; Buntenkoetter et al., 2014). Furthermore, a recent meta-analysis of risk factors for MRSA in grower-finisher herds determined no difference between organic and conventional herds but did report group treatment with antimicrobials (aggregated data) as a risk factor (Fromm et al., 2014). Regional differences in MRSA carriage by pigs are also paradoxical; Denmark and The Netherlands, which have legislated restraints for antimicrobial use in livestock production, report a considerably higher prevalence than the Midwestern United States (Broens et al., 2011; Agersø et al., 2012; Smith et al., 2013). In addition, further research has demonstrated no difference in the presence of MRSA in conventional and antimicrobial-free pork products (O'Brien et al., 2012). Further research is needed to identify unconventional antimicrobials or antimicrobial-independent factors which may be contributing to the selection and persistence of MRSA in swine production.

1.4.3 – The risk of LA-MRSA to public health

There is concern that LA-MRSA poses a risk to human health. Research has demonstrated that MRSA is transferable between humans and pigs (Voss et al., 2005; Khanna et al., 2008) and that pig handlers or people living on pig farms are at a much greater risk of carrying MRSA (Graveland et al., 2011; Bisdorff et al., 2012). It has been demonstrated that the prevalence of MRSA in people exposed to livestock (24%) is significantly greater than the prevalence of MRSA in the unexposed population (1%) in Germany (Bisdorff et al., 2012). Colonization with MRSA for livestock farmers is such a risk that the Netherland's "search and destroy" policy has been amended to specifically screen for livestock handling upon hospital admission (van Rijen and Kluytmans, 2009). However, LA-MRSA colonization in humans is very transient and transmissibility

between humans is very poor compared to non-LA-MRSA (Hetem et al., 2013). A shift in host specificity and the loss of several virulence factors is likely responsible for the attenuated pathogenicity of LA-MRSA in humans (Price et al., 2012; Jamrozy et al., 2012).

Infections arising from LA-MRSA have also been reported, including skin and soft tissue infections (SSTIs), pneumonia, and endocarditis (Golding et al., 2010; Wulf et al., 2012; Omland and Hoffmann, 2012), although whether the clinical strain of MRSA originated from animals is not always clear (Davies, 2013). Since many strains of *S. aureus* are shared between humans and animals, making an inference based on the clinical strain alone is not always appropriate; further epidemiological investigation into previous contact with livestock and more discriminatory molecular techniques are warranted (Davies, 2013). For example, although *S. aureus* ST398 is commonly associated with livestock, this type contains 2 distinct clades of human- and livestock-origin (McCarthy et al., 2012; Uhlemann et al., 2012; Stegger et al., 2013), and therefore infection with ST398 does not necessitate livestock involvement. Nevertheless, there are genetic markers (*scn, tetM*) that can distinguish between livestock-associated and non-livestock-associated *S. aureus* ST398 with reasonable certainty (McCarthy et al., 2012; Stegger et al., 2013). Given this preface, the next section will review clinical infections caused by MRSA ST398, the most common strain isolated from livestock in many regions.

A laboratory serving 800,000 people in a high density pig farming area of the Netherlands reported 30 clinical cases of MRSA ST398 infections in a 2-year period and only 11 of these patients reported contact with livestock (Wulf et al., 2012). This study

demonstrates an incidence rate of 1.9 cases of MRSA ST398 infections per 100,000 people per year. Another study of MRSA ST398 infections in the North Denmark Region (580,515 people) reported 3 infections (all had contact with livestock) over a 2-year period which is equivalent to an incidence rate 0.26 cases of MRSA ST398 infections per 100,000 people per year (Omland and Hoffmann, 2012). In Canada, screening of 3,687 isolates of MRSA revealed 4 clinical isolates of MRSA ST398 (Golding et al., 2010). The 4 clinical infections occurred over a 20-month period in Saskatchewan (1.1 million people), and given that this collection of isolates represents ~66% of MRSA cases in that province (Golding et al., 2010), a reasonable estimate of the incidence rate of MRSA ST398 infections in Saskatchewan would be approximately 0.33 cases per 100,000 people per year. Compared to the annual rate of all MRSA infections in Canada (28.4 cases per 100,000 people), for every 1 person infected by a livestock-associated strain there are 85 people infected with a non-livestock-associated strain of MRSA (Laupland et al., 2013). The higher incidence rate of MRSA ST398 infections in the Netherlands (1.9 cases per 100,000 people per year) compared to Saskatchewan, Canada (0.33 cases per 100,000 people per year) may be related to the pig density in these regions; 1,244 pigs/km^2 in the Netherlands compared to 6 pigs/km^2 in Saskatchewan, Canada (Golding et al., 2010). This is supported by recent findings which have demonstrated that there may be an association between livestock density and MRSA infections in the community (Casey et al., 2013). Lastly, LA-MRSA infections are not without consequence; a recent report in the literature confirmed a fatal MRSA infection where exposure to livestock was likely a contributing factor (Lozano et al., 2011)

In addition to the threat of LA-MRSA infections, there is also concern that the use of prophylactic antimicrobials in veterinary medicine, particularly as a feed or water additive in livestock production, may create bacterial resistance to antimicrobials that are important to human medicine. Presently, given the widespread prevalence of MRSA in swine, the use of virginiamycin and trimethoprim is concerning as it is possible that exposure to these antimicrobials may cause resistance to important anti-staphylococcal agents used in human medicine, including quinupristin, dalfopristin and trimethoprim. Virginiamycin M (streptogramin A) and virginiamycin S (streptogramin B) are very similar to dalfopristin and quinupristin, respectively. The combined quinupristin – dalfopristin therapy is considered one of the last defenses for multidrug-resistant staphylococci and conserving the anti-staphylococcal activity of these drugs is paramount (Sibbald, 2012). Trimethoprim-sulphamethoxazole is an old antimicrobial combination that is an effective antimicrobial therapy for many MRSA infections (Goldberg et al., 2010), but the use of trimethoprim in livestock production may compromise the usefulness of this antimicrobial. However, the use of virginiamycin and trimethoprim on Canadian swine farms is uncommon (Rajic et al., 2006; Glass-Kastra et al., 2013) and *S. aureus* from Canadian pigs remains very susceptible to trimethoprim (Park et al., 2013). Nevertheless, further investigation and surveillance is needed to monitor for resistance to trimethoprim, quinupristin, and dalfopristin in staphylococci of livestock origin.

1.5 – The microbiome

Humans and animals are composed of two distinct genomes; the nuclear genome and the mitochondrial genome – both of which play a vital role in host health. However,

in 2001, a third genetic component was described in the literature as the "microbiome"; that is, the totality of microorganisms and their genetic material within the host (Lederberg and McCray, 2001). It has long been recognized that indigenous microbes are important for host health; however, progressive methodology has only recently given researchers the opportunity to examine the composition and functionality of these microbial communities in significant detail.

It is estimated that the bacterial microbiome of mammals contains over 10^{14} cells and 3 million genes (Savage, 1977; Qin et al., 2010). That is, the bacteria that populate the body outnumber eukaryotic cells by 10-fold and collectively harbor 100-fold more genes than the human genome. It has been theorized that a host contains a healthy core microbiome with variation occurring in lower taxa. However, the core microbiome has proven to be more elusive than previously expected as next-generation methodology has unveiled extraordinary host-specific diversity (Huse et al., 2012). Nevertheless, there are some dominant taxa that are found almost universally at specific niches, such as *Lactobacillus* which dominates the vaginal site in over 90% of women (Huse et al., 2012). The identification of key taxa associated with a healthy microbiome is of vital importance for future research and development in this field.

Overall, the extensiveness of the microbiome precipitates a complex and diverse system of host-microbe interactions. The microorganisms that populate the host are important drivers of metabolism and health, and when these microcosms become dysfunctional, an intervention to restore the microbial population may be warranted. However, the understanding of the microbiome is lacking, especially among livestock animals, and there is a considerable wealth of untapped knowledge. The following

section will review the current understanding of microbial ecology and the microbiome with an emphasis on domesticated pigs.

1.5.1 – Microbial ecology and population dynamics

It is estimated that the earth is home to 10^{30} prokaryotes which collectively contain about 10^{14} kg of carbon (Whitman et al., 1998). In other words, there are more prokaryotes than stars in the universe, and their combined carbon weight is 1,000-times greater than the combined mass of the human population. Given the numerical immensity of prokaryotes, their rapid propagation, and their propensity for mutations, it is no wonder that there is extraordinary genetic diversity among these microorganisms. The number of prokaryotic species that exist is estimated to be 100,000 – 10,000,000, although we have only identified about 1% of these existing species (Madigan et al., 2008). Hence, to say science has only begun to explore the frontiers of the microbial world is an overstatement.

Microbial ecology is the study of microorganisms and their interaction with each other and their environment. To survive, microorganisms often form symbiotic relationships with one another. There are several observed symbiotic relationships; parasitism is when one microbe benefits while the other is harmed, mutualism is when both microbes benefit, and commensalism is when one benefits and the other is neither helped nor harmed (Madigan et al., 2008). Oftentimes microorganisms will form metabolically related communities known as guilds whereby the community of microbes converts a common energy source into new organic matter. Such communities are found

in every ecosystem in nature as they are vital catalysts of the earth's nutrient cycle (Madigan et al., 2008).

The body also harbours an abundant and diverse collection of microorganisms. The sum of all microorganisms that populate a niche within the body is known as the microbiota, whereas the microbiome may be more expansively defined as the sum of all microorganisms and their genetic content. Autochthonous bacteria are the indigenous microbes which have adapted to form a stable and balanced community that compliments the host. Allochthonous bacteria are foreign or transient microbes that are not a part of the resident population and these 'opportunistic colonizers' can disrupt the functionality of resident microbes leading to disease in the host (Isaacson and Kim, 2012). Previously it has been theorized that there exists a "core microbiome"; that is, a defined population of bacterial taxa associated with the optimal health of that particular ecosystem. In the gastrointestinal system, researchers have identified several types of core microbiomes, defined as enterotypes, which utilize different combinations of bacterial pathways to achieve the same function (Huse et al., 2012). However, further discrimination at lower taxonomical levels has revealed incredible host-specific populations of bacteria that are unable to be differentiated into definitive biotypes (Huse et al., 2012). The individuality and adaptability of the host microbiome has become an important topic in the study of personalized medicine and is an area that requires further research in both humans and animals.

Microbial communities are often characterized by the richness and abundance of microbial taxa. The 3 domains of cellular life consist of the eukaryotic microorganisms of Eukaryota and the prokaryotic microorganisms of Bacteria and Archaea. Within the

bacterial lineage, a taxonomical hierarchy system is used to classify bacteria. The taxa, ordered from highest to lowest, include phylum, class, order, family, genus, and species (Barton and Northup, 2011). In microbial ecology, species richness is the number of different species within an ecosystem whereas species abundance refers to the commonness of a particular species relative to the other species within the ecosystem. Richness and abundance of species may be further quantified into species evenness which is a measurement of the equal distribution of species within an ecological community. An ecosystem is said to be even when there is equal abundance of all types of species. These measures of richness, abundance, and evenness are used for describing species diversity; effectively, the differences that exist within a population of microorganisms at a particular taxonomical level (Barton and Northup, 2011). However, what constitutes a species is still a subject of debate in microbiology. Previously, species was determined by DNA-DNA hybridization whereby two microbes were said to be the same species if >70% of their genomic DNA hybridized. At present, species is defined by relatedness of the 16S rRNA gene; two microbes are described as the same species if they share a 16S rRNA gene sequence homology of ≥97%, although some researchers believe a 99% threshold is more appropriate. Since species remains an ambiguous term that is still debated, researchers will often defer to the concept of operational taxonomic units (OTUs) whereby microorganisms are assigned to groups based on defined parameters (e.g. 97% sequence homology) (Barton and Northup, 2011).

Determining microbial diversity in ecological communities requires methodology that can reliably measure and quantify microbial populations. Prior to the 1980s, this was primarily completed using cultivation techniques; however, cultivation of bacteria only

provided a narrow and biased understanding of microbial ecology as less than 1% of bacteria are actually cultivatable. The field of microbial ecology was greatly advanced by the work of Carl Woese and colleagues whose research in small subunit ribosomal RNAs initiated a new era of modern microbial ecology (Hugenholtz, 2002). Their research allowed for the quantification of microbial diversity based on the dissimilarity between sequences of small subunit rRNAs, and using this methodology they were able to determine the presence of 11 distinct phyla within the bacterial domain by sequencing and comparing the 16S rRNA gene from cultivated sources (Woese, 1987). Since then, advancements in methodology, notably the development of culture-independent metagenetic 16S rRNA gene sequencing (Lane et al., 1985), have revealed a microbial world that is incredibly diverse. Compared to the 11 phyla identified by Woese et al. in the mid-1980s, in 2002 there were at least 45 phyla of bacteria reported in the literature (Hugenholtz, 2002) and at present there are over 80 phyla of bacteria that have been discovered (Madigan et al., 2008). However, only 29 bacterial phyla are recognized by the International Code of Nomenclature of Bacteria (ICNB) as at least one representative bacterial species has been cultivated from each of these phyla. Bacterial phyla that have been identified exclusively by 16S rRNA gene metagenetic analysis are referred to as candidate (candidatus) lineages.

Overall, the mass identification of bacterial taxa is largely owing to the revolution of small subunit rRNA metagenetics and subsequent advancements in this field. Current technology allows for high-throughput extraction and sequencing of nucleic acids to such an extent that standard data analysis requires super computing power. The advent and

future of next-generation DNA sequencing as a tool in microbial ecology will be discussed in a forthcoming section.

1.5.2 – Gastrointestinal microbiota of the domestic pig

The gut health of pigs is of paramount importance for swine production systems as gastrointestinal diseases can have severe consequences including poor productivity and economic loss (McOrist et al., 1997; Hampson et al., 2006). Therefore, an understanding of factors that contribute to the susceptibility to or protection from gastrointestinal disease is an asset to the industry. One area of research has focused on the association between gut health and the microbes that populate this niche. However, advancements in next-generation sequencing technology have only recently allowed researchers to explore these microbial communities in significantly more detail and there is a considerable gap in our understanding of how the microbiota is related to porcine gut health.

Previous research exploring the gut microbiota in adult pigs using next-generation sequencing methods has revealed an incredibly diverse population of bacteria (Kim et al., 2011). The adult porcine gut microbiota has been found to contain at least 7 identifiable bacterial phyla, the predominant being Firmicutes and Bacteroidetes, and at least 171 genera of bacteria (Kim et al., 2011). Interestingly, Kim et al. observed an age-related shift in the relative abundance of gut phyla; Firmicutes became more dominant with time while Bacteroidetes became less dominant (2011). Some studies suggest that age is the most influential driver of the gastrointestinal microbiota, even more so than diet and antimicrobial consumption (Levesque et al., 2014).

Additional factors that contribute to the balance of the gut microbiota include environmental exposures, the immune system (Thompson et al., 2008), and antimicrobials (Looft et al., 2014). Research by Thompson et al. suggests that pigs have a "developmental window" during early life (14-21 days) when their gut microbiota is more susceptible to changes that may persist until adulthood (2008). However, knowledge of the early-age microbiota of pigs is still lacking, and further research is needed to characterize the gastrointestinal microbiota using next-generation sequencing methods. This may provide a further understanding of susceptibility to gastrointestinal disease and lead to the development of interventions that increase resilience to these diseases.

One limitation of this research is the ability to extrapolate the fecal microbiota to the rest of the gastrointestinal tract. While it has been demonstrated that the fecal microbiota is representative of the distal gastrointestinal tract (cecum, colon), its representation of the proximal gastrointestinal tract (stomach, ileum) is minimal (Looft et al., 2014).

1.5.2 − Nasal microbiota of the domestic pig

Respiratory disease is the most prevalent causes of morbidity and mortality in pigs, especially in young pigs. Immunity and herd management are well studied factors that can influence susceptibility to disease, but the nasal microbiota can also play a key role in respiratory diseases as it is known that competition and interaction between bacteria can protect a host from becoming colonized with particular respiratory pathogens (Pettigrew et al., 2008).

Previous research has used culture-based techniques to describe the nasal microbiota of young pigs (Woods et al., 1972); however, such studies only describe less than 1% of bacteria that may be present. Recent characterization of the nasal microbiota in adult pigs using next-generation sequencing has revealed a very rich and diverse community of microbes (Weese et al., 2014). Nine phyla were identified in the nares of the adult pigs which was dominated by Proteobacteria but also showed considerable abundance of Firmicutes and Spirochaetes. Additionally, the nares harboured over 124 different genera, although the majority of sequences consisted of *Moraxella* (34%), *Psychrobacter* (21%), *Pseudomonas* (15%) and *Acinetobacter* (5%) (Weese et al., 2014). Similar results were reported for the tonsils of healthy adult pigs whereby Proteobacteria and Firmicutes were the dominant phyla, but differences at the genera level showed dominances of *Actinobacillus* and *Pasteurella* (Lowe et al., 2012).

Changes in the porcine nasal microbiome have been associated with diet and antimicrobial consumption (Weese et al., 2014). Understanding factors that can influence the porcine nasal microbiome may be important for developing interventions that protect pigs from colonization with swine pathogens and zoonotic agents. However, before interventions can be developed, further understanding of the nasal microbiota of young pigs is needed as it is still poorly described in the literature. Further research should characterize the development of the resident bacteria in the nasal cavity of young pigs and determine production parameters, such as weaning or antimicrobial use, which may impact the porcine nasal microbiome.

1.6 – Metagenetics and next-generation DNA sequencing

Metagenetics is the analysis of microbial communities using targeted nucleotide sequencing as a means to describe the entire population of microorganisms. Metagenetic studies often utilize a single target gene (e.g. 16S rRNA gene) to characterize microbial populations and such studies are different from metagenomics which is the study of the sum of genomes within an ecosystem. Generating sequencing data for microbial populations has been revolutionized by the advancement of next-generation sequencing technology which typically utilizes the variable regions of the 16S rRNA gene. The 16S rRNA gene is universal among prokaryotes as it is essential for the translation of mRNA into protein (Madigan et al., 2008). It contains 9 hyper-variable regions (V1-V9) which may be sequenced and used for metagenetic analysis, although the performance of these regions varies depending on the sample and sequencing methods. Phylogenetic analysis of the sequenced variable region(s) can allow for taxonomical classification that is accurate to the genus level (Hugenholtz, 2002; Mizrahi-Man et al., 2013).

When performing a metagenetic study using next-generation sequencing, there are a number of parameters which can influence the final results. The first of these parameters is the method of DNA extraction which is a critical step as systematic bias can arise from different methodology, particularly for mechanical or enzymatic lysis methods (Lozupone et al., 2013). Secondly, choosing an appropriate hyper-variable region to sequence is one of the most important determinants of the quality of the results as each region is associated with particular biases, error rates, and depth of coverage, and the optimal region can vary by sample type or sequencing platform (Mizrahi-Man et al., 2013). The length of sequence which is amplified is also important as increasing the

sequence length can reduce error rate and increase discriminatory power but will also reduce throughput and increase cost (Mizrahi-Man et al., 2013). Lastly, selecting appropriate primers for amplifying the target region is incredibly important as poorly designed primers can cause biased representation of some bacterial taxa (Polz and Cavanaugh, 1998).

For high-throughput short nucleotide sequencing, evidence from the literature suggests that the V4 region performs optimally as this region demonstrates: (i) the best coverage at a lower false prediction rate (Mizrahi-Man et al., 2013), (ii) the lowest error rates (Kozich et al., 2013; Mizrahi-Man et al., 2013), (iii) excellent consistency in ecological measures when compared to full length sequencing (Youssef et al., 2009), and (iv) the least amplification bias which results in better representation of microbial communities (Bergmann et al., 2011). The next section of this chapter will review the use of the Illumina MiSeq platform for rapid sequencing of short nucleotides.

1.6.1 – Illumina MiSeq Platform

The Illumina MiSeq is a next-generation sequencing platform that is used for culture-independent determination of nucleic acid sequences. It was released in 2011 as an accessible bench-top device for sequencing of fewer samples with a faster turn-around time compared to its counterpart the HiSeq 2000 (Quail et al., 2012).

The Illumina MiSeq is able to generate sequences ina 2-step process. The first step is cluster generation whereby each DNA fragment is isothermically amplified on a flow cell. The flow cell contains a lawn of oligonucleotides that adhere to the adapter region of the DNA fragment. The DNA fragment forms a bridge with the other oligonucleotides on the flow cell and then it is amplified. This generates millions of

clusters of amplified DNA fragments. The second step is the sequence by synthesis (SBS) process. A universal primer binds to the single-stranded DNA fragment and then fluorescently-labelled nucleotides are added. Each nucleotide competes for a position in the amplification of the double-stranded DNA fragment. When a nucleotide is incorporated into the growing double-stranded DNA it releases a unique fluorescent signal. The emitted signal is then recorded by a computer and translated into a sequence. Through this process, which is known as sequencing by synthesis (SBS), hundreds of millions of fragments can be sequenced simultaneously in approximately 3 hours (Quail et al., 2012; Illumina, 2014).

The advantages of the MiSeq platform are that it requires a very small amount on DNA (sub-nanomolar) (Quail et al., 2012), it has an increased read depth (up to x10 when compared to 454), it is far cheaper than many other platforms, and preparation is less labour-intensive allowing for a more streamlined workflow (Quail et al., 2012). When compared to some of the other next-generation sequencing platforms, the Illumina MiSeq demonstrates the highest throughput and lowest error rates (Loman et al., 2012). It is also backwards compatible with some molecular typing methods; that is, sequencing on the MiSeq is able to produce accurate MLST types (Loman et al., 2012). However, one of the limitations of the Illumina MiSeq is the size of the sequence that it can generate. The MiSeq is able to generate very accurate, short sequences but this can cause difficulties when assembling a scaffold as there are usually many gaps and many repeat reads (Loman et al., 2012).

1.7 – Summary and research objectives

The use of a high-dose therapeutic zinc diet to enhance growth and prevent post-weaning diarrhea has become commonplace on Canadian swine farms. This may be due to the increasing pressure to reduce the use of antimicrobials in livestock production and the appeal of zinc therapy as an alternative to antimicrobials. However, as previously described throughout the review of the literature, the effects of zinc therapy on the bacteria that populate the host are not well understood. Therefore, based on the knowledge gaps in the currently available scientific literature, the research objectives and rationale for this thesis are as follows:

First objective: To determine whether zinc administration could inadvertently co-select for antimicrobial-resistant *S. aureus* given the co-location of the zinc-resistance gene (*czrC*) within a bacterial resistance island.

- Due to the co-location of *czrC* and other antimicrobial-resistance genes within the SCC*mec* (Cavaco et al., 2010), exposure to high levels of in-feed zinc may inadvertently cause co-selection of antimicrobial-resistance genes in the absence of antimicrobial exposure. It will be determined whether high levels of in-feed zinc can affect the prevalence and persistence of MRSA among swine through a randomized-controlled trial.
- The previously described hypothesis will also be investigated under field conditions. A multi-farm cohort study will determine whether the presence of MRSA among swine herds is associated with any particular risk factors, including the usage of high levels of in-feed zinc and antimicrobials.

Second objective: To further develop the understanding of the early-life porcine microbiota and to determine whether zinc impacts the bacterial ecology of the porcine nasal microbiota.

- Since the early-life porcine microbiota is poorly understood, the next phase of this investigation will attempt to characterize the development of the fecal and nasal microbiotas of commercially-raised pigs from birth to 7-weeks of life without exposure to any therapeutic compounds. This study will use next-generation sequencing to provide detailed insight into the evolution of the early-life fecal and nasal microbiotas of young pigs.
- Lastly, the impact of zinc therapy on the developing nasal microbiota of swine is unknown. An experimental field trial will be completed to determine the effects of a high-dose zinc therapy on the porcine nasal microbiota.

Overall, it is expected that the results of this research project will provide a better understanding as to the potential risks and benefits of using high levels of in-feed zinc as a therapy in swine production.

1.8 – References

Agersø Y, Hasman H, Cavaco LM, Pedersen K, Aarestrup FM. 2012. Study of methicillin resistant *Staphylococcus aureus* (MRSA) in danish pigs at slaughter and in imported retail meat reveals a novel MRSA type in slaughter pigs. Vet Microbiol. 2012; 157(1):246-50.

Argudin M, Rodicio M, Guerra B. The emerging methicillin-resistant *Staphylococcus aureus* ST398 clone can easily be typed using the Cfr9I SmaI-neoschizomer. Lett Appl Microbiol. 2010; 50(1):127-30.

Arriola CS, Güere ME, Larsen J, Skov RL, Gilman RH, Gonzalez AE, Silbergeld EK. Presence of methicillin-resistant *Staphylococcus aureus* in pigs in peru. PloS One. 2011; 6(12):e28529.

Baker-Austin C, Wright MS, Stepanauskas R, McArthur J. Co-selection of antibiotic and metal resistance. Trends Microbiol. 2006; 14(4):176-82.

Bannerman TL, Hancock GA, Tenover FC, Miller JM. Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. J Clin Microbiol. 1995; 33(3):551-5.

Barton LL and Northup DE. Microbial ecology. New Jersey: Wiley-Blackwell; 2011.

Berglund C and Söderquist B. The origin of a methicillin-resistant *Staphylococcus aureus* isolate at a neonatal ward in Sweden—possible horizontal transfer of a staphylococcal cassette chromosome mec between methicillin-resistant staphylococcus haemolyticus and *Staphylococcus aureus*. Clin Microbiol Infect. 2008; 14(11):1048-56.

Bergmann GT, Bates ST, Eilers KG, Lauber CL, Caporaso JG, Walters WA, Knight R, Fierer N. The under-recognized dominance of *Verrucomicrobia* in soil bacterial communities. Soil Biol Biochem. 2011; 43(7):1450-5.

Bisdorff B, Scholhölter J, Claussen K, Pulz M, Nowak D, Radon K. MRSA-ST398 in livestock farmers and neighbouring residents in a rural area in germany. Epidemiol Infect. 2012; 140(10):1800-8.

Brink M, Becker D, Terrill S, Jensen A. Zinc toxicity in the weanling pig. J Anim Sci, 1959; 18(2):836-42.

Broens E, Graat E, Van Der Wolf P, Van De Giessen A, De Jong M. Prevalence and risk factor analysis of livestock associated MRSA-positive pig herds in the Netherlands. Prev Vet Med. 2011; 102(1):41-9.

Buntenkoetter V, Blaha T, Tegeler R, Fetsch A, Hartmann M, Kreienbrock L, Meemken D. Comparison of the phenotypic antimicrobial resistances and spa-types of

methicillin-resistant *Staphylococcus aureus* (MRSA) isolates derived from pigs in conventional and in organic husbandry systems. Berl Munch Tierarztl Wochenschr. 2014; 127(3-4):135-43.

Buzby JC and Roberts T. Economic costs and trade impacts of microbial foodborne illness. World Health Stat Q. 1997; 50(1-2):57-66.

Calomiris JJ, Armstrong JL, Seidler RJ. Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. Appl Environ Microbiol. 1984; 47(6):1238-42.

Casey JA, Curriero FC, Cosgrove SE, Nachman KE, Schwartz BS. High-density livestock operations, crop field application of manure, and risk of community-associated methicillin-resistant *Staphylococcus aureus* infection in Pennsylvania. JAMA Internal Medicine. 2013; 173(21):1980-90.

Cavaco LM, Hasman H, Aarestrup FM. Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance. Vet Microbiol. 2011; 150(3):344-8.

Cavaco LM, Hasman H, Stegger M, Andersen PS, Skov R, Fluit AC, Ito T, Aarestrup FM. Cloning and occurrence of *czrC*, a gene conferring cadmium and zinc resistance in methicillin-resistant *Staphylococcus aureus* CC398 isolates. Antimicrob Agents Chemother. 2010; 54(9):3605-8.

Centers for Disease Control and Prevention (CDC). *Staphylococcus aureus* resistant to vancomycin--United States, 2002. MMWR Morb Mortal Wkly Rep. 2002; 51(26):565-7.

Crombé F, Willems G, Dispas M, Hallin M, Denis O, Suetens C, Gordts B, Struelens M, Butaye P. Prevalence and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* among pigs in Belgium. Microbial Drug Resistance. 2012; 18(2):125-31.

Cui S, Li J, Hu C, Jin S, Li F, Guo Y, Ran L, Ma Y. Isolation and characterization of methicillin-resistant *Staphylococcus aureus* from swine and workers in China. J Antimicrob Chemother. 2009; 64(4):680-3.

Cuny C, Friedrich A, Kozytska S, Layer F, Nübel U, Ohlsen K, Strommenger B, Walther B, Wieler L, Witte W. Emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in different animal species. International Journal of Medical Microbiology. 2010; 300(2):109-17.

Davies P. Livestock associated MRSA: Tiger or pussycat? Allen D. Leman swine conference 2013 proceedings. Minnesota, USA: University of Minnesota. 2013.

Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN, Beach M, SENTRY Partcipants Group. Survey of infections due to staphylococcus species: Frequency of

occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY antimicrobial surveillance program, 1997-1999. Clin Infect Dis. 2001; 32 Suppl 2:S114-32.

Feßler AT, Kadlec K, Schwarz S. Novel apramycin resistance gene apmA in bovine and porcine methicillin-resistant *Staphylococcus aureus* ST398 isolates. Antimicrob Agents Chemother. 2011; 55(1):373-5.

Fluit A. Livestock-associated *Staphylococcus aureus*. Clin Microbiol Infect. 2012; 18(8):735-44.

Fromm S, Beißwanger E, Käsbohrer A, Tenhagen B. Risk factors for MRSA in fattening pig herds–A meta-analysis using pooled data. Prev Vet Med. 2014; 117(1):180-8.

García-Álvarez L, Holden MT, Lindsay H, Webb CR, Brown DF, Curran MD, Walpole E, Brooks K, Pickard DJ, Teale C. Meticillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: A descriptive study. Lancet Infect Dis. 2011; 11(8):595-603.

Glass-Kaastra SK, Pearl DL, Reid-Smith R, McEwen B, McEwen SA, Amezcua R, Friendship RM. Describing antimicrobial use and reported treatment efficacy in ontario swine using the Ontario swine veterinary-based surveillance program. BMC Vet Res. 2013; 9(1):238.

Goldberg E, Paul M, Talker O, Samra Z, Raskin M, Hazzan R, Leibovici L, Bishara J. Co-trimoxazole versus vancomycin for the treatment of methicillin-resistant *Staphylococcus aureus* bacteraemia: A retrospective cohort study. J Antimicrob Chemother. 2010; 65(8):1779-83.

Golding GR, Bryden L, Levett PN, McDonald RR, Wong A, Wylie J, Graham MR, Tyler S, Van Domselaar G, Simor AE. Livestock-associated methicillin-resistant *Staphylococcus aureus* sequence type 398 in humans, Canada. Emerging Infect Dis. 2010; 16(4):587-94.

Gomez-Sanz E, Kadlec K, Fessler AT, Zarazaga M, Torres C, Schwarz S. Novel *erm*(T)-carrying multiresistance plasmids from porcine and human isolates of methicillin-resistant *Staphylococcus aureus* ST398 that also harbor cadmium and copper resistance determinants. Antimicrob Agents Chemother. 2013; 57(7):3275-82.

Gómez-Sanz E, Torres C, Lozano C, Fernández-Pérez R, Aspiroz C, Ruiz-Larrea F, Zarazaga M. Detection, molecular characterization, and clonal diversity of methicillin-resistant *Staphylococcus aureus* CC398 and CC97 in Spanish slaughter pigs of different age groups. Food Patho Dis. 2010; 7(10):1269-77.

Hampson DJ, Fellstrom C, Thomson JR. Swine dysentery. In: Diseases of swine. Straw BE, Zimmerman JJ, D'Allaire S, et al, editors. 9th ed. Ames, Iowa: Blackwell Publishing; 2006.

Harmsen D, Claus H, Witte W, Rothganger J, Claus H, Turnwald D, Vogel U. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J Clin Microbiol. 2003; 41(12):5442-8.

Hasman H and Aarestrup FM. *tcrB*, a gene conferring transferable copper resistance in enterococcus faecium: Occurrence, transferability, and linkage to macrolide and glycopeptide resistance. Antimicrob Agents Chemother. 2002; 46(5):1410-6.

Hetem DJ, Bootsma MC, Troelstra A, Bonten MJ. Transmissibility of livestock-associated methicillin-resistant *Staphylococcus aureus*. Emerg Infect Dis. 2013; 19(11):1797-802.

Hill GM, Cromwell GL, Crenshaw TD, Dove CR, Ewan RC, Knabe DA, Lewis AJ, Libal GW, Mahan DC, Shurson GC, et al. Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (regional study). J Anim Sci. 2000; 78(4):1010-6.

Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, Hori S, Fukuchi Y, Kobayashi I. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. The Lancet. 1997; 350(9092):1670-3.

Hooper DC. Fluoroquinolone resistance among gram-positive cocci. The Lancet Infectious Diseases. 2002; 2(9):530-8.

Hugenholtz P. Exploring prokaryotic diversity in the genomic era. Genome Biol. 2002; 3(2):1,0003.8.

Huse SM, Ye Y, Zhou Y, Fodor AA. A core human microbiome as viewed through 16S rRNA sequence clusters. PloS One. 2002; 7(6):e34242.

International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). Classification of staphylococcal cassette chromosome mec (SCCmec): Guidelines for reporting novel SCCmec elements. Antimicrob Agents Chemother. 2009; 53(12):4961-7.

Isaacson R and Kim HB. The intestinal microbiome of the pig. Animal Health Research Reviews. 2012; 13(01):100-9.

Ito T, Katayama Y, Hiramatsu K. Cloning and nucleotide sequence determination of the entire mec DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. Antimicrob Agents Chemother. 1999; 43(6):1449-58.

Jacela JY, DeRouchey JM, Tokach MD, Goodband RD, Nelssen JL, Renter DG, Dritz SS. Feed additives for swine: Fact sheets–high dietary levels of copper and zinc for young pigs, and phytase. Journal of Swine Health and Production. 2010;18(2):87-91.

Jamrozy DM, Fielder MD, Butaye P, Coldham NG. Comparative genotypic and phenotypic characterisation of methicillin-resistant *Staphylococcus aureus* ST398 isolated from animals and humans. PloS One. 2012; 7(7):e40458.

Jevons M. "Celbenin"-resistant staphylococci. British Medical Journal. 1961; 1(5219):125.

Ji G, Silver S. Reduction of arsenate to arsenite by the ArsC protein of the arsenic resistance operon of Staphylococcus aureus plasmid pI258. Proceedings of the National Academy of Sciences. 1992;89(20):9474-8.

Jones RN, Flonta M, Gurler N, Cepparulo M, Mendes RE, Castanheira M. Resistance surveillance program report for selected European nations (2011). Diagn Microbiol Infect Dis. 2014; 78(4):429-36.

Jones RN, Sader HS, Flamm RK. Update of dalbavancin spectrum and potency in the USA: Report from the SENTRY antimicrobial surveillance program (2011). Diagn Microbiol Infect Dis. 2013; 75(3):304-7.

Kaatz GW, Seo SM, Ruble CA. Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 1993; 37(5):1086-94.

Kadlec K and Schwarz S. Identification of a novel trimethoprim resistance gene, *dfrK*, in a methicillin-resistant *Staphylococcus aureus* ST398 strain and its physical linkage to the tetracycline resistance gene *tet*(L). Antimicrob Agents Chemother. 2009; 53(2):776-8.

Kadlec K and Schwarz S. Identification of a plasmid-borne resistance gene cluster comprising the resistance genes *erm*(T), dfrK, and *tet*(L) in a porcine methicillin-resistant *Staphylococcus aureus* ST398 strain. Antimicrob Agents Chemother. 2010; 54(2):915-8.

Kadlec K and Schwarz S. Novel ABC transporter gene, *vga*(C), located on a multiresistance plasmid from a porcine methicillin-resistant *Staphylococcus aureus* ST398 strain. Antimicrob Agents Chemother. 2009; 53(8):3589-91.

Katayama Y, Ito T, Hiramatsu K. A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 2000; 44(6):1549-55.

Khanna T, Friendship R, Dewey C, Weese J. Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. Vet Microbiol. 2008; 128(3):298-303.

Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ, Isaacson RE. Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. Vet Microbiol. 2011; 153(1):124-33.

Klein E, Smith DL, Laxminarayan R. Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999-2005. Emerg Infect Dis. 2007; 13(12):1840-6.

Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of *Staphylococcus aureus*: Epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev. 1997; 10(3):505-20.

Köck R, Harlizius J, Bressan N, Laerberg R, Wieler LH, Witte W, Deurenberg R, Voss A, Becker K, Friedrich AW. Prevalence and molecular characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) among pigs on german farms and import of livestock-related MRSA into hospitals. European Journal of Clinical Microbiology & Infectious Diseases. 2009; 28(11):1375-82.

Koreen L, Ramaswamy SV, Graviss EA, Naidich S, Musser JM, Kreiswirth BN. Spa typing method for discriminating among *Staphylococcus aureus* isolates: Implications for use of a single marker to detect genetic micro- and macrovariation. J Clin Microbiol. 2004; 42(2):792-9.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq illumina sequencing platform. Appl Environ Microbiol. 2014; 79(17):5112-20.

Kraushaar B, Thanh MD, Hammerl JA, Reetz J, Fetsch A, Hertwig S. Isolation and characterization of phages with lytic activity against methicillin-resistant *Staphylococcus aureus* strains belonging to clonal complex 398. Arch Virol. 2013; 158(11):2341-50.

Kreausukon K, Fetsch A, Kraushaar B, Alt K, Müller K, Krömker V, Zessin K, Käsbohrer A, Tenhagen B. Prevalence, antimicrobial resistance, and molecular characterization of methicillin-resistant *Staphylococcus aureus* from bulk tank milk of dairy herds. J Dairy Sci. 2012; 95(8):4382-8.

Kuroda M, Hayashi H, Ohta T. Chromosome-Determined Zinc-Responsible operon *czr* in *Staphylococcus aureus* strain 912. Microbiol Immunol. 1999; 43(2):115-25.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci U S A. 1985; 82(20):6955-9.

Laupland KB, Church DL, Mucenski M, Sutherland LR, Davies HD. Population-based study of the epidemiology of and the risk factors for invasive *Staphylococcus aureus* infections. J Infect Dis. 2003; 187(9):1452-9.

Leclercq R. Mechanisms of resistance to macrolides and lincosamides: Nature of the resistance elements and their clinical implications. Clin Infect Dis. 2002; 34(4):482-92.

Lederberg J, McCray AT. Ome SweetOmics--A Genealogical Treasury of Words. The Scientist. 2001 Apr 2;15(7):8.

Li M, Du X, Villaruz AE, Diep BA, Wang D, Song Y, Tian Y, Hu J, Yu F, Lu Y. MRSA epidemic linked to a quickly spreading colonization and virulence determinant. Nat Med. 2012; 18(5):816-9.

Li S, Skov RL, Han X, Larsen AR, Larsen J, Sorum M, Wulf M, Voss A, Hiramatsu K, Ito T. Novel types of staphylococcal cassette chromosome *mec* elements identified in clonal complex 398 methicillin-resistant *Staphylococcus aureus* strains. Antimicrob Agents Chemother. 2011; 55(6):3046-50.

Loeffler A, McCarthy A, Lloyd DH, Musilová E, Pfeiffer DU, Lindsay JA. Whole-genome comparison of meticillin-resistant *Staphylococcus aureus* CC22 SCCmecIV from people and their in-contact pets. Vet Dermatol. 2013; 24(5):538-e128.

Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J, Pallen MJ. Performance comparison of benchtop high-throughput sequencing platforms. Nat Biotechnol. 2012; 30(5):434-9.

Looft T, Allen HK, Cantarel BL, Levine UY, Bayles DO, Alt DP, Henrissat B, Stanton TB. Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. ISME J. 2014; 8(8):1566-76.

Lowe BA, Marsh TL, Isaacs-Cosgrove N, Kirkwood RN, Kiupel M, Mulks MH. Defining the "core microbiome" of the microbial communities in the tonsils of healthy pigs. BMC Microbiol. 2012; 12:20,2180-12-20.

Lozano C, Aspiroz C, Ezpeleta AI, Gomez-Sanz E, Zarazaga M, Torres C. Empyema caused by MRSA ST398 with atypical resistance profile, spain. Emerg Infect Dis. 2011; 17(1):138-40.

Lozupone CA, Stombaugh J, Gonzalez A, Ackermann G, Wendel D, Vazquez-Baeza Y, Jansson JK, Gordon JI, Knight R. Meta-analyses of studies of the human microbiota. Genome Res. 2013; 23(10):1704-14.

Madigan MT, Martinko JM, Dunlap PV, Clark DP. Brock Biology of microorganisms 12th edn. International Microbiology. 2008;11:65-73.

Maple P, Hamilton-Miller J, Brumfitt W. World-wide antibiotic resistance in methicillin-resistant *Staphylococcus aureus*. The Lancet. 1989; 333(8637):537-40.

McCarthy AJ, Lindsay JA, Loeffler A. Are all meticillin-resistant *Staphylococcus aureus* (MRSA) equal in all hosts? epidemiological and genetic comparison between animal and human MRSA. Vet Dermatol. 2012; 23(4):267-e54.

McOrist S, Smith S, Green L. Estimate of direct financial losses due to porcine proliferative enteropathy. Vet Rec. 1997; 140(22):579-81.

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV. Food-related illness and death in the United States. Emerg Infect Dis. 1999; 5(5):607-25.

Medardus JJ, Molla BZ, Nicol M, Morrow WM, Rajala-Schultz PJ, Kazwala R, Gebreyes WA. In-feed use of heavy metal micronutrients in U.S. swine production systems and its role in persistence of multidrug-resistant salmonellae. Appl Environ Microbiol. 2014; 80(7):2317-25.

Mediavilla JR, Chen L, Mathema B, Kreiswirth BN. 2012. Global epidemiology of community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA). Curr Opin Microbiol. 2012; 15(5):588-95.

Mendes RE, Sader HS, Farrell DJ, Jones RN. Telavancin activity tested against a contemporary collection of gram-positive pathogens from USA hospitals (2007–2009). Diagn Microbiol Infect Dis. 2012; 72(1):113-7.

Mizrahi-Man O, Davenport ER, Gilad Y. Taxonomic classification of bacterial 16S rRNA genes using short sequencing reads: Evaluation of effective study designs. PloS One. 2013; 8(1):e53608.

Moellering RC. Discovering new antimicrobial agents. Int J Antimicrob Agents. 2011; 37(1):2-9.

Moellering RC. MRSA: The first half century. J Antimicrob Chemother. 2012; 67(1):4-11.

Nichol KA, Adam HJ, Roscoe DL, Golding GR, Lagace-Wiens PR, Hoban DJ, Zhanel GG, Canadian Antimicrobial Resistance Alliance. Changing epidemiology of methicillin-resistant *Staphylococcus aureus* in Canada. J Antimicrob Chemother. 2013; 68 Suppl 1:i47-55.

Nimmo GR, Bell JM, Mitchell D, Gosbell IB, Pearman JW, Turnidge JD. Antimicrobial resistance in *Staphylococcus aureus* in australian teaching hospitals, 1989-1999. Microbial Drug Resistance. 2003; 9(2):155-60.

Noskin GA, Rubin RJ, Schentag JJ, Kluytmans J, Hedblom EC, Jacobson C, Smulders M, Gemmen E, Bharmal M. National trends in *Staphylococcus aureus* infection rates: Impact on economic burden and mortality over a 6-year period (1998-2003). Clin Infect Dis. 2007; 45(9):1132-40.

O'Brien AM, Hanson BM, Farina SA, Wu JY, Simmering JE, Wardyn SE, Forshey BM, Kulick ME, Wallinga DB, Smith TC. MRSA in conventional and alternative retail pork products. PLoS One. 2012; 7(1):e30092.

Oksuz L and Gurler N. Susceptibility of clinical methicillin-resistant staphylococci isolates to new antibiotics. The Journal of Infection in Developing Countries. 2013; 7(11):825-31.

Olde Riekerink R, Barkema H, Kelton D, Scholl D. Incidence rate of clinical mastitis on Canadian dairy farms. J Dairy Sci. 2008; 91(4):1366-77.

Omland O and Hoffmann L. Occupational acquisition of methicillin-resistant *Staphylococcus aureus* in humans--a description of MRSA carrier and infected cases from the region of north Jutland in Denmark. Ann Agric Environ Med. 2012; 19(4):637-40.

Osadebe L, Hanson B, Smith T, Heimer R. Prevalence and characteristics of *Staphylococcus aureus* in connecticut swine and swine farmers. Zoonoses and Public Health. 2013; 60(3):234-43.

Osadebe L, Hanson B, Smith T, Heimer R. Prevalence and characteristics of *Staphylococcus aureus* in connecticut swine and swine farmers. Zoonoses and Public Health. 2013; 60(3):234-43.

Pantosti A, Sanchini A, Monaco M. Mechanisms of antibiotic resistance in *Staphylococcus aureus*. Future Medicine. 2007; 2(3):323-34.

Park J, Friendship RM, Poljak Z, Weese JS, Dewey CE. An investigation of exudative epidermitis (greasy pig disease) and antimicrobial resistance patterns of *Staphylococcus hyicus* and *Staphylococcus aureus* isolated from clinical cases. The Can Vet J. 2013; 54(2):139.

Paterson GK, Harrison EM, Holmes MA. The emergence of *mecC* methicillin-resistant *Staphylococcus aureus*. Trends Microbiol. 2014; 22(1):42-7.

Peleg AY, Monga D, Pillai S, Mylonakis E, Moellering RC,Jr, Eliopoulos GM. Reduced susceptibility to vancomycin influences pathogenicity in *Staphylococcus aureus* infection. J Infect Dis. 2009; 199(4):532-6.

Pettigrew MM, Gent JF, Revai K, Patel JA, Chonmaitree T. Microbial interactions during upper respiratory tract infections. Emerg Infect Dis. 2008; 14(10):1584-91.

Pletinckx LJ, Verhegghe M, Crombé F, Dewulf J, De Bleecker Y, Rasschaert G, Butaye P, Goddeeris BM, De Man I. Evidence of possible methicillin-resistant *Staphylococcus aureus* ST398 spread between pigs and other animals and people residing on the same farm. Prev Vet Med. 2013; 109(3):293-303.

Pluske JR. Feed-and feed additives-related aspects of gut health and development in weanling pigs. J Anim Sci Biotechnol. 2013; 4(1):1.

Polz MF and Cavanaugh CM. Bias in template-to-product ratios in multitemplate PCR. Appl Environ Microbiol. 1998; 64(10):3724-30.

Price LB, Stegger M, Hasman H, Aziz M, Larsen J, Andersen PS, Pearson T, Waters AE, Foster JT, Schupp J, et al. *Staphylococcus aureus* CC398: Host adaptation and

emergence of methicillin resistance in livestock. Mbio. 2012; 3(1):10.1128/mBio.00305,11. Print 2012.

Public Health Ontario (PHO). Snapshots: Ontario: Reportable Burdensome Infectious Disease Incidence – crude rate (both sexes combined). Toronto, ON: Ontario Agency for Health Protection and Promotion; 2013.

Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T. A human gut microbial gene catalogue established by metagenetic sequencing. Nature. 2010; 464(7285):59-65.

Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR, Bertoni A, Swerdlow HP, Gu YA tale of three next generation sequencing platforms: Comparison of ion torrent, pacific biosciences and illumina MiSeq sequencers. BMC Genomics. 2012; 13:341,2164-13-341.

Rajic A, Reid-Smith R, Deckert AE, Dewey CE, McEwen SA. Reported antibiotic use in 90 swine farms in alberta. Can Vet J. 2006; 47(5):446-52.

Richter SS, Heilmann KP, Dohrn CL, Riahi F, Costello AJ, Kroeger JS, Biek D, Critchley IA, Diekema DJ, Doern GV. Activity of ceftaroline and epidemiologic trends in *Staphylococcus aureus* isolates collected from 43 medical centers in the United States in 2009. Antimicrob Agents Chemother. 2001; 55(9):4154-60.

Rivera AM, Boucher HW. Current concepts in antimicrobial therapy against select gram-positive organisms: methicillin-resistant *Staphylococcus aureus*, penicillin-resistant pneumococci, and vancomycin-resistant enterococci. Mayo Clinic Proceedings. 2011; 86(12):1230-43.

Ross JI, Eady EA, Cove JH, Baumberg S. Minimal functional system required for expression of erythromycin resistance by *msrA* in *Staphylococcus aureus* RN4220. Gene. 1996; 183(1):143-8.

Rossi F, Diaz L, Wollam A, Panesso D, Zhou Y, Rincon S, Narechania A, Xing G, Di Gioia TS, Doi A. 2014. Transferable vancomycin resistance in a community-associated MRSA lineage. N Engl J Med 370(16):1524-31.

Rubin J and Chirino-Trejo M. Antimicrobial susceptibility of canine and human *Staphylococcus aureus* collected in Saskatoon, Canada. Zoonoses and Public Health. 2011; 58(7):454-62.

Sader HS, Watters AA, Fritsche TR, Jones RN. Daptomycin antimicrobial activity tested against methicillin-resistant staphylococci and vancomycin-resistant enterococci isolated in European medical centers (2005). BMC Infect Dis. 2007; 7:29.

Sakoulas G and Moellering RC. Increasing antibiotic resistance among methicillin-resistant *Staphylococcus aureus* strains. Clin Infect Dis. 2008; 46 Suppl 5:S360-7.

Saravolatz LD, Pawlak J, Johnson LB. In vitro susceptibilities and molecular analysis of vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus* isolates. Clin Infect Dis. 2012; 55(4):582-6.

Savage DC. Microbial ecology of the gastrointestinal tract. Annual Reviews in Microbiology. 1977; 31(1):107-33.

Schmitz FJ, Fluit AC, Gondolf M, Beyrau R, Lindenlauf E, Verhoef J, Heinz HP, Jones ME. The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of staphylococci from 19 European hospitals. J Antimicrob Chemother. 1999; 43(2):253-9.

Schwendener S and Perreten V. New transposon Tn6133 in methicillin-resistant *Staphylococcus aureus* ST398 contains *vga*(E), a novel streptogramin A, pleuromutilin, and lincosamide resistance gene. Antimicrob Agents Chemother. 2011; 55(10):4900-4.

Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol Rev. 1993; 57(1):138-63.

Shlaes DM, Etter L, Gutmann L. Synergistic killing of vancomycin-resistant enterococci of classes A, B, and C by combinations of vancomycin, penicillin, and gentamicin. Antimicrob Agents Chemother. 1991; 35(4):776-9.

Shopsin B, Gomez M, Montgomery SO, Smith DH, Waddington M, Dodge DE, Bost DA, Riehman M, Naidich S, Kreiswirth BN. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J Clin Microbiol. 1999; 37(11):3556-63.

Sibbald B. Farm-grown superbugs: while the world acts, Canada dawdles. CMAJ. 2012; 184(14):1553.

Sitthisak S, Howieson K, Amezola C, Jayaswal RK. Characterization of a multicopper oxidase gene from *Staphylococcus aureus*. Appl Environ Microbiol. 2005; 71(9):5650-3.

Smith TC, Gebreyes WA, Abley MJ, Harper AL, Forshey BM, Male MJ, Martin HW, Molla BZ, Sreevatsan S, Thakur S. Methicillin-resistant *Staphylococcus aureus* in pigs and farm workers on conventional and antibiotic-free swine farms in the USA. PloS One. 2013; 8(5):e63704.

Smyth DS, Wong A, Robinson DA. Cross-species spread of SCC *mec* IV subtypes in staphylococci. Infection, Genetics and Evolution. 2011; 11(2):446-53.

Stefani S, Chung DR, Lindsay JA, Friedrich AW, Kearns AM, Westh H, MacKenzie FM. Meticillin-resistant *Staphylococcus aureus* (MRSA): Global epidemiology and harmonisation of typing methods. Int J Antimicrob Agents. 2012; 39(4):273-82.

Stegger M, Liu CM, Larsen J, Soldanova K, Aziz M, Contente-Cuomo T, Petersen A, Vandendriessche S, Jiménez JN, Mammina C. Rapid differentiation between livestock-associated and livestock-independent *Staphylococcus aureus* CC398 clades. PloS One. 2013; 8(11):e79645.

Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, King CJ, McArthur J. Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. Environ Microbiol. 2006; 8(9):1510-4.

Stevens D, Parimon T, Bryant A. MRSA: Genetics, virulence factors, and toxin expression. In: MRSA. Weigelt J, editor. 2nd ed. New York, NY: Informa Healthcare USA, Inc; 2010.

Thompson CL, Wang B, Holmes AJ. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. The ISME Journal. 2008; 2(7):739-48.

Tulinski P, Fluit AC, Wagenaar JA, Mevius D, van de Vijver L, Duim B. Methicillin-resistant coagulase-negative staphylococci on pig farms as a reservoir of heterogeneous staphylococcal cassette chromosome mec elements. Appl Environ Microbiol. 2012; 78(2):299-304.

Ug A and Ceylan Ö. Occurrence of resistance to antibiotics, metals, and plasmids in clinical strains of *Staphylococcus* spp. Arch Med Res. 2003; 34(2):130-6.

Uhlemann AC, Porcella SF, Trivedi S, Sullivan SB, Hafer C, Kennedy AD, Barbian KD, McCarthy AJ, Street C, Hirschberg DL, Lipkin WI. Identification of a highly transmissible animal-independent *Staphylococcus aureus* ST398 clone with distinct genomic and cell adhesion properties. MBio. 2012 May 2;3(2):e00027-12.

Van Duijkeren E, Ikawaty R, Broekhuizen-Stins M, Jansen M, Spalburg E, De Neeling A, Allaart J, Van Nes A, Wagenaar J, Fluit A. Transmission of methicillin-resistant *Staphylococcus aureus* strains between different kinds of pig farms. Vet Microbiol. 2008; 126(4):383-9.

Van Rijen M and Kluytmans J. Costs and benefits of the MRSA search and destroy policy in a Dutch hospital. European Journal of Clinical Microbiology & Infectious Diseases. 2009; 28(10):1245-52.

Vandendriessche S, Vanderhaeghen W, Larsen J, de Mendonca R, Hallin M, Butaye P, Hermans K, Haesebrouck F, Denis O. High genetic diversity of methicillin-susceptible *Staphylococcus aureus* (MSSA) from humans and animals on livestock farms and presence of SCC*mec* remnant DNA in MSSA CC398. J Antimicrob Chemother. 2014; 69(2):355-62.

Vanderhaeghen W, Vandendriessche S, Crombé F, Dispas M, Denis O, Hermans K, Haesebrouck F, Butaye P. Species and staphylococcal cassette chromosome *mec*

(SCC *mec*) diversity among methicillin-resistant non-*Staphylococcus aureus* staphylococci isolated from pigs. Vet Microbiol. 2012; 158(1):123-8.

Verhegghe M, Crombé F, De Man I, Haesebrouck F, Butaye P, Heyndrickx M, Rasschaert G. Preliminary study of the effect of sow washing, as performed on the farm, on livestock-associated methicillin-resistant *Staphylococcus aureus* skin status and strain diversity. Journal of Swine Health and Production. 2013; 21(6):313-9.

Vijver LP, Tulinski P, Bondt N, Mevius D, Verwer C. Prevalence and Molecular Characteristics of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Organic Pig Herds in The Netherlands. Zoonoses and Public Health. 2014; 61(5):338-45.

Vos MC, Ott A, Verbrugh HA. Successful search-and-destroy policy for methicillin-resistant *Staphylococcus aureus* in the Netherlands. J Clin Microbiol. 2005; 43(4):2034; author reply 2034-5.

Voss A, Loeffen F, Bakker J, Klaassen C, Wulf M. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerging Infect Dis. 2005; 11(12):1965-6.

Votintseva AA, Fung R, Miller RR, Knox K, Godwin H, Wyllie DH, Bowden R, Crook DW, Walker AS. Prevalence of *Staphylococcus aureus* protein A (*spa*) mutants in the community and hospitals in Oxfordshire. BMC Microbiology. 2014; 14(1):63.

Weese J, Zwambag A, Rosendal T, Reid-Smith R, Friendship R. Longitudinal investigation of Methicillin-Resistant *Staphylococcus aureus* in piglets. Zoonoses and Public Health. 2011; 58(4):238-43.

Weese JS, Rousseau J, Deckert A, Gow S, Reid-Smith RJ. *Clostridium difficile* and methicillin-resistant *Staphylococcus aureus* shedding by slaughter-age pigs. BMC Veterinary Research. 2011; 7(1):41.

Weese JS, Slifierz M, Jalali M, Friendship R. Evaluation of the nasal microbiota in slaughter-age pigs and the impact on nasal methicillin-resistant *Staphylococcus aureus* (MRSA) carriage. BMC Veterinary Research. 2014; 10(1):69.

Weese JS. Methicillin-resistant *Staphylococcus aureus* in animals. ILAR J. 2010; 51(3):233-44.

Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, Kolonay JF, Shetty J, Killgore GE, Tenover FC. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. Science. 2003; 302(5650):1569-71.

Whitman WB, Coleman DC, Wiebe WJ. Prokaryotes: The unseen majority. Proc Natl Acad Sci U S A. 1998; 95(12):6578-83.

Williams RE. Healthy carriage of *Staphylococcus aureus*: its prevalence and importance. Bacteriol Rev. 1963; 27:56-71.

Woods GT, Jensen AH, Gossling J, Rhoades HE, Nickelson WF. The effect of medicated feed on the nasal microflora and weight gain of pigs. Can J Comp Med. 1972; 36(1):49-54.

Wulf M, Verduin C, Van Nes A, Huijsdens X, Voss A. Infection and colonization with methicillin resistant *Staphylococcus aureus* ST398 versus other MRSA in an area with a high density of pig farms. European Journal of Clinical Microbiology & Infectious Diseases. 2012; 31(1):61-5.

Xiong A and Jayaswal RK. 1998. Molecular characterization of a chromosomal determinant conferring resistance to zinc and cobalt ions in *Staphylococcus aureus*. J Bacteriol. 1998; 180(16):4024-9.

Yilmaz F, Orman N, Serim G, Kochan C, Ergene A, Icgen B. Surface water-borne multidrug and heavy metal-resistant staphylococcus isolates characterized by 16S rDNA sequencing. Bull Environ Contam Toxicol. 2013; 91(6):697-703.

Youssef N, Sheik CS, Krumholz LR, Najar FZ, Roe BA, Elshahed MS. Comparison of species richness estimates obtained using nearly complete fragments and simulated pyrosequencing-generated fragments in 16S rRNA gene-based environmental surveys. Appl Environ Microbiol. 2009; 75(16):5227-36.

Zhanel GG, Adam HJ, Baxter MR, Fuller J, Nichol KA, Denisuik AJ, Lagace-Wiens PR, Walkty A, Karlowsky JA, Schweizer F, et al. Antimicrobial susceptibility of 22746 pathogens from canadian hospitals: Results of the CANWARD 2007-11 study. J Antimicrob Chemother. 2013; 68 Suppl 1:i7-22.

1.9 – Tables

Table 1.1. Antimicrobial activity and resistance in human clinical isolates of methicillin-resistant *Staphylococcus aureus*.

Antimicrobial (class)	**Antimicrobial mechanism**	**Resistance factor(s)**	**Mechanism of resistance**	**Prevalence of resistance in MRSA (Region, Year)**
Ceftaroline ("fifth generation" cephalosporin)	Inhibits cell wall synthesis	Unknown	Unknown	2% (USA, 2009)[1]
Ciprofloxacin ("second generation" fluoroquinolone)	Blocks DNA synthesis by topoisomerase interferance	QRDR, *norA*	Point mutation causing topoisomerase modification; efflux pump	86% (Canada, 2007-11)[2] 67% (Australia, 2011)[3] 81% (EU, 2012)[4]
Clindamycin (lincosamide)	Inhibits protein synthesis	*erm*, *lnu*(A)	Modification of target site (ribosome methylation); drug inactivation	27% (USA, 2009)[1] 52% (Canada, 2007-11)[2] 30% (Australia, 2011)[3] 50% (EU, 2005)[5]
Dalbavancin (lipoglycopeptide)	Inhibits cell wall synthesis	Unknown	Unknown	0% (USA, 2011)[6]
Daptomycin (lipopeptide)	Disrupts cell membrane	Unknown	Unknown	0.4% (USA, 2009)[1] 0% (Canada, 2007-11)[2] 1% (Australia, 2011)[3] 0% (EU, 2005)[5]
Erythromycin, Clarithromycin (macrolides)	Inhibits protein synthesis	*erm*, *mrs*	Modification of target site (ribosome methylation); efflux pump	92% (USA, 2009)[1] 88% (Canada, 2007-11)[2] 64% (Australia, 2011)[3] 75% (EU, 2005)[5]
Gentamicin (aminoglycoside)	Disrupts protein synthesis (translation)	*aac*, *aph*	Aminoglycoside-inactivating enzymes; ribosomal modification; cell permeability	9% (Canada, 2007-11)[2] 3% (USA, 2007-9)[7]
Levofloxacin ("third generation" fluoroquinolone)	Blocks DNA synthesis by topoisomerase interferance	QRDR, *norA*	Point mutation causing topoisomerase modification; efflux pump	65% (USA, 2009)[1] 86% (Canada, 2007-11)[2] 91% (EU, 2005)[5]
Linezolid (oxazolidonone)	Inhibits protein synthesis	*cfr*	Modification of the target site (ribosome methylation)	0.1% (USA, 2009)[1] 0% (Canada, 2007-11)[2] 0% (EU, 2011)[8]
Methicillin, oxacillin (β-lactam)	Inhibits cell wall synthesis	*mecA* *mecC* *blaZ*	Encoded penicillin-binding protein with low β-lactam affinity	100%
Penicillin (β-lactam)	Inhibits cell wall synthesis	*blaZ* operon	Produces β-lactamase causing drug inactivation	100% (cross-resistance)

Quinupristin (streptogramin B) Dalfopristin (streptogramin A)	Inhibits protein synthesis Inhibits protein synthesis; enhances activity of quinupristin	*erm*, *mrs* *vat*, *vga*	Modification of target site (ribosome methylation); efflux pump Drug inactivation; efflux pump	1% (EU, 2005)[5] 0.1% (USA, 2007-9)[7] 0% (Canada, 2008-9)[9]
Rifampicin (rifamycins)	Inhibits RNA synthesis	*rpoB*	Point mutation causing RNA polymerase with low drug affinity	2% (Australia, 2011)[3] 6% (EU, 2012)[4] 0% (Canada, 2008-9)[9]
Telavancin (lipoglycopeptide)	Inhibits cell wall synthesis	Unknown	Unknown	0% (Canada, 2007-11)[2]
Tetracycline (tetracyclines)	Inhibits protein synthesis	*tetO*, *tetM*, *tetK*, *tetL*	Protection of the ribosome; efflux pump	5% (USA, 2009)[1] 34% (Australia, 2011)[3] 16% (Canada, 2008-9)[9]
Tigecycline (glycylcycline)	Inhibits protein synthesis	*mepA*, unknown	Efflux pump; unknown	0% (USA, 2009)[1] 0% (EU, 2011)[8]
Trimethoprim (DHFR inhibitor) Sulphamethoxazole (sulfonamides)	Inhibits DNA synthesis Inhibits DNA synthesis	*dfr* *sul*	Modified target with reduced affinity Modified target with reduced affinity	2% (USA, 2009)[1] 8% (Canada, 2007-11)[2] 6% (EU, 2005)[5]
Vancomycin (glycopeptide)	Inhibits cell wall synthesis	*vanA*	Modification of target site by encoded ligase	0% (USA, 2009)[1] 0% (Canada, 2007-11)[2] 0% (EU, 2011)[8]

References: [1] Richter et al., 2011. [2] Zhanel et al., 2013. [3] Nimmo et al., 2011. [4] ECDC, 2013. [5] Sader et al., 2007. [6] Jones et al., 2013. [7] Mendes et al., 2012. [8] Jones et al., 2014. [9] Rubin and Chirino-Trejo, 2011.

Table 1.2. Resistance to heavy metals in methicillin-resistant *Staphylococcus aureus* (MRSA).

Metal	**Resistance factors**	**Mechanism of resistance**	**Characteristics of resistant isolates**
Arsenic	*ars* operon	Membrane bound transporter[1].	MRSA ST398 (SCC*mec* IX and X)[2]; MRSA ST22 (SCC*mec* IV)[2]
Cadmium	*czrA, czrB, czrC*	Membrane bound transporter[3,4].	MRSA ST398 (SCC*mec* V)[4].
	cadA, cadC, cadD, cadX	Metal efflux pump[5].	MRSA ST398 (SCC*mec* IVa, V, IX, and X)[5]; MRSA ST22 (SCC*mec* IV)[6].
Copper	*mco*	Enzymatic oxidation of copper[7].	MRSA ST398 (SCC*mec* IVa, V, IX, and X)[5]; MRSA ST22 (SCC*mec* IV)[6].
	copA, copB, copC	ATPase transporter[5].	MRSA ST398 (SCC*mec* IVa, V, IX, and X)[5]; MRSA ST22 (SCC*mec* IV)[6].
Zinc	*czrA, czrB, czrC*	Membrane bound transporter[3,4].	MRSA ST398 (SCC*mec* V)[4].
	zntA, zntR	Membrane bound transporter[8].	Various laboratory strains[8].

References: [1] Ji and Silver, 1992. [2] Li et al., 2011. [3] Kuroda et al., 1999. [4] Cavaco et al., 2010. [5] Gómez-Sanz et al., 2013. [6] Loeffler et al., 2013. [7] Sitthisak et al., 2005. [8] Xiong and Jayaswal, 1998.

Table 1.3. Reported prevalence of methicillin-resistant *Staphylococcus aureus* in swine production among top pork producing countries.

Country	**Pig-level prevalence**	**Herd-level prevalence**	**Sample**	**Predominant molecular types**	**Source**
Canada	SP: 20% (17/85) W: 28% (27/95) GF: 26% (27/105)	45% (9/20)	Nasal, Rectal	t034, t1255, t002	Khanna et al., 2008
China	M: 11.4% (58/509)	42% (13/31)	Nasal	t899 / ST9	Cui et al., 2009.
Belgium	SO: 26% (88/340) SP/W: 41% (141/340) GF: 26% (87/340)	68% (34/50)	Nasal	t011, t034, t567, t2970	Crombé et al., 2012.
Germany	W: 53% (169/320)	70% (28/40)	Pooled Nasal	t011, t2510, t034	Kock et al., 2009.
Netherlands	SO: 38.3% (282/737) SP: 53.4% (172/322) W: 53% (202/382) GF: 38.7% (43/111)	67.8% (137/202)	Pooled Nasal	t011, t108	Broens et al., 2011.
South America (Peru)	GF: 6.7% (8/120)	17% (1/6)	Nasal	t571	Arriola et al., 2011.
Spain	SP: 49% (26/53) GF: 21% (11/53)	83% (5/6)	Nasal	t011, t108, t1197, t2346	Gomez-Sanz et al., 2010.
United States	W: 4.6% (50/1085)	9% (4/45)	Nasal	t034, t3446, t002	Smith et al., 2013.

SP – suckling pigs; SO – sows; W – weaned/nursery pigs; GF – grower-finisher pigs; M – undefined mixed samples.

1.10 – Figures

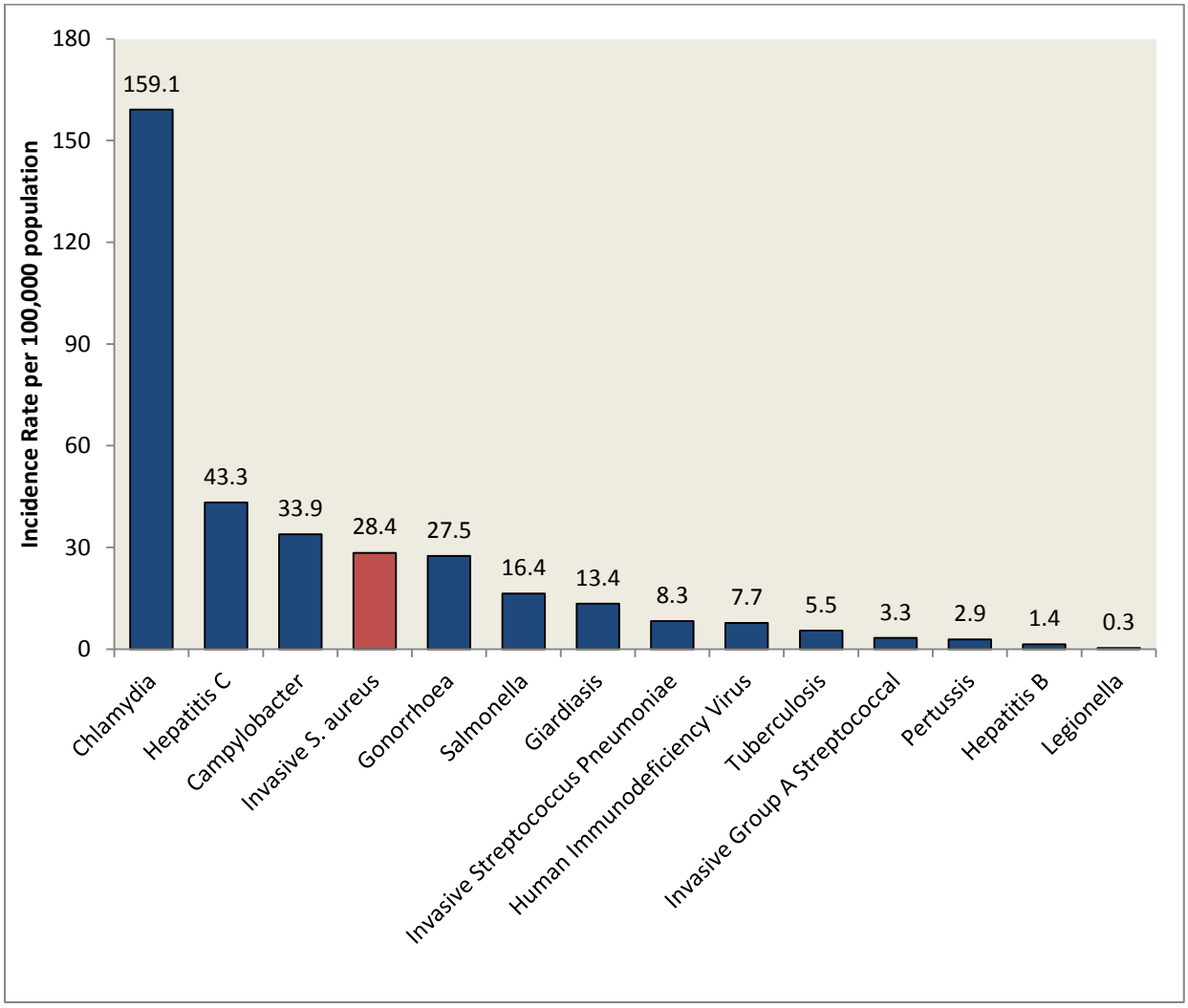


Figure 1.1. The incidence rate of invasive *Staphylococcus aureus* infections and other reportable infectious diseases in Ontario (2003). The incidence rate for invasive *S. aureus* infections was determined by Laupland et al., 2003, and the incidence rates for the other diseases were reported by Public Health Ontario (PHO, 2016).

CHAPTER 2: The effects of zinc therapy on the prevalence and persistence of methicillin-resistant *Staphylococcus aureus* (MRSA) in pigs: a randomized-controlled trial

This chapter was published as: Slifierz MJ, Friendship R, Weese JS. Zinc oxide therapy increases prevalence and persistence of methicillin-resistant *Staphylococcus aureus* in pigs: a randomized controlled trial. Zoonoses Public Health. 2015 Jun;62(4):301-8.

2.1 – Abstract

There is concern that therapeutic use of zinc oxide (ZnO) in swine production may select for methicillin-resistant *Staphylococcus aureus* (MRSA) due to co-location of the zinc-resistance gene (*czrC*) and methicillin-resistance gene (*mecA*) within the staphylococcal cassette chromosome *mec* (SCC*mec*). The objective of this investigation was to determine whether MRSA carriage in pigs is influenced by exposure to therapeutic doses of in-feed ZnO (3,000 mg/kg) when compared to the recommended dietary levels (100 mg/kg). A randomized-controlled trial was completed using 110 pigs that were naturally colonized with *czrC*-positive MRSA. The pigs were followed from birth to weaning (21 d), at which point they were randomized into 8 pens and exposed to either a control feed (100 mg ZnO/kg feed; n=49 pigs) or a treatment feed (3,000 mg ZnO/kg feed; n=50 pigs); neither feed contained additional antimicrobials. MRSA carriage was monitored weekly in each group for 4 weeks post-weaning. The prevalence of MRSA was significantly higher in the treatment group at 1-week (OR=18.1; $P<0.01$) and 2-weeks (OR=3.01; $P=0.01$) post-weaning when compared to the control group, but there was no difference later in the nursery phase. Persistent MRSA carriage (testing positive ≥2 times post-weaning) was observed in 2% (1/49) of control pigs and 22%

(11/50) of treated pigs ($P<0.01$). All MRSA isolates (spa types t034 and t3075) carried *czrC* and showed uniform resistance to zinc. These findings demonstrate that the prevalence and persistence of MRSA in nursery pigs can be affected by high levels of in-feed ZnO in the absence of antibiotics.

2.2 – Background

Staphylococcus aureus is the leading cause of skin and soft tissue infections, pneumonia, and bacteremia in humans in almost all regions of Canada, the United States, Europe, Latin America, and the West Pacific (Diekema et al., 2001). The procurement of multidrug resistance by methicillin-resistant *S. aureus* (MRSA) has further increased the burden on healthcare systems due to treatment difficulties associated with antimicrobial therapy (Klein et al., 2007). In the mid-2000s, MRSA was identified in pigs (Voss et al., 2005), and since this first recognition there has been an almost global emergence of specific lineages in pigs, particularly sequence types (ST) 398 and 9 (Cuny et al., 2010). Although MRSA is rarely a clinically relevant pathogen in swine, MRSA carriage in pigs may pose a risk to human health (Casey et al., 2013) and in some regions, livestock-associated MRSA is the predominate type of MRSA carried by humans (van Rijen et al., 2008; Haenen et al., 2010).

It is speculated that methicillin-sensitive *S. aureus* (MSSA) ST398 originating from humans colonized pigs and acquired resistance through exposure to antimicrobial agents routinely used in swine production (Price et al., 2012), with subsequent evolution so that genetic markers of pig- or human- origin are now identifiable (Sung et al., 2008). Although antimicrobial use in livestock production has been associated with MRSA carriage in pigs and humans (Smith et al., 2013; Rinsky et al., 2013), the commonness and persistence of MRSA in pigs raised without exposure to conventional antimicrobials indicates that there are other contributing factors (Weese et al., 2011a; Park et al., 2013).

One area of recent interest is resistance to zinc and cadmium, mediated by *czrC*, which can be found in MRSA isolated from pigs (Cavaco et al., 2010). The *czrC* gene is

located within the *Staphylococcus* Cassette Chromosome *mec* (SCC*mec*); a mobile genetic element that harbours additional antimicrobial-resistance genes including *mecA* which confers resistance to beta-lactam antimicrobials (Ito et al., 1999; Cavaco et al., 2010). Due to the co-location of *czrC* and *mecA* within the SCC*mec*, exposure to zinc has the potential to co-select and cause the persistence of methicillin resistance in staphylococci in the absence of beta-lactam antimicrobial exposure (Cavaco et al., 2011). This situation is concerning as high concentrations (≥2500 mg/kg) of in-feed zinc oxide (ZnO) are often used therapeutically in swine production to control *E. coli* diarrhea (colibacillosis) in young pigs (Amezcua et al., 2008), and this practice has emerged as an alternative to conventional in-feed antimicrobials (Pluske, 2013). However, evidence of the effects of in-feed ZnO dosage on the selection and persistence of MRSA in swine production systems is lacking. Therefore, the objective of this study was to determine whether the herd prevalence of MRSA among nursery pigs is affected by exposure to therapeutic levels of in-feed ZnO (3,000 mg/kg) when compared to the recommended dietary levels of in-feed ZnO (100 mg/kg). Nursery pigs were also monitored for any adverse physiological responses to therapeutic ZnO exposure, including changes in growth performance and serum zinc concentrations.

2.3 – Methods

The use of animals in this study was approved by the Animal Care Committee at the University of Guelph, following the Canadian Council on Animal Care guidelines. This parallel-group randomized-controlled trial was conducted at the University of Guelph swine research station, a 300-sow farrowing operation. The swine herd at the research facility was determined to be naturally colonized with *czrC*-positive MRSA

prior to the beginning of the trial; MRSA was detected in pooled nasal swabs from nursery pigs and sows, and *czrC* was identified by PCR as described below.

Using *a priori* estimates (p_1=0.10, p_2=0.35) based on previous findings (Khanna et al., 2008), the required sample size to detect a 25% difference in prevalence with 95% confidence and 80% power was determined to be 51 pigs per group. The total sample size was increased to 110 pigs to account for mortalities and culls. Eligibility for enrollment in the trial required a birth weight >1.0 kg, with no more than 6 pigs enrolled per litter. In total, 110 pigs from 23 sows were enrolled into the trial at birth (0 d) and followed for 49 days. The pigs were not given creep feed prior to weaning. At weaning (21 d), stratified randomization was used to ensure pigs testing positive for MRSA prior to weaning were equally distributed among 8 pens. Stratified randomization of pigs was completed as follows: (i) numbered identification tags were randomly drawn from a bag immediately before pigs were tagged; (ii) pigs were stratified based on pre-weaning MRSA carriage; (iii) the identification tag numbers were sequentially assigned a pen and pigs were placed in the respective pen at weaning. Each pen housed 11-13 pigs and all pens were located within the same nursery room, containing individual feeders and solid partitions. Four pens of control pigs received a two-phase nursery ration with a basal level of 100 mg/kg ZnO and the remaining 4 pens of treated pigs received an identical two-phase ration supplemented with an additional 2,900 mg/kg ZnO. The basal formulation for the two-phase nursery ration is shown in Table 2.1. All pigs were fed ad libitum and raised without exposure to any other antimicrobial agents. There was no formal blinding procedure used in this trial.

Sampling occurred at 1, 3, 7, 14, 21, 28, 35, 42, and 49 days of age. Nasal swabs were collected from both nares of individual pigs at every sampling and from sows on day 14. At weaning (21 d), pigs were sampled immediately prior to being exposed to their assigned ration. The nasal samples were transported at 4°C and processed on the same day they were collected. Personal protective equipment was changed after sampling each pen to prevent contamination and transfer of MRSA between pens. Body weight was measured on days 1, 21, 35, and 49, and feed in-take was monitored throughout the post-weaning period. Serum was collected from a random subset of pigs (n=17 per group) on days 21 and 49. Standard toxicological testing was completed by the Animal Health Laboratory at the University of Guelph to quantify serum zinc concentrations. Serum zinc concentrations of 0.70−2.0 μg/mL were considered normal and values <0.70 μg/mL were considered low and suboptimal for development (Hahn and Baker, 1993).

Nasal swabs were tested for MRSA using an enrichment culturing procedure. Each nasal swab was inoculated into 2 mL of enrichment broth containing tryptone (10 g/L), sodium chloride (75 g/mL), mannitol (10 g/L), and yeast extract (2.5 g/L), and then incubated at 35°C overnight. One loop-full of broth was then inoculated onto MRSA Chromogenic agar (BBL CHROMagar MRSA, Becton, Dickinson and Company, Sparks, MD, USA) and read after 24 h and 48 h of incubation at 35°C. Suspected isolates were confirmed by colony morphology, hemolysis, coagulase reaction, and *S. aureus* latex agglutination assay (Pastorex Staph-plus; Bio-Rad, Marnes-la-Coquette, France). Resistance to methicillin was identified by a penicillin binding protein 2a latex agglutination assay (MRSA latex agglutination test; Oxoid Ltd., Hants, UK). One MRSA isolate was stored from each positive nasal swab. The presence of *czrC*, staphylococcal

protein A (spa) type, and minimum inhibitory concentration (MIC) for zinc chloride ($ZnCl_2$) were determined for all MRSA isolates from suckling pigs and sows on day 14, and nursery pigs on day 35. The *czrC* gene was detected by PCR amplification (Slifierz et al., 2014) using primers previously designed by Cavaco et al. (2010). The MRSA isolates were typed by sequencing the polymorphic X region of the spa gene (Shopsin et al., 1999) and assigned a Ridom type through the SpaSever database (http://www.spaserver.ridom.de/). The MIC for zinc was determined by plating isolates on Muller-Hinton-II agar supplemented with $ZnCl_2$ (0.25-16 mM) (Aarestrup et al., 2004).

Demographics, growth performance, feed in-take, serum zinc concentrations, and distribution of MRSA spa types were first assessed with univariate regression to determine if these variables were significantly different between the treatment and control groups. The association between ZnO dosage and post-weaning persistence of MRSA (testing positive ≥2 occasions after weaning) was assessed using Fisher's exact test. Some of these associations were then further evaluated with multivariate regression methods to control for any potential confounding effects and to explore two-way interactions.

The effect of in-feed ZnO concentration on MRSA carriage was assessed by multivariate logistic regression for each time point after weaning. Two covariates were manually retained in each model: pre-weaning carriage of MRSA and exposure to an MRSA-positive sow. Other covariates, including sow parity, live births per litter, and sex, were also explored for significance or a confounding effect (>30% change in coefficient). The model building process also assessed the significance of sow level clustering of data.

The final modelled data were determined to have a binomial covariate pattern and thus the Pearson residuals were assessed to establish the goodness of fit of the model. The association between MRSA carriage in sows and suckling pigs was also assessed using the same multivariable logistic regression approach.

Multivariate linear regression was used to determine if early nursery ADG (21 – 35 d) and late nursery ADG (35 – 49 d) was affected by in-feed zinc dosage. In addition, sex, birth weight, pre-weaning ADG, parity, and live births per litter were also assessed as covariates in each model. The variables were assessed for linearity using a smoothed locally weighted scatterplot and screened for co-linearity (R^2>0.8). Each variable was then screened with univariate regression for inclusion in the initial model (P<0.15). Any excluded variables were then evaluated for a confounding effect (>30% change in coefficient). Clustering at the sow level was explored by including it as a fixed effect in the model. Two-way interactions were also assessed for variables included in the initial model. The homoscedasticity and normality of residuals was evaluated for each model using the Cook-Weisberg test and the Shapiro-Wilk test, respectively.

All statistical analysis was completed using STATA 10 (Stata Corporation, College Station, TX). Associations where P<0.05 were considered statistically significant and non-significant associations with a P<0.10 were considered to demonstrate a tendency towards an association.

2.4 – Results

Fifty pigs received the treatment feed containing therapeutic levels of ZnO (3,000 mg/kg) and 49 pigs received the control feed containing the recommended dietary levels of ZnO (100 mg/kg) at weaning. There was a pre-weaning mortality and culling rate of

10% (11/110) but there were no mortalities or culls after weaning. The cause of the mortalities is unknown, but clinical signs of *E. coli* diarrhea (colibacillosis) were absent throughout the trial. No adverse reactions to the therapeutic levels of ZnO were observed and no evidence of clinical MRSA infection was noted in any pig.

Demographic information, growth performance, and daily feed in-take are summarized in Table 2.2. For the early nursery ADG, multivariate analysis revealed no significant difference between the treated pigs and control pigs (P=0.978). However, early nursery ADG was associated with live births per litter and birth weight (Table 3). Similarly, late nursery ADG was not significantly different between the treated pigs and control pigs (P=0.244), but it was significantly associated with live births per litter, birth weight, and pre-weaning ADG (Table 3). Parity had a confounding effect and was retained in both models. Clustering of data at the sow level was not present in this study. Furthermore, there was no significant difference in feed in-take between treatment and control pens (P=0.629).

The results for zinc serum concentrations are described in Table 2.2. The concentration of zinc in sera was not significantly different between treated pigs (n=17) and control pigs (n=17) at weaning (P=0.789). However, serum zinc concentrations were significantly higher in treated pigs at 4 weeks post-weaning when compared to control pigs (P=0.020). It was also observed that 26.5% (9/34) of the pigs had a suboptimal concentration of serum zinc (<0.70 µg/mL) at weaning but at 4 weeks post-weaning only one control pig had a low concentration of zinc in serum (0.66 µg/mL).

The pre-weaning prevalence of MRSA in suckling pigs on days 1, 3, 7, 14, and 21 was 4.8% (5/105), 5.7% (6/105), 5.7% (6/105), 5.9% (6/101), and 6.0% (6/100),

respectively. The average pre-weaning prevalence of MRSA was 5.6 ± 0.5%. There were 25 different suckling pigs that carried MRSA before weaning and only 4 of these pigs tested positive on more than one occasion. Additionally, 34.8% (8/23) of the sows carried MRSA. Suckling pigs raised on a MRSA-positive sow were 2.71 times more likely to carry MRSA than suckling pigs raised on a MRSA-negative sow (95% CI: 1.07 – 6.84; *P*=0.035). However, repeated MRSA positivity in suckling pigs (n=4) was not associated with MRSA carriage in sows (*P*=0.380). Parity, farrowing room, and live births per litter did not have a confounding effect on these associations and were not significantly associated with MRSA carriage in suckling pigs in this study. The results for *czrC*, $ZnCl_2$ susceptibility, and spa typing of all isolates recovered from suckling pigs and sows on day 14 are summarized in Table 2.4.

The prevalence of MRSA from birth to 4-weeks post-weaning for the treatment and control groups is shown in Figure 2.1. At weaning – just prior to being exposed to in-feed ZnO – 4.1% (2/49) of the control pigs and 8.0% (4/50) of the treated pigs carried MRSA (*P*=0.422). The post-weaning prevalence of MRSA in the control group on days 28, 35, 42, and 49 was 2.0% (1/49), 24.5% (12/49), 2.0% (1/49), and 0% (0/49), respectively. In the treatment group, the post-weaning prevalence of MRSA was 26% (13/50), 52% (26/50), 8.0% (4/50), and 2.0% (1/50) on days 28, 35, 42, and 49, respectively. After controlling for pre-weaning MRSA carriage and exposure to MRSA-positive sows, the treated pigs were significantly more likely to carry MRSA on day 28 (OR=18.1, 95% CI: 2.2-147.6; *P*=0.007) and day 35 (OR=3.01, 95% CI: 1.24-7.28; *P*=0.015) when compared to the control pigs. However, there was no significant difference between groups on day 42 (*P*=0.19) and day 49 (*P*=0.99). Parity, live births

per litter, and sex did not confound the relationship between MRSA prevalence and ZnO dosage.

Post-weaning persistence of MRSA in pigs (those carrying MRSA on 2 or more occasions after weaning) was observed in 2% (1/49) of control pigs and 22% (11/50) of treated pigs (P=0.004). In addition, throughout the post-weaning period 73.5% (36/49) of control pigs and 34% (17/50) of treated pigs never carried MRSA (P<0.001). The characteristics of MRSA isolates from nursery pigs (35 d), including *czrC*, $ZnCl_2$ susceptibility, and distribution of spa types (Table 2.4), did not differ between treated and control pigs (P>0.05). Despite the circulation of two spa types within this herd (t034 and t3075), all isolates of MRSA harboured the zinc-resistance gene *czrC* and showed uniform resistance to $ZnCl_2$ (MIC >2 mM).

2.5 – Discussion

The finding that therapeutic levels of ZnO can increase the prevalence and persistence of MRSA in nursery pigs in the absence of other antimicrobials is concerning as swine feed is commonly supplemented with therapeutic ZnO to control colibacillosis (Amezcua et al., 2008). This may provide an explanation for the observed persistence of MRSA in swine herds raised without exposure to antibiotics as therapeutic ZnO has become a leading alternative to antibiotics in some countries (Pluske, 2013). It is possible that therapeutic levels of ZnO can disrupt the normal composition of the resident nasal microbiota resulting in susceptibility to MRSA colonization. Exposure to ZnO may also cause co-selection of methicillin-resistance in staphylococci in the absence of antimicrobials due to co-location of *mecA* and *czrC* within the SCC*mec* of MRSA (Cavaco et al., 2010), although further research is needed to investigate this claim.

Nevertheless, these results highlight the complex and multifactorial nature of antimicrobial resistance of staphylococci in swine production systems.

The association between herd-level prevalence of MRSA and ZnO dosage observed in this study is similar to previous reports of zinc tolerance in porcine MRSA isolates (Cavaco et al., 2011) and a study that demonstrated that young pigs fed therapeutic ZnO (2,500 mg/kg) have a higher MRSA load in the nasal passage when compared to untreated pigs (Moodley et al., 2011). It is unclear how in-feed ZnO dosage exerts a selective pressure on MRSA in the nares, but it may result from accumulated zinc in the tissues or through ration entering the nasal cavity at feeding. Low doses of ZnO, such as the 100 mg/kg dose used in the control group, may also have an impact on MRSA carriage; however, this was not explored in the present study as the absence of a zinc supplementation from the ration would not be a practical management strategy for swine production systems. Nevertheless, the absence of a group fed a ration lacking ZnO cannot allow for determination of the impact of low doses of ZnO (100 mg/kg) on MRSA carriage and presents an understandable limitation of the study.

The dramatic increase in MRSA prevalence immediately after weaning is similar to other longitudinal observations of MRSA in nursery pigs raised without exposure to conventional antimicrobials (Weese et al., 2011a). This increase is likely the result of exposing susceptible pigs to MRSA carriers when pigs are sorted and mixed at weaning but other factors such as environmental contamination, stress, or changes in the nasal microbiota cannot be excluded (Weese et al., 2011a). Furthermore, after peaking at two weeks post-weaning, the prevalence of MRSA rapidly declined in both the treatment and control groups. The cause of this decline is unclear but it may be attributed to a change of

the nasal microbiota or development of natural immunity, although this requires further investigation. At the end of the nursery phase, MRSA was undetectable in control pigs but was still detected in a small proportion of treated pigs which may be indicative of an established baseline prevalence that persists until slaughter (Weese et al., 2011b). Additionally, the number of pigs in each pen was not representative of commercial swine production and perhaps the number of pen mates or stocking density affects the dynamics of MRSA transmission and herd persistence. The total sample size used in this study may also be a limiting factor and replication of this trial using a larger number of pigs may allow for identification of differences in MRSA carriage during the late nursery phase with greater power.

The lack of association of *czrC*, zinc susceptibility, and spa type with in-feed ZnO dosage is likely indicative of the homogeneity of the MRSA circulating within this herd. Observational research that encompasses a multitude of swine herds is needed to further identify specific characteristics of MRSA that may be associated with ZnO dosage. Nevertheless, the only two spa types identified in this study, t034 and t3075, are frequently isolated from pigs (Khanna et al., 2008; Dressler et al., 2012) and the identification of *czrC* in these MRSA spa types was unsurprising as *czrC* is common among MRSA isolates of porcine origin (Cavaco et al., 2011).

The positive association observed between MRSA carriage in suckling pigs and sows in this trial has also been reported by Weese et al. (2011a). Although MRSA persistence in suckling pigs was not associated with MRSA carriage in sows in the present study, this should be treated with caution as the sample size was limited due to the relatively low baseline prevalence of MRSA prior to weaning. As a further matter,

MRSA was relatively transient and uncommitted to persist in individual suckling pigs, with no evidence of long-term shedders, yet MRSA still maintained uniform persistence among the collective herd, presumably from ready circulation of MRSA between pigs and the lack of development of natural immunity to colonization following exposure. This transient behaviour may also be the result of the absence of an adequate selective pressure as it is similar to the transient nature of MRSA among the control pigs in this trial.

The finding that therapeutic ZnO supplementation does not affect growth performance or feed intake of healthy nursery pigs when compared to the required dietary levels is consistent with previous research (Fryer et al., 1992; Tokach et al., 1992; Schell et al., 1996; Smith et al., 1997) although some studies have found that therapeutic doses of in-feed ZnO can enhance the growth performance and feed intake of nursery pigs even in the absence of colibacillosis (Hahn et al., 1993; Hill et al., 2000; Han et al., 2010). The effectiveness of therapeutic ZnO supplementation as a growth promoter in healthy nursery pigs is likely dependent on additional herd-level factors as there is considerable variation in its effectiveness between herds (Hill et al., 2000). Nevertheless, the absence of any negative health or production effects in the group that did not receive therapeutic levels of ZnO (or antimicrobials) indicates that these prophylactic therapies are not always needed. In addition, ZnO dosage was also associated with serum zinc concentration which was anticipated and in agreement with previous findings (Hahn et al., 1993; Hill et al., 2000).

Overall, exposure to therapeutic levels of in-feed ZnO is associated with an increase in the prevalence and persistence of MRSA among pigs, particularly during the

early phase of the nursery. This raises concerns that ZnO may co-select for methicillin resistance in the absence of beta-lactam antimicrobials and that the common use of ZnO as a growth promoter and therapy for colibacillosis may indiscriminately promote the persistence of MRSA in swine production systems. Zinc-mediated selection of antimicrobial resistance genes in MRSA may also be of concern in human medicine as many widely used products contain zinc, including lozenges, nasal sprays, topical skin ointments, cosmetics, and antiseptic solutions used in healthcare settings. Minimizing the spread of antimicrobial resistance genes will require encouraging responsible use of ZnO therapy in agriculture and it may be prudent for policymakers to create regulations for ZnO usage, particularly in countries that allow for the unrestricted application of this therapy. Further studies are also needed to determine if other sources of inorganic or organic zinc can contribute to MRSA carriage in pigs, and to determine the risk of using these agents in agricultural production systems given the potential to select for antimicrobial resistance.

2.6 – References

Aarestrup FM, Hasman H. Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride and antimicrobial substances used for disinfection. Vet. Microbiol. 2004; 100:83-89.

Amezcua R, Friendship RM, Dewey CE. An investigation of the presence of *Escherichia coli* O149:K91:F4 on pig farms in southern Ontario and the use of antimicrobials and risk factors associated with the presence of this serogroup. Can. Vet. J. 2008; 49:39–45.

Casey JA, Curriero FC, Cosgrove SE, Nachman KE, Schwartz BS. High-Density Livestock Operations, Crop Field Application of Manure, and Risk of Community-Associated Methicillin-Resistant *Staphylococcus aureus* Infection in Pennsylvania. JAMA Intern. Med. 2013; 173:1980-1990.

Cavaco LM, Hasman H, Aarestrup FM. Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance. Vet. Microbiol. 2011; 150:344-348.

Cavaco LM, Hasman H, Stegger M, Andersen PS, Skov R, Fluit AC, Ito T, Aarestrup FM. Cloning and occurrence of *czrC*, a gene conferring cadmium and zinc resistance in methicillin-resistant *Staphylococcus aureus* CC398 isolates. Antimicrob. Agents Chemother. 2010; 54:3605-3608.

Cuny C, Friedrich A, Kozytska S, Layer F, Nübel U, Ohlsen K, Strommenger B, Walther B, Wieler L, Witte W. Emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in different animal species. Int. J. Med. Microbiol. 2005; 300:109-117.

Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN, Beach M, SENTRY Partcipants Group. Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. Clin. Infect. Dis. 2001; 32:114-32.

Dressler AE, Scheibel RP, Wardyn S, Harper AL, Hanson BM, Kroeger JS, Diekema DJ, Bender JB, Gray GC, Smith TC. Prevalence, antibiotic resistance and molecular characterisation of *Staphylococcus aureus* in pigs at agricultural fairs in the USA. Vet. Rec. 2012; 170:495.

Fryer A, Miller ER, Ku PK, Ullrey DE. Effect of elevated dietary zinc on growth performance of weanling swine. Michigan State Univ. Rep. of Swine Res. 1992; 520:128-132.

Haenen A, Huijsdens X, Pluister C, van Luit M, van Luit T, Bosch T, van Santen-Verheuvel MG, Spalburg E, Heck MEOC, de Neeling AJ, Mulders MN.

Surveillance van MRSA in Nederland in 2008. Infectieziekten Bulletin. 2010; 21:162–169.

Hahn JD, Baker DH. Growth and plasma zinc responses of young pigs fed pharmacologic levels of zinc. J. Anim. Sci. 1993; 71:3020-3024.

Han YK, Thacker PA. Effect of antibiotics, zinc oxide and rare earth mineral yeast on performance, nutrient digestibility and blood parameters in weaned pigs. Asian-Aust. J. Anim. Sci. 2010; 23:1057–1065.

Hill G, Cromwell MGL, Crenshaw TD, Dove CR, Ewan RC, Knabe DA, Lewis AJ, Libal GW, Mahan DC, Shurson GC, Southern LL, Veum TL. Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (regional study). J. Anim. Sci. 2000; 78:1010-1016.

Ito T, Katayama Y, Hiramatsu K. Cloning and nucleotide sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. Antimicrob. Agents Chemother. 1999; 43:1449-1458.

Khanna T, Friendship R, Dewey C, Weese JS. Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. Vet. Microbiol. 2008; 128:298-303.

Klein E, Smith DL, Laxminarayan R. Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999-2005. Emerg. Infect. Dis. 2007; 13:1840-1846.

Moodley A, Nielsen SS, Guardabassi L. Effects of tetracycline and zinc on selection of methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type 398 in pigs. Vet. Microbiol. 2011; 152:420-423.

Park J, Friendship RM, Poljak Z, Weese JS, Dewey CE. An investigation of exudative epidermitis (greasy pig disease) and antimicrobial resistance patterns of *Staphylococcus hyicus* and *Staphylococcus aureus* isolated from clinical cases. Can. Vet. J. 2013; 54:139-144.

Pluske JR. Feed- and feed additives-related aspects of gut health and development in weanling pigs. J. Anim. Sci Biotechnol. 2013; 4:1.

Price LB, Stegger M, Hasman H, Aziz M, Larsen J, Andersen PS, Pearson T, Waters AE, Foster JT, Schupp J, Gillece J, Driebe E, Liu CM, Springer B, Zdovc I, Battisti A, Franco A, Zmudzki J, Schwarz S, Butaye P, Jouy E, Pomba C, Porrero MC, Ruimy R, Smith TC, Robinson DA, Weese JS, Arriola CS, Yu F, Laurent F, Keim P, Skov R, Aarestrup FM. *Staphylococcus aureus* CC398: host adaptation and emergence of methicillin resistance in livestock. MBio. 2012; 3.

Rinsky JL, Nadimpalli M, Wing S, Hall D, Baron D, Price LB, Larsen J, Stegger M, Stewart J, Heaney CD. Livestock-associated methicillin and multidrug resistant

Staphylococcus aureus is present among industrial, not antibiotic-free livestock operation workers in North Carolina. PLoS One. 2013; 8:e67641.

Schell TC, Kornegay ET. Zinc concentration in tissues and performance of weanling pigs fed pharmacological levels of zinc from ZnO, Zn-methionine, Zn-lysine, or $ZnSO_4$. J. Anim. Sci. 1996; 74:1584-1593.

Shopsin B, Gomez M, Montgomery SO, Smith DH, Waddington M, Dodge DE, Bost DA, Riehman M, Naidich S, Kreiswirth BN. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. 1999; 37:3556–3563.

Slifierz MJ, Park J, Friendship R, Weese JS. Zinc-resistance gene *czrC* identified in methicillin-resistant *Staphylococcus hyicus* isolated from pigs with exudative epidermitis. Can. Vet. J. 2014; 55:489-490.

Smith JW, Tokach MD, Goodband RD, Nelssen JL, Richert BT. Effects of the interrelationship between zinc oxide and copper sulphate on growth performance of early weaned pigs. J. Anim. Sci. 1997; 75:1861-1866.

Smith TC, Gebreyes WA, Abley MJ, Harper AL, Forshey BM, Male MJ, Martin HW, Molla BZ, Sreevatsan S, Thakur S, Thiruvengadam M, Davies PR. Methicillin-resistant *Staphylococcus aureus* in pigs and farm workers on conventional and antibiotic-free swine farms in the USA. PLoS One. 2013; 8:e63704.

Sung JM, Lloyd DH, Lindsay JA. *Staphylococcus aureus* host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. Microbiol. 2008; 154:1949-1959.

Tokach LM, Tokach MD, Goodband RD, Nelssen JL, Henry SC, Marsteller TA. Influence of zinc oxide in starter diets on pig performance. Proc. Am. Assoc. Swine Prac. 1992; 1:411.

van Rijen MM, Van Keulen PH, Kluytmans JA. Increase in a Dutch hospital of methicillin-resistant *Staphylococcus aureus* related to animal farming. Clin. Infect. Dis. 2008; 46:261-263.

Voss A, Loeffen F, Bakker J, Klaassen C, Wulf M. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerg. Infect. Dis. 11:1965-1966.

Weese JS, Zwambag A, Rosendal T, Reid-Smith R, Friendship R. Longitudinal investigation of methicillin-resistant *Staphylococcus aureus* in piglets. Zoonoses Public Health. 2011; 58:238-243.

Weese JS, Rousseau J, Deckert A, Gow S, Reid-Smith RJ. *Clostridium difficile* and methicillin-resistant *Staphylococcus aureus* shedding by slaughter-age pigs. BMC Vet. Res. 2011; 7:41.

2.7 – Tables

Table 2.1. Basal formulation (%) of the two-phase starter ration fed to nursery pigs.

Ingredient	Phase I	Phase II
Soybean meal, 47%	25.5	25.8
Corn chop	35.3	33.5
Whey permeate	10	8
Bakery meal	10	10
Wheat chop	7.8	7.5
Wheat shorts	0	5
Fish meal	3	2
Blood meal	2.5	2.5
Tallow	1.4	1.5
Monocalcium phosphate	1.1	1.05
Limestone	0.95	1
L-lysine, 50%	0.62	0.55
DL-methionine, 88%	0.28	0.25
Maple Butter	0.2	0.1
Fine salt	0.2	0.13
Threonine	0.19	0.16
Selenium	0.1	0.1
Choline chloride, 70%	0.05	0.05
Vitamin E (50 KIU/kg)	0.05	0.05
PellTech	0.4	0.4
Integral	0.05	0.05
Superzyme-CS	0.05	0.05

IntelliBond C (58% Cu)	0.017	0.017
Vitamin and mineral supplement[a]	0.2	0.2

[a] Composition per kg of starter ration: 100 mg zinc oxide, 270 mg iron, 28 mg manganese, 124 mg copper, 0.60 mg iodine, 0.50 mg selenium, 0.16 mg cobalt, 20 mg fluorine, 10 KIU vitamin A, 1.5 KIU vitamin D3, 70 IU vitamin E, 2.0 mg vitamin K, 20 µg vitamin B12, 6.5 mg riboflavin, 30 mg niacin, 1.34 g choline, 24 mg D-pantothenic acid, 2.5 mg pyridoxine, 2.0 mg thiamine, 3.0 mg folic acid, 200 µg biotin.

Table 2.2. Characteristics of pigs from control and treatment groups.

Parameter	Control Pigs (n=49)	Treated Pigs (n=50)	*P*-value[a]
Demographics			
Males	26	33	0.191
Live births per litter	10.5 ± 3.6	10.4 ± 4.1	0.866
Parity	2.65 ± 0.69	2.94 ± 0.82	0.068
Pigs with MRSA before weaning	8	11	0.323
Pigs exposed to an MRSA-positive sow	14	21	0.164
Body Weight (kg)			
Birth	1.55 ± 0.31	1.61 ± 0.29	0.318
Weaning	6.37 ± 1.19	6.45 ± 1.32	0.760
2 weeks post-weaning	9.25 ± 1.58	9.39 ± 1.42	0.641
4 weeks post-weaning	17.50 ± 2.45	17.25 ± 2.48	0.620
Average Daily Gain (g/day)			
Pre-weaning (1 – 21 d)	241.2 ± 56.1	242.0 ± 62.8	0.944
Early Nursery (21 – 35 d)	205.6 ± 53.7	210.1 ± 45.6	0.649
Late Nursery (35 – 49 d)	589.1 ± 80.3	561.7 ± 116.6	0.177
Average Daily Feed Intake (g/day)	578.7 ± 56.2	595.2 ± 32.4	0.629
Average Daily Zinc Intake (g/day)	0.058 ± 0.01	1.786 ± 0.09	<0.001
Serum Zinc Concentration[b] (ug/ml)			
Weaning	0.821 ± 0.16	0.805 ± 0.19	0.789
4 weeks post-weaning	0.925 ± 0.22	1.089 ± 0.18	0.020

[a] Univariate regression analysis.
[b] Subsample of 17 pigs per group.
MRSA − methicillin-resistant *Staphylococcus aureus*

Table 2.3. Effects of in-feed ZnO on the growth performance of nursery pigs (multivariate linear regression models).

Variable	Coefficient[a]	95% CI	*P*-value
Early Nursery ADG (g/day)			
Therapeutic ZnO (3,000 mg/kg)	-0.25[b]	-18.6 − 18.2	0.978
Live births per litter	5.63	3.2 − 8.1	<0.001
Birth weight (g)	0.068	0.037 – 0.099	<0.001
Late Nursery ADG (g/day)			
Therapeutic ZnO (3,000 mg/kg)	-22.6[b]	-60.8 − 15.7	0.244
Live births per litter	8.77	3.6 − 13.9	0.001
Birth weight (g)	0.080	0.015 – 0.14	0.016
Pre-weaning ADG (g/day)	0.632	0.298 − 0.968	<0.001

[a] The predicted change in ADG (g/day) if the corresponding predictor is increased by one unit (ie. the model predicts an increase of 0.068 g/day during the early nursery phase for every 1 g increase in birth weight).
[b] Referent: 100 mg of ZnO/kg ration

Table 2.4. Methicillin-resistant *Staphylococcus aureus* isolates from suckling pigs (14 d), sows (14 d), and nursery pigs (35 d).

	MRSA Isolates (%)			
Characteristic	Suckling Pigs (n=6)	Sows (n=8)	Control Nursery Pigs (n=12)	Treated Nursery Pigs (n=26)
czrC	6 (100)	8 (100)	12 (100)	26 (100)
$ZnCl_2$ MIC (mM)				
0.25	–	–	–	–
0.5	–	–	–	–
1.0	–	–	–	–
2.0	–	–	–	–
4.0	–	–	–	–
8.0	6 (100)	8 (100)	12 (100)	26 (100)
16.0	–	–	–	–
Spa type				
t034	6 (100)	4 (50)	8 (66.7)	10 (38.5)
t3075	0 (0)	4 (50)	4 (33.3)	16 (61.5)

MRSA − methicillin-resistant *Staphylococcus aureus*

2.8 – Figures

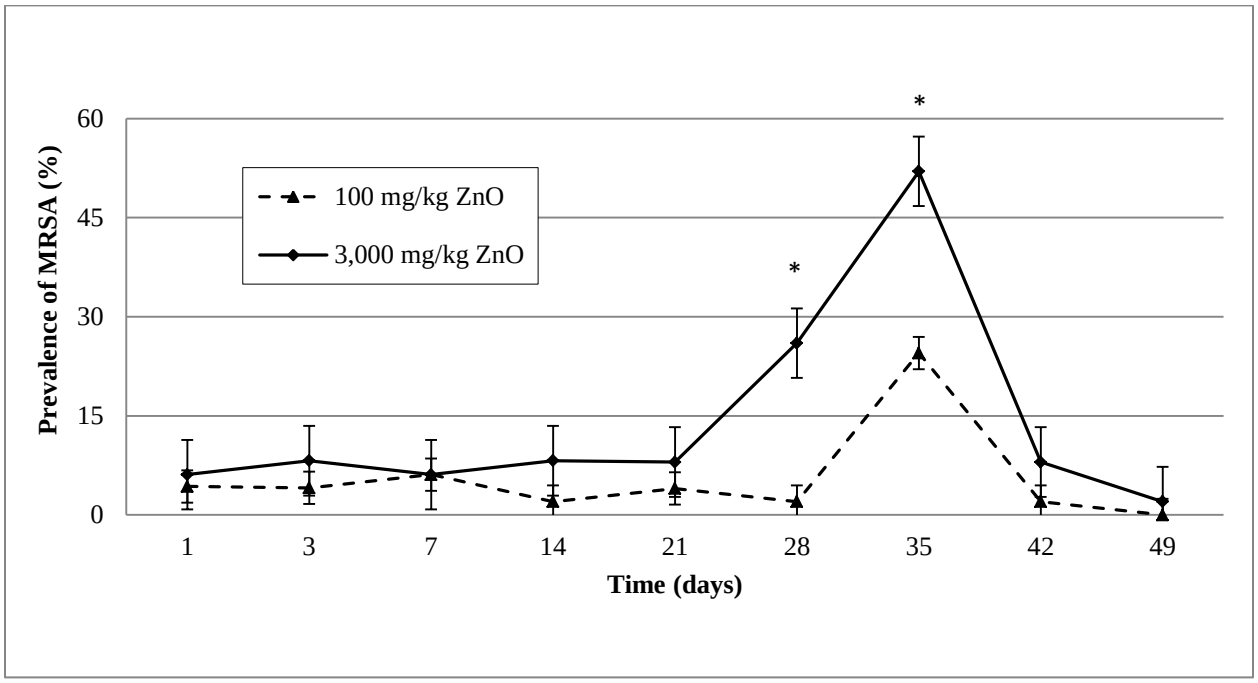


Figure 2.1. Prevalence of methicillin-resistant *Staphylococcus aureus* in control and treated pigs from birth to 4-weeks post-weaning. Exposure to starter ration containing zinc oxide began just after sampling on day 21.
* Statisically significant difference (P<0.05) between the two groups.

CHAPTER 3: Epidemiological investigation of farm-level parameters associated with nasal carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) in nursery pigs: a multi-farm cohort study

This chapter was published as: Slifierz MJ, Friendship RM, Weese JS. Methicillin-resistant *Staphylococcus aureus* in commercial swine herds is associated with disinfectant and zinc usage. Appl Environ Microbiol. 2015 Apr;81(8):2690-5.

3.1 – Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) originating from swine is concerning for public health but an understanding of the emergence and persistence of MRSA in nursery herds is lacking. The aim of this study was to determine whether MRSA in nursery pigs is associated with particular herd-level parameters, including the use of antimicrobials, disinfectants, and heavy metals, which may be driving the selection and persistence of antimicrobial-resistance. Nasal cultures for MRSA were completed for 390 pigs from 26 farms at the end of the suckling phase and again at 3-weeks post-weaning. Herd-level information was collected and a random subset of MRSA isolates were screened for resistance to zinc and quaternary ammonium compounds (QACs). Multivariate analysis revealed that in-feed concentrations of zinc ($P<0.001$) and frequent disinfection of nursery pens ($P<0.001$) are associated with MRSA shedding in nursery pigs. Furthermore, 62.5% (25/40) of MRSA isolates carried the zinc-resistance gene *czrC* and demonstrated decreased susceptibility to zinc. All MRSA isolates carried at least 1 QAC-resistance gene. The most common genotype was *qacG-qacH-smr* which occurred in 32.5% (13/40) of isolates. Seven isolates (17.5%) demonstrated a significant tolerance to benzalkonium chloride, indicating a potential to survive commercial QAC exposure in

the presence of organic matter. Overall, these findings indicate that high levels of in-feed zinc and QAC-based disinfectants may be important drivers in the selection and persistence of MRSA in commercial swine herds and these agents may potentially be co-selecting for other antimicrobial-resistance genes.

3.2 – Background

Staphylococcus aureus is one of the leading causes of opportunistic infections in humans. Those strains with multi-resistant phenotypes, particularly methicillin-resistant *Staphylococcus aureus* (MRSA), are of concern due to the risk of treatment failure, increased hospitalization, and increased use of medical resources (Engemann et al., 2003). Hence, it is understandable that since the first report of MRSA in pigs and the implication of pigs as a source of human infections (Voss et al., 2005), there has been escalating concern about the use of antimicrobials in livestock production systems and the potential public health risk of MRSA originating from animals.

However, previous investigations of the association between antimicrobial usage and the presence of MRSA in swine production systems have yielded conflicting results. Several studies have documented the commonness and long-term persistence of MRSA in pigs raised without exposure to antimicrobials (Weese et al., 2011; Osadebe et al., 2013; Park et al., 2013) and in organic husbandry (Vijver et al., 2013; Buntenkoetter et al., 2014). Furthermore, a Dutch study of 202 pig herds and a German study of 291 pig herds were unable to find an association between MRSA and antimicrobial usage (Alt et al., 2011; Broens et al., 2011), and a recent meta-analysis of risk factors for MRSA in grower-finisher herds determined no difference between organic and conventional herds, but did report group treatment with antimicrobials as a risk factor (Fromm et al., 2014).

Regional differences in MRSA carriage by pigs are also paradoxical; Denmark and the Netherlands, which have legislated restraints for antimicrobial use in livestock production, report a considerably higher prevalence of MRSA among pigs than the Midwestern United States (Broens et al., 2011; Agersø et al., 2012; Smith et al., 2013). In addition, further research has demonstrated no difference in the presence of MRSA in conventional and antibiotic-free pork products (O'Brien et al., 2012).

The lack of a clear association between antimicrobial usage and MRSA in swine production indicates that there may be additional factors which may play a synergistic or independent role in the selection and perpetuation of antimicrobial-resistance in staphylococci. Some recent evidence suggests that the use of disinfectants and zinc may be potential risk factors for MRSA as these compounds are associated with the co-selection of resistance genes (Whitehead et al., 2011; Cavaco et al., 2011). However, these factors have not been thoroughly investigated in nursery pig herds despite the relatively high prevalence of MRSA and commonness of antimicrobial use during this phase of production. Therefore, the objective of the present study was to investigate risk factors for MRSA shedding in pigs in commercial nursery herds with a particular focus on antimicrobials, heavy metals, disinfectants, biosecurity, and management practices.

3.3 – Methods

The use of animals in this study was approved by the Animal Care Committee at the University of Guelph. Twenty-two cohorts of pigs (n=390) were followed from farrowing to 3-weeks post-weaning on 26 participating farms (4 farrow-to-wean, 4 wean-to-finish, and 18 farrow-to-finish) located throughout 10 counties in southern Ontario (November 2013 – October 2014). A variety of different farm sizes and management

types were enrolled in the study from a sampling frame of southern Ontario swine farms that have associations with the University, although the composition of this sampling frame does not represent a probability selection of all southern Ontario swine herds. At each farm, 1-3 pigs were selected from each available litter (maximum of 20 pigs enrolled per farm) and assigned a unique identifier. There were no selection criteria for either the commercial farms or the pigs.

A survey of production parameters, biosecurity, and herd health was completed for each farm. The investigator completed an additional observational survey of the biosecurity and management practices on the farm. Documentation of antimicrobial usage and diet were also collected for each nursery herd. Lastly, a nasal swab of both nares was collected from each pig just prior to weaning and once again at 3-weeks post-weaning. The samples were transported and stored at 4°C before being processed on the same day.

Methicillin-resistant *S. aureus* was detected using a previously described enrichment protocol (Slifierz et al., 2015). Briefly, nasal swabs were inoculated into an enrichment broth (10 g/L tryptone, 75 g/L NaCl, 10 g/L mannitol, 2.5 g/L yeast extract) and incubated at 35°C overnight. The enrichment was then inoculated onto MRSA Chromogenic agar (BBL CHROMagar MRSA, Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 35°C for 48 h. Suspect colonies were confirmed to be MRSA by coagulase test, *S. aureus* latex agglutination assay (Pastorex Staph-plus; Bio-Rad, Marnes-la-Coquette, France), and a penicillin binding protein 2a latex agglutination assay (MRSA latex agglutination test; Oxoid Ltd., Hants, UK). One isolate from each MRSA-positive pig was stored for further analysis.

Five isolates of MRSA were randomly chosen from each MRSA-positive nursery herd for further characterization. Extraction of DNA was completed with InstaGene Matrix (Bio-Rad Laboratories) in accordance with the instructions of the manufacturer. Multiplex PCR was used for detection of *mecA* and *mecC* (Stegger et al., 2012), and PCR detection of Panton-Valentine Leukocidin (PVL) genes and *scn* was completed as previously described (Roberts et al., 2005; van Wamel et al., 2006). The staphylococcal protein A gene (*spa*) was also amplified and sequenced (Shopsin et al., 1999), and isolates were assigned a *spa* type from the Ridom SpaSever database (http://www.spaserver.ridom.de).

Susceptibility to zinc was determined by agar dilution, with inoculation of a McFarland 0.5 suspension of each isolate on Muller-Hinton II agar plates containing $ZnCl_2$ (0.25 – 16 mM) (Aarestrup et al., 2004). Plates were read after 24 h incubation at 35°C and the minimum inhibitory concentration (MIC) of $ZnCl_2$ was recorded. Isolates with an MIC >2 mM were considered zinc-resistant (Cavaco et al., 2011). Identification of the zinc-resistance gene *czrC* was completed by PCR amplification (Cavaco et al., 2010).

A selection of genes responsible for tolerance to quaternary ammonium compounds (QACs) (*qacAB, qacG, qacH, qacJ, smr*) were evaluated using a real-time PCR protocol (Prag et al., 2014). The amplification and melting curves were assessed for agreement with controls and the products were confirmed by sequencing. Phenotypic susceptibility to QACs was completed using the protocol developed by Sundheim et al. (Sundheim et al., 1992). Bacteria were suspended (final concentration of 10^5 cells/ml) in microdilutions of Muller-Hinton broth containing benzalkonium chloride (0.5 – 12.0

μg/ml). The microtitre plates were incubated for 48 h and read on a spectrophotometer using a 630 nm filter. The minimum inhibitory concentration (MIC) was recorded as the concentration needed to completely prevent regrowth. Two technical replicates were performed for this protocol.

The group-level nursery data were initially analyzed using univariate methods. Fisher's exact test was used for dichotomous predictors of MRSA status and Wilcoxon rank sum test was used for continuous predictors of MRSA status. Further individual-based analysis was performed to investigate the associations observed in the group-level data. A multivariate random-effect logistic regression model was constructed using individual data from 311 pigs that were not shedding MRSA prior to weaning. The model was built manually and *a priori* decisions were made to assess confounding as a >30% alteration in the coefficient and to assess two-way interactions between variables demonstrating a tendency towards significance ($P<0.10$). Additional descriptive univariate statistics were used to validate the presence or lack of confounding variables. The co-linearity of variables was also assessed. Variables were only retained in the final model if they were confounders, were part of an interaction term, or demonstrated statistical significance. Standardized residuals and the best linear unbiased predictions of the random effect were used to assess the fit of the model, and the final model was only accepted if the assumptions of normality and homoscedasticity were met. Statistical analysis was completed using STATA 10.0 I/C and the null hypothesis was rejected at $P<0.05$ for all statistical tests used in this study.

3.4 – Results

The swine herds participating in the study had an average of 524 sows (SD=440, Range: 25–1,500) and weaned an average of 951 pigs per month (SD=758, Range: 30−2,600). Ten (45.4%) of the 22 nursery herds identified as operating as continuous flow while the remaining 12 nursery herds identified as being all-in/all-out operations. Two of the farrow-to-finish herds raise pigs without exposure to antimicrobials and receive a premium at slaughter.

The prevalence of MRSA among suckling pigs was 24.1% (94/390) and MRSA was detected in 27.3% (6/22) of the cohorts at the end of the suckling phase. At 3-weeks post-weaning, 23.3% (90/387) of pigs carried MRSA and 36.4% (8/22) of the cohorts were MRSA-positive (Table 3.1). All cohorts testing positive prior to weaning were also positive at 3-weeks post-weaning. The swine operations testing positive for MRSA (2 farrow-to-wean, 2 wean-to-finish, and 6 farrow-to-finish sites) were located in 7 of the 10 counties visited in southern Ontario.

Univariate analysis of risk factors associated with MRSA carriage in nursery herds is presented in Table 3.2. Nursery herds testing positive for MRSA reported more frequent use of zinc therapy and disinfectants, as well as having a higher stocking density. Interestingly, the presence of companion animals (dogs, cats) on the farm was negatively associated with MRSA status. The presence of MRSA was not associated with any particular antimicrobial therapy, the number of antimicrobials used, or the route of administration in the nursery herds.

After analyzing the data with a multivariate random-effect logistic regression model, only two variables remained significantly associated with MRSA in nursery

herds: in-feed zinc concentration (mg zinc per kg feed; OR=1.000915, 95% CI: 1.000405 − 1.001425, *P*<0.001) and disinfection of the nursery for each new group of incoming pigs (OR=14.12, 95% CI: 4.36 − 45.77, *P*<0.001). Based on the predictions of this model, the odds of MRSA carriage in pigs consuming a ration containing 3,000 mg zinc/kg feed was 12.4 times greater than the odds of MRSA carriage in pigs consuming a ration containing 250 mg zinc/kg feed (95% CI: 3.04 − 50.25, *P*<0.001) which was the lowest in-feed zinc concentration observed in this study. The average concentration of zinc used in nursery feed was 2,284 mg zinc/kg feed (range: 250 – 7,000 mg zinc/kg feed) and 15 nursery herds (68.2%) used a therapeutic concentration (2,000 – 3,000 mg zinc/kg feed) (Table 1). There were no significant two-way interactions terms or confounding variables detected in the final multivariable model.

Twenty-five (62.5%) of the 40 isolates of MRSA, originating from 6 (75%) of the 8 MRSA-positive nursery herds, carried the *czrC* gene, but 90% (36/40) of isolates were phenotypically resistant to zinc chloride (MIC >2 mM) (see Table 3.3). Two nursery herds carried both *czrC*+ and *czrC*− genotypes of MRSA. The minimum inhibitory concentrations towards $ZnCl_2$ are presented in Table 2.3 for *czrC*+ MRSA and *czrC*− MRSA isolates.

Each MRSA isolate harboured at least 1 resistance gene for QACs and 30 (75%) of the 40 isolates harboured 2 or more QAC-resistance genes (see Table 3.4). The most common genotype was *qacG-qacH-smr* which was detected in 32.5% (13/40) of the tested isolates which originated from 6 (75%) of the 8 MRSA-positive nursery herds. One MRSA isolate (*spa* type t571) carried *qacAB* along with the *qacG*, *qacH*, and *smr* genes. The *qacJ* gene was not detected in any of the tested isolates. In addition, there were 7

isolates (17.5%) from 4 farms with a MIC ≥4.5 μg/ml, indicating a potential to survive exposure to commercial QAC preparations in the presence of organic matter. A summary of the QAC susceptibility profiles is presented in detail in Table 2.4.

The MRSA isolates tested in this study primarily belonged to the clonal complex (CC) 398-associated *spa* types t034 (57.5%) and t571 (37.5%), along with single isolates of t3075 (CC398-associated) and t002 (CC5-associated). The *spa* types t034 and t571 were from 5 (22.7%) and 3 (13.6%) nursery herds, respectively. The *spa* types t002 and t3075 were from two different farms, each co-existing with *spa* type t034. All of the tested MRSA isolates were negative for *mecC*, *scn* and PVL. The molecular characteristics of each *spa* type are summarized in Table 3.5. Lastly, it was observed that isolates belonging to *spa* type t034 were more likely to carry *czrC* ($P<0.001$) and demonstrated an increased tolerance to zinc ($P<0.001$) when compared to isolates of *spa* type t571.

3.5 – Discussion

The finding that MRSA in nursery pigs is associated with the concentration of zinc in the nursery ration and the frequent use of disinfectants to clean the nursery, in addition to the commonness of resistance to zinc and QACs among MRSA isolates, indicates that these compounds may be important drivers in the selection and persistence of MRSA in swine production systems. Resistance genes for zinc and QACs have been found to co-locate with antibiotic-resistance genes in mobile genetic elements in the chromosomal genome and on plasmids (Sidhu et al., 2001; Cavaco et al., 2011), forming a biological basis to the observed statistical association in this investigation. This is concerning as exposure to these compounds may cause co-selection or co-retention of

antibiotic resistance genes in environments with low or no antibiotic exposure, as has been demonstrated in laboratory experiments (Braoudaki et al., 2004; Whitehead et al., 2011; Moodley et al., 2011; Slifierz et al., 2015).

The association between frequent disinfection of the nursery and MRSA carriage in nursery pigs is consistent with the findings of a meta-analysis which also determined that regular disinfection of fattening pig holdings was associated with MRSA carriage (Fromm et al., 2014). The use of therapeutic levels of in-feed zinc has also been shown to affect the shedding of MRSA in nursery pigs in previous controlled experiments (Moodley et al., 2011; Slifierz et al., 2015). The evidence indicates that therapeutic levels of zinc and routine use of QAC-based disinfectants may be exerting a selective pressure on MRSA in commercial nursery herds. The means by which such a selective advantage leads to within-herd changes in the persistence of MRSA is still unclear, but it may be due to a combination of increased survival in the environment and increased host susceptibility to colonization.

Supplementation of the diets of pigs in the post-weaning period with high levels of zinc is a common and an increasingly used approach to prevent post-weaning diarrhea (most often associated with *E. coli*). Increased public concern about the use of antimicrobials in pork production and the premiums paid for antibiotic-free pork are presumably important driving factors. This study demonstrates that the use of rations containing therapeutic levels of zinc is commonplace in Ontario nursery herds and highlights the complexity of antimicrobial resistance. These data cannot be taken as indicating that movement towards antibiotic-free pork production is harmful; however, they indicate that replacing traditional antimicrobials with compounds that are not

considered to be classical anti-infective drugs (yet are being used for their antimicrobial ability) may not be an effective means of reducing antimicrobial resistance pressure.

Interestingly, all qualitative questions relating to the reason for using therapeutic levels of zinc were left unanswered by the producers (data not shown). This suggests that producers may have a minimal role in setting the in-feed zinc dosage and further studies should investigate the role of the herd veterinarian and nutritionist with respect to setting the level of in-feed zinc as it is essential that these findings be communicated to the appropriate target group. Furthermore, it was observed that a single production system was using a nursery ration containing 7,000 mg zinc/kg feed which is surprising and indicates that there may be an opportunity to better educate the industry on appropriate use of zinc in swine feed.

Quaternary ammonium compounds are routinely used on swine farms and in food processing facilities as disinfectants primarily due to the bactericidal activity of these compounds in the presence of organic burdens and the low cost (Amass et al., 2000). It has been demonstrated that MRSA isolates with a benzalkonium chloride MIC ≥4.5 μg/ml, as exhibited by 17.5% of isolates in the current study, are able to survive exposure to commercial QAC-based disinfectants in the presence of organic matter (Sundheim et al., 2004). Hence, the finding that QAC-resistance genes are common among MRSA of porcine origin is concerning and may provide explanations for the observed association between frequent disinfection and MRSA in the present study and the reported ineffectiveness of QAC-based disinfectants for elimination of MRSA from swine holdings (Merialdi et al., 2013). Additionally, genes conferring resistance to QACs,

particularly *qacG* and *smr*, have also been identified in MRSA ST9 originating from pig carcasses in Hong Kong (Wong et al., 2013).

Herd size is a documented risk factor in several studies (Alts et al., 2011; Broens et al., 2011; Fromm et al., 2014), but in this study it was not significantly associated with MRSA status; however, it did approach significance. This is potentially due to limited sample size and the decision not to categorize herd size during analysis, contrary to the approach used in other studies (Alts et al., 2011; Broens et al., 2011; Fromm et al., 2014). Stocking density was associated with MRSA carriage in nursery pigs according to univariate statistics and, although not statistically significant in the final model, it should be investigated further as the rate of contact between pigs can play an important role in MRSA transmission (Broens et al., 2012). Additionally, the inverse association between cohort-level MRSA status and the presence of companion animals is similar to reports of multi-species farms acting as a protective factor (Fromm et al., 2014), although the reason for this relationship is still unclear.

The lack of an association between MRSA and various measures of antibiotic usage is consistent with previous reports (Alt et al., 2011; Broens et al., 2011). One explanation for this lack of association may be due to the use of therapeutic concentrations of in-feed zinc as a replacement for antibiotic therapy. As observed in this study, MRSA-positive cohorts appeared to use fewer antibiotics and higher levels of in-feed zinc than the MRSA-negative cohorts. However, although no confounding effect was detected in the present data set, future studies should consider the possibility for zinc to act as a confounder that can influence the association between antibiotic usage and MRSA status of swine herds. It is also worth mentioning that high fitness and superior

host adaptation may contribute to long-term persistent of MRSA in the absence of antimicrobials (Crombé et al., 2012).

One of the limitations of the present study is the limited number of cohorts that were enrolled. This sample size did reduce statistical power when detecting differences at the cohort-level and future studies should investigate these relationships using a larger number of swine herds. Another limitation was a potential for bias to be introduced into the survey results due to producers providing answers that would please the surveyor; however, most of the data were validated by documentation and investigator-completed surveys. Lastly, comparing the measurements of the current study to more comprehensive regional data indicates that the cohorts enrolled in this study were representative of the Ontario swine industry.

Overall, resistance determinants for zinc and QACs are widespread among MRSA of porcine origin and, when paired with the epidemiological evidence, it supports the hypothesis that these compounds may be playing an important role in the selection and persistence of MRSA in commercial swine herds. These findings illustrate the multifactorial nature of antimicrobial-resistance among staphylococci and these results may be useful for devising control measures to reduce antimicrobial-resistance in staphylococci on swine farms. However, this issue may be region-specific as resistance to zinc can vary substantially by *spa* type and the dominant *spa* type of pig holdings also differs by region. Therefore, determination of resistance patterns to zinc and QACs in additional MRSA *spa* types from different regions is warranted.

3.6 – References

Aarestrup, FM, Hasman, H. Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride and antimicrobial substances used for disinfection. Vet. Microbiol. 2004;100:83-89.

Agersø, Y, Hasman, H, Cavaco, LM, Pedersen, K, Aarestrup, FM. Study of methicillin resistant *Staphylococcus aureus* (MRSA) in Danish pigs at slaughter and in imported retail meat reveals a novel MRSA type in slaughter pigs. Vet. Microbiol. 2012;157:246-250.

Alt, K, Fetsch, A, Schroeter, A, Guerra, B, Hammerl, JA, Hertwig, S, Senkov, N, Geinets, A, Mueller-Graf, C, Braeunig, J, Kaesbohrer, A, Appel, B, Hensel, A, Tenhagen, BA. Factors associated with the occurrence of MRSA CC398 in herds of fattening pigs in Germany. BMC Vet. Res. 2011;7:69.

Amass, SF, Vyverberg, B, Ragland, D, Dowell, CA, Anderson CD, Stover, JH, Beaudry, DJ. Evaluating the efficacy of boot baths in biosecurity protocols. J. Swine Health Prod. 2000;8:169-173.

Braoudaki, M, Hilton, AC. Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli* O157 and cross-resistance to antimicrobial agents. J. Clin. Microbiol. 2004;42:73-78.

Broens, E, Graat, E, Van Der Wolf, P, Van De Giessen, A, De Jong, M. Prevalence and risk factor analysis of livestock associated MRSA-positive pig herds in The Netherlands. Prev. Vet. Med. 2011;102:41-49.

Broens, EM, Espinosa-Gongora, C, Graat, EA, Vendrig, N, Van Der Wolf, PJ, Guardabassi, L, Butaye, P, Nielsen, JP, De Jong, MC, Van De Giessen, AW. Longitudinal study on transmission of MRSA CC398 within pig herds. BMC Vet. Res. 2012;8:58.

Buntenkoetter, V, Blaha, T, Tegeler, R, Fetsch, A, Hartmann, M, Kreienbrock, L, Meemken, D. Comparison of the phenotypic antimicrobial resistances and *spa*-types of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates derived from pigs in conventional and in organic husbandry systems. Berl. Munch. Tierarztl. Wochenschr. 2014;127:135-143.

Cavaco, LM, Hasman, H, Aarestrup, FM. Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance. Vet. Microbiol. 2011;150:344-348.

Cavaco, LM, Hasman, H, Stegger, M, Andersen, PS, Skov, R, Fluit, AC, Ito, T, Aarestrup, FM. Cloning and occurrence of *czrC*, a gene conferring cadmium and zinc resistance in methicillin-resistant *Staphylococcus aureus* CC398 isolates. Antimicrob. Agents Chemother. 2010;54:3605-3608.

Crombé, F, Vanderhaeghen, W, Dewulf, J, Hermans, K, Haesebrouck, F, Butaye, P. Colonization and transmission of methicillin-resistant *Staphylococcus aureus* ST398 in nursery piglets. Appl. Environ. Microbiol. 2012;78:1631-1634.

Engemann, JJ, Carmeli, Y, Cosgrove, SE, Fowler, VG, Bronstein, MZ, Trivette, SL, Briggs, JP, Sexton, DJ, Kaye, KS. Adverse clinical and economic outcomes attributable to methicillin resistance among patients with *Staphylococcus aureus* surgical site infection. Clin. Infect. Dis. 2003;36:592-598.

Fromm, S, Beißwanger, E, Käsbohrer, A, Tenhagen, B. Risk factors for MRSA in fattening pig herds–A meta-analysis using pooled data. Prev. Vet. Med. 2014;117:180-188.

Merialdi, G, Galletti, E, Guazzetti, S, Rosignoli, C, Alborali, G, Battisti, A, Franco, A, Bonilauri, P, Rugna, G, Martelli, P. Environmental methicillin-resistant *Staphylococcus aureus* contamination in pig herds in relation to the productive phase and application of cleaning and disinfection. Res. Vet. Sci. 2013;94:425-427.

Moodley, A, Nielsen, SS, Guardabassi, L. Effects of tetracycline and zinc on selection of methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type 398 in pigs. Vet. Microbiol. 2011;152:420-423.

O'Brien, AM, Hanson, BM, Farina, SA, Wu, JY, Simmering, JE, Wardyn, SE, Forshey, BM, Kulick, ME, Wallinga, DB, Smith, TC. MRSA in conventional and alternative retail pork products. PLoS One.2012;7:e30092.

Osadebe, L, Hanson, B, Smith, T, Heimer, R. Prevalence and characteristics of *Staphylococcus aureus* in Connecticut swine and swine farmers. Zoonoses Public Health. 2013;60:234-243.

Park, J, Friendship, RM, Poljak, Z, Weese, JS, Dewey, CE. An investigation of exudative epidermitis (greasy pig disease) and antimicrobial resistance patterns of *Staphylococcus hyicus* and *Staphylococcus aureus* isolated from clinical cases. Can. Vet. J. 2013;54:139.

Prag, G, Falk-Brynhildsen, K, Jacobsson, S, Hellmark, B, Unemo, M, Söderquist, B. Decreased susceptibility to chlorhexidine and prevalence of disinfectant resistance genes among clinical isolates of *Staphylococcus epidermidis*. APMIS. 2014;122:961-967.

Roberts, S, O'Shea, K, Morris, D, Robb, A, Morrison, D, Rankin, S. A real-time PCR assay to detect the Panton Valentine Leukocidin toxin in staphylococci: screening *Staphylococcus schleiferi* subspecies *coagulans* strains from companion animals. Vet. Microbiol. 2005;107:139-144.

Shopsin, B, Gomez, M, Montgomery, SO, Smith, DH, Waddington, M, Dodge, DE, Bost, DA, Riehman, M, Naidich, S, Kreiswirth, BN. Evaluation of protein A gene

polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. 1999;37:3556-3563.

Sidhu, MS, Heir, E, Sørum, H, Holck, A. Genetic linkage between resistance to quaternary ammonium compounds and β-lactam antibiotics in food-related *Staphylococcus* spp. Microb. Drug Resist. 2001;7:363-371.

Slifierz, M, Friendship, R, Weese, J. Zinc oxide therapy increases prevalence and persistence of methicillin-resistant *Staphylococcus aureus* in pigs: a randomized controlled trial. Zoonoses Public Health. 2015;62(4):301-8.

Smith, TC, Gebreyes, WA, Abley, MJ, Harper, AL, Forshey, BM, Male, MJ, Martin, HW, Molla, BZ, Sreevatsan, S, Thakur, S. Methicillin-resistant *Staphylococcus aureus* in pigs and farm workers on conventional and antibiotic-free swine farms in the USA. PloS One. 2013;8:e63704.

Stegger, M, Andersen, P, Kearns, A, Pichon, B, Holmes, M, Edwards, G, Laurent, F, Teale, C, Skov, R, Larsen, A. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mecA* or the new *mecA* homologue *mec*ALGA251. Clin. Microbiol. Infect. 2012;18:395-400.

Sundheim, G, Hagtvedt, T, Dainty, R. Resistance of meat associated staphylococci to a quarternary ammonium compound. Food Microbiol. 1992;9:161-167.

van Wamel, WJ, Rooijakkers, SH, Ruyken, M, van Kessel, KP, van Strijp, JA. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting bacteriophages. J. Bacteriol. 2006;188:1310-1315.

Vijver, L, Tulinski, P, Bondt, N, Mevius, D, Verwer, C. Prevalence and molecular characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) in organic pig herds in the Netherlands. Zoonoses Public Health. 2013;61:338-345.

Voss, A, Loeffen, F, Bakker, J, Klaassen, C, Wulf, M. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerging Infect. Dis. 2005;11:1965-1966.

Weese, J, Zwambag, A, Rosendal, T, Reid-Smith, R, Friendship, R. 2011. Longitudinal investigation of methicillin-resistant *Staphylococcus aureus* in piglets. Zoonoses Public Health. 2011;58:238-243.

Whitehead, RN, Overton, TW, Kemp, CL, Webber, MA. Exposure of *Salmonella enterica* serovar Typhimurium to high level biocide challenge can select multidrug resistant mutants in a single step. PloS One.2011;6:e22833.

Wong, T, Zhang, M, O'Donoghue, M, Boost, M. Presence of antiseptic resistance genes in porcine methicillin-resistant *Staphylococcus aureus*. Vet. Microbiol. 2013;162:977-979.

3.7 – Tables

Table 3.1. Observed concentration of zinc in nursery rations and methicillin-resistant *Staphylococcus aureus* carriage in 22 swine cohorts.

In-feed zinc concentration (mg zinc[a] / kg feed)	**Number of cohorts (% of cohorts)**	**MRSA-positive cohorts (% of cohorts)**
<500	1 (4.5)	0
500 – 999	5 (22.7)	0
1,000 – 1,499	0	0
1,500 – 1,999	0	0
2,000 – 2,499	2 (9.1)	1 (4.5)
2,500 – 3,000	13 (59.1)	7 (31.8)
>3,000	1 (4.5)	0

a – all zinc was supplemented in the ration as zinc oxide.

Table 3.2. Factors associated with methicillin-resistant *S. aureus* in nursery herds.

Parameters	**MRSA positive cohorts (n=8)**	**MRSA negative cohorts (n=14)**	***P*-value[a]**
Herd size (sows, SD)	698 (433)	425 (427)	0.076
Nursery was off-site (%)	25	14.3	0.602
Continuous nursery flow (%)	25	57.1	0.204
Average weaning age (days, SD)	22.4 (2.6)	24.5 (4.8)	0.351
Pigs weaned per month (pigs/month, SD)	1224 (696)	795 (711)	0.088
Nursery stocking density (pigs/m^2, SD)	3.22 (1.23)	2.47 (1.17)	0.048
Direct pig-to-pig contact between pens (%)	50	42.9	0.999
Exposure to wooden surfaces (%)	25	21.4	0.999
Temperature upon entrance to nursery (°C, SD)	27.6 (1.23)	26.7 (2.79)	0.273
No outside breeding stock replacements (%)	62.5	71.4	0.999
Danish entry (%)	37.5	42.9	0.999
Bootbath (%)	0	28.6	0.254
Shower-in/shower-out required (%)	62.5	28.6	0.187
Danish entry or bootbath or shower (%)	75	71.4	0.999
Older pigs sometimes mixed with new pigs coming into the nursery (%)	25	35.7	0.999
Nursery pens disinfected for incoming pigs every time (%)	100	50	0.022
Corridors are disinfected on a weekly to monthly basis (%)	87.5	42.9	0.074
Cat(s) and/or Dog(s) on the property (%)	37.5	92.9	0.011
Pets allowed into the barn (%)	0	71.4	0.115
Live rodents observed in barn at sampling (%)	12.5	7.1	0.999
Wild birds observed in barn in past year (%)	12.5	42.9	0.193
Antibiotics administered by feed (%)	87.5	78.6	0.999
Antibiotics administered by water (%)	37.5	42.9	0.999
Antibiotics administered by injection (%)	87.5	71.4	0.613
Tetracycline[b] (in-feed) (%)	62.5	85.7	0.309
Tiamulin[b] (in-feed) (%)	25	42.9	0.649
Penicillin[b] (in-feed/injection) (%)	50	50	0.999
Sulfamethazine[b] (in-feed/in-water) (%)	25	21.4	0.999
Zinc therapy[b] (≥2,000 ppm in-feed) (%)	100	50	0.022
Number of different antibiotics administered at the group-level (n, SD)	1.88 (1.13)	2.43 (1.09)	0.345

a – Univariate statistics (Fisher's exact or Wilcoxon rank sum).

b – group-level exposure to antimicrobial between birth and 3-weeks post-weaning.

Table 3.3. Minimum inhibitory concentration of zinc chloride ($ZnCl_2$) among methicillin-resistant *Staphylococcus aureus* isolates from nursery pigs.

MRSA genotype	**No. of isolates**	**No. of farms**	**No. of isolates with $ZnCl_2$ MIC (mM) of:**								
			0.25	0.5	1.0	2.0	4.0	6.0	8.0	12.0	16.0
czrC+	25	6						6	13	3	3
czrC−	15	4				4	11				

Table 3.4. Minimum inhibitory concentration (MIC) of benzalkonium chloride (BKC) among methicillin-resistant *Staphylococcus aureus* isolates from nursery pigs.

MRSA genotype	No. of isolates	No. of farms	No. of *spa* types				No. of isolates with BKC MIC (µg/ml) of:									
			t034	t571	t3075	t002	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5
qacG	6	3	6						2			1	2	1		
qacH	3	1		3					3							
smr	1	1		1							1					
qacG+qacH	6	4	4	1		1			2				1	1	2	
qacG+smr	10	5	6	3	1				5			1	3		1	
qacG+qacH+smr	13	6	7	6					3		1	3	4	1	1	
qacG+qacH+smr+qacAB	1	1		1									1			

Table 3.5. Distribution of zinc and quaternary ammonium compound (QAC) resistance by methicillin-resistant *Staphylococcus aureus spa* type.

Resistance gene/phenotype	**No. of isolates (%)**				
	t034 (n=23)	t571 (n=15)	t002 (n=1)	t3075 (n=1)	Total (n=40)
qacAB	0 (0)	1 (6.7)	0	0	1 (2.5)
qacG	23 (100)	11 (73.3)	1 (100)	1 (100)	36 (90)
qacH	11 (47.8)	11 (73.3)	1 (100)	0	23 (57.5)
qacJ	0	0	0	0	0
smr	13 (56.5)	11 (73.3)	0	1 (100)	25 (62.5)
≥2 QAC-resistance genes	17 (73.9)	11 (73.3)	1 (100)	1 (100)	30 (75)
Mean MIC of BKC (μg/ml)	3.6	3.0	2	2	3.3
czrC	23 (100)*	1 (6.7)*	0	1 (100)	25 (62.5)
Mean MIC of $ZnCl_2$ (mM)	8.78*	3.73*	2	16	6.9

* Significant difference between *spa* types (P<0.05).

CHAPTER 4: Characterization of the developing fecal and nasal microbiotas of young pigs using high-throughput next-generation sequencing

This chapter was published as: Slifierz MJ, Friendship RM, Weese JS. Longitudinal study of the early-life fecal and nasal microbiotas of the domestic pig. BMC Microbiol. 2015 Sep 21;15(1):184.

4.1 – Abstract

The mammalian microbiota plays a key role in host health and disease susceptibility. However, knowledge of the early-age microbiota of pigs is lacking. The purpose of this study was to use high-throughput next-generation sequencing to characterize the fecal and nasal microbiotas of pigs during early life. Ten commercially-raised pigs were randomly enrolled at birth and sampled throughout the first 7 weeks of life. DNA was extracted from fecal and nasal samples and the hypervariable region V4 of the 16S rRNA gene was amplified. The product was sequenced using the Illumina MiSeq platform and 2x250 chemistry. Sequencing data were processed and analyzed with the mothur algorithms using an operational taxonomic unit approach. In total, 4.7 million and 5.4 million high-quality sequences were recovered from fecal and nasal samples, respectively. Analysis revealed that these microbiotas contain a very rich and diverse population of bacteria that display a remarkable evolution during the first 7 weeks of life. During this developmental period, a pig was exposed to an average of 1,976 and 6,257 species of bacteria by way of the gastrointestinal and respiratory tracts, respectively. Aging was significantly associated with an increasing measure of richness and diversity as well as with distinct changes to the core microbiota. At 2-3 weeks post-weaning, the

rapidly developing microbiotas appeared to reach a developmental milestone as a relative degree of stability was evident. These findings expand the knowledge of the developing porcine microbiota which is important for understanding susceptibility to disease, particularly for vulnerable neonatal pigs.

4.2 – Background

The microorganisms that populate the body, collectively known as the microbiota, are important drivers of host health and metabolism. Characterization of the porcine microbiota using previous (primarily culture-dependent) techniques provides only a narrow understanding of the complexity of these ecosystems due to methodological limitations (Pryde et al., 1999; Leser et al., 2002). Advancements in next-generation sequencing and bioinformatics have only recently given researchers the opportunity to examine the composition and diversity of these microbial populations in significant detail. Presently, high-throughput sequencing of the hypervariable regions of the 16S rRNA gene can provide a depth of coverage that is far greater than any previous method (Shokralla et al., 2012).

The microbiotas of the gastrointestinal and respiratory tracts of pigs are of particular interest due to the association of these body sites with common swine diseases or pathogens. These microbiotas provide a first line of defense against foreign invaders as competition and interaction between bacteria can protect a host from becoming colonized with particular pathogens (Pettigrew et al., 2008). However, the developing microbiota of young pigs is particularly vulnerable to disruptions which can result in long-term impacts that may affect disease susceptibility and growth performance (Thompson et al., 2008). Such variation in the porcine microbiota has been associated with stress, diet,

management practices, and antimicrobial compounds (Thompson et al., 2008; Isaacson and Kim, 2012; Weese et al., 2014).

Previous research exploring the gut microbiota in adult pigs using next-generation sequencing methods has revealed an incredibly diverse population of bacteria (Kim et al., 2011). The adult porcine gut microbiota has been found to contain at least 7 identifiable bacterial phyla, the predominant being Firmicutes and Bacteroidetes, and at least 171 genera of bacteria (Kim et al., 2011). Similarly, the nasal cavity of adult pigs also harbours an incredibly diverse and rich microbial ecosystem which contains an estimated 1,749 species of bacteria from 124 different genera. The porcine nasal microbiota was found to contain 9 phyla of bacteria, of which Proteobacteria, Firmicutes and Spirochaetes predominated (Weese et al., 2014).

However, despite the emerging understanding of the microbiota of adult pigs, characterization of the development of the early-age microbiota using next-generation techniques is lacking. Further understanding of the fecal and nasal microbiotas can provide significant insight into swine health and susceptibility to disease, especially among young pigs who are particularly vulnerable. Therefore, the objective of this longitudinal investigation was to characterize the transformation of the fecal and nasal microbiotas of conventionally-raised pigs throughout the first 7 weeks of life using high-throughput next-generation sequencing.

4.3 – Methods

The use of animals in this study was approved by the Animal Care Committee at the University of Guelph. Ten conventionally-raised Yorkshire-Landrace pigs (4 males, 6 females) at a 300-sow batch-farrowing facility were enrolled on the day of birth from ten

different litters (1 pig per litter). Each pig in the herd had a unique identifier assigned at birth and the pigs enrolled in the present study were randomly selected (random number generator) from a stratified sampling frame of pigs that weighed ≥1 kg at birth and did not demonstrate clinical signs of disease. Each pig was raised with their mother and litter-mates for 21 days without creep feed and, at weaning, randomized into 4 pens (located in the same nursery room) containing 11-13 pen-mates. Pigs were given a first phase ration for 14 days followed by a second phase ration for the remainder of the nursery period (Supplementary Table 4.1). The rations did not contain antimicrobial compounds and pigs were not administered any antimicrobials during the trial.

Samples were collected on days 1, 3, 7, 14, 21 (prior to weaning), 28, 35, 42, and 49. Samples were collected at the same time of day by the same investigator. At each sampling, a swab of the rectum was collected, as well as whole feces when possible, and a swab of the interior of both nares (approximately 10 mm penetration). Samples were transported at 4°C and stored at -80°C before being processed.

Extraction of DNA from fecal and nasal samples was completed using a commercially prepared kit and following the manufacturer's protocol for stool DNA extraction for pathogen detection (E.Z.N.A. Stool DNA Kit, Omega Bio-Tek Inc., Doraville, Georgia, USA). For nasal samples, the whole tip of the swab was processed through the lysis stage of extraction.

The V4 region of the 16S rRNA gene was amplified using forward (5′-AYTGGGYDTAAAGNG-3′) and reverse (5′-TACNVGGGTATCTAATCC-3′) primers previously designed (Caporaso et al., 2010). The 16S primers contained adapter regions (Forward: TCGTCGGCAGCGTCAGATGTGTATAA-GAGACAG, Reverse:

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) for annealing to Illumina universal index sequencing adaptors that were added in a later PCR.

Amplification of the 16S rRNA V4 region was completed in a 25 µl reaction consisting of 12.5 µl of KAPA 2G Fast HotStart ReadyMix 2X (KapaBiosystems), 9.0 µl of molecular-grade water, 2.5 µl template DNA, and 0.5 µl each of both the forward and reverse 16S rRNA V4 primers (10.0 µM). The reaction conditions for PCR were 94°C for 10 min, and 27 cycles of 94°C for 45 s, 53°C for 60 s, and 72°C for 90 s, followed by a final period of 72°C for 10 min. The product was then purified using AMPure X (Beckman Coulter Inc, Mississauga, Ontario, Canada). The AMPure (20 µl) was mixed with the amplicon (25 µl) and incubated at room temperature for 2 min. After applying a magnetic field, the supernatant was discarded and the beads were washed twice with 80% ethanol. The beads were then incubated at room temperature for 10 min before eluting with 50 µl of 10 mM Tris buffer.

Illumina universal adapters (Forward: AATGATACGGCGACCACCGAGATCTACAC-index-TCGTCGGCAGCGTC, Reverse: CAAGCAGAAGACGGCATACGAGAT-index-GTCTCGTGGGCTCGG) were then added to the purified 16S rRNA gene product by PCR using a 25 µl reaction consisting of 12.5 µl KAPA 2G Fast HotStart ReadyMix 2X (KapaBiosystems), 8.0 µl of molecular-grade water, 2.5 µl template DNA, and 1.0 µl each of the forward and reverse sample-specific Illumina universal adapters. The PCR conditions were as follows; a single cycle of 94°C for 3 min, 8 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, followed by a final cycle of 72°C for 10 min. The product was purified with AMPure X (Beckman Coulter Inc, Mississauga, Ontario, Canada) as previously described and DNA

was eluted into 30 µl of 10 mM Tris buffer. The samples were then quantified by spectrophotometry (Nanodrop, Roche, Mississauga, Canada) and normalized to a final concentration of 2 nM. Sequencing of the library pool was performed using an Illumina MiSeq (San Diego, USA) and 2×250 chemistry at the University of Guelph's Advanced Analysis Centre.

Sequencing data were analyzed using the mothur software package v.1.33.0 (Schloss et al., 2009). The paired-end reads were aligned and screened to remove sequences with the following irregularities: contiguous sequence lengths >245 bp or <239 bp, ambiguous base calls, stretches of homopolymers >8 bp, and misalignment with the target region. Sequences were then screened for chimeras using the UCHIME tool (Edgar et al., 2011) and sequences belonging to non-bacterial domains, including chloroplasts, mitochondria, Archaea and Eukaryotes, were removed. Operational taxonomic units (OTUs) were created using a 3.0% dissimilarity threshold and average neighbour algorithm, then assigned taxonomy using the RDP reference database (http://rdp.cme.msu.edu). The observed relative abundance of taxa was analyzed prior to subsampling and the mean relative abundance of the 10 pigs was plotted graphically and in a chart to demonstrate changes in phyla, classes, and genera over time. Random subsampling of sequences from each individual sample was completed to normalize the sequence count. Community diversity (inverse Simpson index) and richness (CatchAll: Bunge et al., 2012) were computed and sampling coverage was assessed by Good's coverage value. The core microbiota (OTUs with >1% relative abundance and shared amongst ≥80% of pigs) was also explored for each sample type at different ages. The Jaccard index of dissimilarity was used as an assessment of community membership and

the Yue and Clayton measure of dissimilarity was used to as an assessment of community structure. These indices were visualized by principle coordinate analysis, and compared between groups using analysis of molecular variance (AMOVA). Probabilistic modelling using Dirichlet multinomial mixtures (DMM) was utilized in examining community types associated with phase of production (Holmes et al., 2012). Additionally, the linear discriminant analysis (LDA) effect size (LEfSe) algorithm was used to identify OTUs associated with pre-weaning and post-weaning microbiotas (Segata et al., 2011) and cladograms were produced using the online LEfSe tool (http://huttenhower.sph.harvard.edu/galaxy/). Non-parametric statistical analysis (Spearman's rank correlation) was performed in STATA 10.0 I/C (Stata Corporation, College Station, TX) to determine age-related differences in taxonomical abundance, diversity, and richness. The null hypothesis for all statistical tests was rejected at $P<0.05$.

4.4 – Results

There were a total of 4,711,191 sequences recovered from 90 fecal samples after removal of erroneous and poor quality reads. The median number of sequences per sample was 47,628 (Range: 9,725 – 100,859). Sequences recovered from the feces of 10 pigs over the 7-week period clustered into 6,714 OTUs. The median number of OTUs recovered per pig during the study period was 1976 (Range: 1,600 – 2,112). The OTUs were classified into 19 bacterial phyla and 489 genera, although only five phyla had >1% overall relative abundance: Firmicutes (70%), Proteobacteria (16%), Bacteroidetes (4.3%), Fusobacteria (1.6%), and Actinobacteria (1.4%). Only 1.4% of sequences were unclassified at the phylum level. However, the relative abundance of bacterial phyla varied considerably with age (Figure 4.1). Specifically, aging was associated with a

greater relative abundance of Firmicutes ($P<0.001$), and lower relative abundance of Proteobacteria ($P<0.001$), Fusobacteria ($P<0.001$), and Actinobacteria ($P<0.001$). The most dominant genera during the pre-weaning phase were Clostridium and Escherichia, while Megasphaera and Lactobacillus dominated the post-weaning phase (Table 4.1). Random subsampling of 9,725 sequences was completed for each fecal sample to normalize sequence numbers. The median sample richness was 665 OTUs (Range: 73 – 1,562), although there was significant age-related variation as richness was positively correlated with age ($\rho=0.79$, $P<0.001$). The median inverse Simpson's diversity index was 19.9 (Range: 1.1 – 72.1) and diversity positively correlated with age ($\rho=0.48$, $P<0.001$). The age-related change in the number of observed and core OTUs is presented in Figure 4.2, while measures of richness and diversity are shown in Figure 4.3.

The community membership (Jaccard measure of dissimilarity) of the fecal microbiota is visually represented in Figure 4.4. There was distinctive clustering of samples according to age group and AMOVA analysis of the Jaccard indices demonstrated significantly differences between all age groups ($P<0.05$). However, while statistically different, by 2-weeks post-weaning there was identifiable overlap between age groups suggesting development of a relatively more stable community membership. Additionally, community structure (Yue and Clayton measure of dissimilarity) is visualized as a dendrogram in Supplementary Figure 4.1. Yue and Clayton indices were significantly different between all age groups ($P<0.05$) with the exception of 35-day-old and 42-day-old pigs ($P=0.293$). There was also strong clustering of samples according to phase of production (pre-weaning and post-weaning) for both community membership ($P<0.001$) and community structure ($P<0.001$).

The core fecal microbiota was notably dichotomized between the pre-weaning and post-weaning phases of production (Table 4.2). Probabilistic modelling using DDM demonstrated the presence of two metacommunities (enterotypes), with 96% (48/50) of fecal samples from suckling pigs being partitioned into one community type and 95% (38/40) of fecal samples from weaned pigs being partitioned into another community type ($P<0.001$). Analysis using the LEfSe algorithm (LDA log score threshold = 2) also revealed 26 OTUs characteristic of the pre-weaning stage and 24 OTUs characteristic of the post-weaning stage. The pre-weaning indicator OTUs clustered into the bacterial classes Bacilli, Negativicutes, Actinobacteria and Lentisphaeria, while the post-weaning indicator OTUs clustered into the classes Betaproteobacteria, Fibrobacteria, and Chlamydiae (Figure 4.5).

The most common core OTUs (>1% relative abundance in ≥80% of pigs) prior to weaning were from Clostridia (Clostridium sensu stricto and Clostridium cluster XIVa). Interestingly, no core OTUs with >1% relative abundance could be identified at 21 days of age. However, after weaning, 3 core OTUs were present throughout the entire nursery phase at >1% relative abundance in at least 80% of pigs: Megasphaera, Lactobacillus, and Erysipelotrichaceae incertae sedis (Table 4.2).

Processing of 90 nasal samples yielded a total of 5,429,616 sequences that passed the quality control measures. There was a median of 61,323 (Range: 5,381 − 104,886) sequences per sample. During the 7-week period, the nasal microbiota of 10 pigs yielded sequences that clustered into 49,458 OTUs. There was a median of 6,257 OTUs recovered per pig throughout the period of study (Range: 5,133 – 7,233). The OTUs were classified into 22 bacterial phyla and 676 genera. However, only three phyla had >1%

overall relative abundance: Proteobacteria (51.5%), Firmicutes (41.0%), and Actinobacteria (5.1%). Only 0.38% of sequences were unclassified at the phylum level. The temporal shift in the relative abundance of bacterial phyla in the nasal microbiota is presented in Figure 4.6. Aging was associated with a reduction in Actinobacteria ($P<0.001$) and an increase in Proteobacteria ($P<0.001$), although a disruption in this trend was observed around the time of weaning causing the relative abundance of Proteobacteria to rebound. The dominant genus at each phase of development was Moraxella, with an average relative abundance ranging between 24.2% and 56.5%. Additionally, a high relative abundance of Clostridium (10.4−16.5%) was present before weaning but a shift in the microbiota after weaning led to an increased abundance of Lactobacillus (11.1−21.2%) and a corresponding decrease in Clostridium (Table 4.1).

A random subsample of 5,381 sequences was used for subsequent analysis. Median sample richness was 1,152 OTUs (Range: 467 – 5,793), and no age-related association was observed ($\rho=0.15$, $P=0.164$). The median inverse Simpson's diversity index was 16.7 (Range: 6.7 – 72.7) and, unlike richness, there was a positive correlation with age ($\rho=0.22$, $P=0.035$), although aging only accounted for a small proportion of the variation in diversity. A summary of the number of observed and core OTUs is shown in Figure 4.2, and the age-related change in richness and diversity is presented in Supplementary Figure 4.3.

Community membership (Jaccard measure of dissimilarity) is presented in Figure 4.7. Interestingly, samples appear to cluster into 3 age-dependent categories; day 1-3, day 7-21, and day 28-49. However, when analyzed by AMOVA, the Jaccard indices differed significantly between all age groups ($P<0.05$). Visualization of community structure (Yue

and Clayton measure of dissimilarity) is displayed in Supplementary Figure 4.2 as a dendrogram. Yue and Clayton indices differed between all age groups ($P<0.05$) with the exception of 7-day-old and 14-day-old pigs ($P>0.05$), and 35-day-old, 42-day-old, and 49-day-old pigs ($P>0.05$). There were also significant differences between the pre-weaning and post-weaning phases for both the Jaccard ($P<0.001$) and Yue and Clayton indices ($P<0.001$).

There was a noticeable difference in the pre-weaning and post-weaning nasal microbiota. The LEfSe algorithm (LDA log score threshold = 2) demonstrated the presence of 51 OTUs characteristic to pre-weaning pigs and 25 OTUs characteristic to post-weaning pigs. The pre-weaning indicator OTUs clustered into the bacterial classes Bacilli, Flavobacteria, Alphaproteobacteria, and Betaproteobacteria, and the post-weaning indicator OTUs clustered into the classes Clostridia, Fusobacteria, Actinobacteria, Chlamydiae, and Deferribacteres (Figure 4.8).

The evolving core nasal microbiota is described in Table 4.2. During the first 3 days of life, the genera Haemophilus, Globicatella, and Rothia formed the core microbiota in addition to Clostridium sensu stricto which was also present throughout the entire pre-weaning period. Following weaning, Lactobacillus was the only genus present throughout the entire nursery phase at >1% abundance in at least 80% of pigs.

4.5 – Discussion

The fecal and nasal microbiotas of young pigs display rich and diverse bacterial communities which undergo a very rapid and profound evolution during the first 7 weeks of life. During this developmental period a herd of pigs is exposed to thousands of bacterial species which play an important pioneering role in establishing a stable

microbiota that is evident at 2-3 weeks post-weaning, particularly for the fecal microbiota. However, the instability of the microbiota during early life means it may be more susceptible to dysbiosis which can create predispositions to other illnesses as has been reported in human studies (Munyaka et al., 2014). This has created the framework for the role of microorganisms in the 'programming hypothesis' which claims that manifestation of diseases later in life can be predetermined by exposure to environmental stimuli during early life (Azad et al., 2010). Some research with swine have demonstrated that antimicrobials, excessive hygiene, stress, and management practices have a long-term impact on the gut microbiota which may contribute to disease susceptibility or resistance (Thompson et al., 2008; Schmidt et al., 2011; Isaacson and Kim, 2012; Weese et al., 2014; Schokker et al., 2015). For example, it has been suggested that the abundance of lactobacilli may influence host immunity and disease susceptibility due to the immunomodulatory activity of these bacteria (Schokker et al., 2013). Further research exploring the relationship between the microbiota and the early-life programming of health in swine is warranted.

The fecal microbiota described in the present study is similar to previous studies which have found Firmicutes and Proteobacteria to be the predominant phyla pre-weaning (Schmidt et al., 2011; Schokker et al., 2015). However, studies of the fecal microbiota at 3-months of age demonstrate that a taxonomical shift results in the predominance of Firmicutes and Bacteroidetes (Kim et al., 2011; Looft et al., 2014). The beginning of this shift was also observed in the present study; Proteobacteria are very dominant during early life (39% relative abundance) but undergo a considerable decline into adulthood (5% relative abundance), and the transition into adulthood is marked by an

establishment of core OTUs belonging to Bacteroidetes (particularly *Prevotella* spp.). Proteobacteria, mainly comprising of *Escherichia*, are common early colonizers of the colon and the decline in this taxa is likely due to increased competition from obligate anaerobes (*Clostridium*, *Megasphaera*, *Prevotella*), changes in the colon epithelium,

Some notable OTUs that were abundant and formed the core microbiota after weaning belonged to butyrate-producing genera including *Megasphaera, Butyricicoccus*, and *Roseburia*. Butyrate is an essential energy source for enterocytes and it supports gut health by inhibiting inflammation, reducing oxidative stress, and promoting gut-barrier defense (Hamer et al., 2008). Additionally, the predominance of *Megasphaera* after weaning may be related to its extensive metabolic capabilities which include the production of vitamins, amino acids, and short-chain fatty acids (Shetty et al., 2013).

The microbiota of the anterior nasal passage of young pigs described in the current investigation demonstrates similarities to the nasal microbiota of adult pigs that has previously been studied using next-generation sequencing methods (Weese et al., 2014). During early-life, there was an apparent co-dominance between Proteobacteria and Firmicutes, but after weaning there was a noticeable shift in dominance towards Proteobacteria which is also the predominant phylum observed in adult pigs (Weese et al., 2014). This transition appeared to coincide with weaning and was marked by an increase in Gammaproteobacteria (particularly *Moraxella*) and a decrease in Clostridium and Bacilli. The genus *Moraxella* is of particular interest due to its high abundance in pigs throughout life. Previous research has found that *Moraxella* is inversely associated with *Haemophilus* colonization in humans (Pettigrew et al., 2008). It is possible that *Moraxella* plays a protective role in the nasal cavity of pigs by preventing colonization

with certain swine pathogens (such as *Haemophilus parasuis*); although this requires much further research.

Aging appeared to be the most significant driver of development of the fecal and nasal microbiotas, although weaning also played an important role. The reported changes in richness, diversity, relative abundance, and community structure and membership all appeared to follow trends that were independent of weaning, but that is not to say weaning was insignificant. The microbiota did respond to this event with noticeable changes in core OTUs and the detection of specific biomarker OTUs. However, this study was unable to determine whether these changes at weaning were associated with the change in diet, mixing of pigs, stress, or other variables encountered during this event. Furthermore, the rapidly evolving fecal microbiota of young pigs appears to reach a developmental milestone at approximately 2-3 weeks post-weaning. This is demonstrated by the decelerating change in relative abundance of taxa, the clustering of community membership and structure, the plateauing of richness and diversity, and the change in core OTUs. This is less evident for the nasal microbiota, but there are still indications of stability or convergence towards stability during this period.

The present characterization of the early-life microbiota of conventionally-raised pigs using next-generation sequencing provides a broader view of the microbial landscape than previous studies that have employed culture-based methods, fingerprinting, Sanger sequencing, microarrays, and 454-sequencing (Pryde et al., 1999; Leser et al., 2002; Thompson et al., 2008; Schmidt et al., 2011; Looft et al., 2014; Schokker et al., 2015) and is novel in using next-generation sequencing technology to characterize the early-life nasal microbiota, which is poorly described in the literature.

However, one of the limitations of the present study is over- or under-representation of particular taxa due to amplification bias. This study also involved only healthy pigs with no clinical presentation of disease and the study was conducted at only one farm, so inter-farm variability and the impact of farm environment and management could not be assessed. Additionally, the fecal microbiota is only representative of the distal gastrointestinal tract (colon, rectum) and characterization of important bacteria populations from the stomach and small intestine may be absent or under-represented.

4.6 – References

Azad MB, Kozyrskyj AL. Perinatal programming of asthma: the role of gut microbiota. Clin Dev Immunol. 2012;2012:932072.

Bunge J, Woodard L, Bohning D, Foster JA, Connolly S, Allen HK. Estimating population diversity with CatchAll. Bioinformatics. 2012;28:1045–7.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335–6.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27: 2194-200.

Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. The role of butyrate on colonic function. Aliment Pharmacol Ther. 2008;27:104e19.

Holmes I, Harris K, Quince C. "Dirichlet multinomial mixtures: generative models for microbial metagenomics." PLoS One. 2012;7:e30126.

Isaacson R, Kim HB. The intestinal microbiome of the pig. Anim Health Res Rev. 2012;13:100-9.

Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ, Isaacson RE. Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. Vet Microbiol. 2011;153:124-33.

Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M, Møller K. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl Environ Microbiol. 2002;68:673-90.

Looft T, Allen HK, Cantarel BL, Levine UY, Bayles DO, Alt DP, Henrissat B, Stanton TB. Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. ISME J. 2014;8:1566-76.

Munyaka PM, Khafipour E, Ghia JE. External influence of early childhood establishment of gut microbiota and subsequent health implications. Front Pediatr. 2014;2:109.

Pettigrew MM, Gent JF, Revai K, Patel JA, Chonmaitree T. Microbial interactions during upper respiratory tract infections. Emerg Infect Dis. 2008;14:1584-91.

Pryde SE, Richardson AJ, Stewart CS, Flint HJ. Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen, and cecal lumen of a pig. Appl Environ Microbiol. 1999;65:5372-7.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75:7537–41.

Schmidt B, Mulder IE, Musk CC, Aminov RI, Lewis M, Stokes CR, et al. Establishment of normal gut microbiota is compromised under excessive hygiene conditions. PloS One. 2011;6:e28284.

Schokker D, Zhang J, Vastenhouw SA, Heilig HG, Smidt H, Rebel JMJ, Smits MA. Long-Lasting Effects of Early-Life Antibiotic Treatment and Routine Animal Handling on Gut Microbiota Composition and Immune System in Pigs. PloS One. 2015;10:e0116523.

Schokker D, Zhang J, Zhang L, Vastenhouw SA, Heilig HGHJ, Smidt H, Rebel JMJ, Smits MA. Early-life environmental variation affects intestinal microbiota and immune development in new-born piglets. PloS One. 2014;9:e100040.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12:R60.

Shetty SA, Marathe NP, Lanjekar V, Ranade D, and Shouche YS. Comparative genome analysis of Megasphaera sp. reveals niche specialization and its potential role in the human gut. PloS One. 2013;8:e79353.

Shokralla S, Spall JL, Gibson JF, Hajibabaei M. Next-generation sequencing technologies for environmental DNA research. Mol Ecol. 2012;21:1794-1805.

Thompson CL, Wang B, Holmes AJ. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. ISME J. 2008;2:739-48.

Weese JS, Slifierz M, Jalali M, Friendship R. Evaluation of the nasal microbiota in slaughter-age pigs and the impact on nasal methicillin-resistant *Staphylococcus aureus* (MRSA) carriage. BMC Vet Res. 2014;15:69.

4.7 – Tables

Table 4.1. Relative abundance of the top five predominant classes and genera during each period of development.

Age Range (days)	Dominant classes (Mean Relative Abundance, %)		Dominant genera (Mean Relative Abundance, %)	
	Fecal	**Nasal**	**Fecal**	**Nasal**
1-3	Clostridia (44.5) Gammaproteobacteria (27.1) Fusobacteria (14.0) Bacilli (6.0) Bacteroidia (2.8)	Gammaproteobacteria (37.7) Bacilli (27.5) Actinobacteria (17.6) Clostridia (13.7) Betaproteobacteria (1.5)	*Clostridium* (17.9) *Escherichia* (15.0) *Fusobacterium* (10.5) *Clostridium* XIVa (4.3) *Lactobacillus* (4.2)	*Moraxella* (24.2) *Rothia* (14.9) *Clostridium* (10.4) *Globicatella* (9.9) *Actinobacillus* (5.3)
7-21	Clostridia (44.5) Gammaproteobacteria (10.8) Bacilli (10.4) Bacteroidia (5.9) Erysipelotrichia (4.1)	Gammaproteobacteria (49.3) Clostridia (27.9) Bacilli (11.1) Betaproteobacteria (3.2) Actinobacteria (2.8)	*Clostridium* (8.8) *Escherichia* (8.6) *Lactobacillus* (8.2) *Clostridium* XIVa (7.4) unclassified Firmicutes (5.2)	*Moraxella* (46.7) *Clostridium* (16.5) *Lactobacillus* (3.9) unclassified Firmicutes (3.4) *Kingella* (2.8)
28-35	Clostridia (29.1) Negativicutes (26.5) Bacilli (15.6) Gammaproteobacteria (6.7) Erysipelotrichia (4.2)	Gammaproteobacteria (47.5) Bacilli (27.9) Clostridia (17.1) Erysipelotrichia (2.9) Betaproteobacteria (1.0)	*Megasphaera* (14.0) *Lactobacillus* (12.3) *Clostridium* (4.6) unclassified Firmicutes (4.2) *Succinivibrio* (4.1)	*Moraxella* (40.0) *Lactobacillus* (21.2) *Blautia* (3.0) Erysipelotrichaceae incertae sedis (2.9) *Staphylococcus* (2.4)
42-49	Negativicutes (32.4) Clostridia (31.7) Bacilli (17.4) Erysipelotrichia (5.4) Bacteroidia (3.7)	Gammaproteobacteria (59.5) Bacilli (15.7) Clostridia (15.1) Betaproteobacteria (3.4) Erysipelotrichia (2.2)	*Megasphaera* (21.2) *Lactobacillus* (12.3) *Roseburia* (4.2) unclassified Firmicutes (3.9) Erysipelotrichaceae incertae sedis (3.9)	*Moraxella* (56.5) *Lactobacillus* (11.1) *Kingella* (3.2) unclassified Firmicutes (2.9) *Blautia* (2.3)

Table 4.2. The genus-level taxonomy of abundant core OTUs of the porcine fecal and nasal microbiotas

Age (days)	Genera of the core OTUs* of the porcine microbiotas (% of pigs with OTU)	
	Fecal Microbiota	**Nasal Microbiota**
1	*Escherichia* (Shigella) (100), *Clostridium sensu stricto* (100)	*Globicatella* (100), *Haemophilus* (100), *Clostridium sensu stricto* (100), *Rothia* (80), *Staphylococcus* (80)
3	*Clostridium sensu stricto* (100), *Clostridium* XIVa (80), *Haemophilus* (80), *Lachnospiracea incertae sedis* (80)	*Globicatella* (100), *Rothia* (100), *Haemophilus* (80), *Clostridium sensu stricto* (80)
7	*Clostridium sensu stricto* (80), *Clostridium* XIVa (80), *Desulfovibrio* (80)	*Clostridium sensu stricto* (80)
14	*Escherichia* (Shigella) (100), *Clostridium* XIVa (80)	*Clostridium sensu stricto* (80), *Kingella* (80), unclassified Firmicutes (80)
21	None	*Clostridium sensu stricto* (100), *Kingella* (80), unclassified Firmicutes (80)
28	*Megasphaera* (80), *Lactobacillus* (80), *Acidaminococcus* (80)	*Lactobacillus* (100), *Erysipelotrichaceae incertae sedis* (100), *Enterococcus* (100), *Haemophilus* (80), *Blautia* (80)
35	*Megasphaera* (100), *Lactobacillus* (100), *Butyricicoccus* (100), *Erysipelotrichaceae incertae sedis* (80), *Selenomonas* (80), *Roseburia* (80), *Acidaminococcus* (80), *Faecalibacterium* (80), unclassified Firmicutes (80)	*Lactobacillus* (100)
42	*Megasphaera* (100), *Butyricicoccus* (100), *Erysipelotrichaceae incertae sedis* (100), *Roseburia* (100), *Acidaminococcus* (100), *Faecalibacterium* (100), *Lactobacillus* (80), *Selenomonas* (80), *Streptococcus* (80), *Prevotella* (80), *Clostridium* XI, unclassified Firmicutes (80)	*Lactobacillus* (100), *Sarcina* (100), *Erysipelotrichaceae incertae sedis* (80), *Kingella* (80), unclassified Firmicutes (80)
49	*Megasphaera* (100), *Butyricicoccus* (100), *Erysipelotrichaceae incertae sedis* (100), *Roseburia* (100), *Lactobacillus* (80), *Streptococcus* (80), *Prevotella* (80), *Gemmiger*(80)	*Lactobacillus* (80), unclassified Firmicutes (80)

***** Present at >1% relative abundance.

Supplementary Table 4.1. Two-phase starter ration fed to nursery pigs.

Ingredients	Composition (%)	
	Phase I Ration	**Phase II Ration**
Soybean meal, 47%	25.5	25.8
Corn chop	35.3	33.5
Whey permeate	10	8
Bakery meal	10	10
Wheat chop	7.8	7.5
Wheat shorts	0	5
Fish meal	3	2
Blood meal	2.5	2.5
Tallow	1.4	1.5
Monocalcium phosphate	1.1	1.05
Limestone	0.95	1
L-lysine, 50%	0.62	0.55
DL-methionine, 88%	0.28	0.25
Maple Butter	0.2	0.1
Fine salt	0.2	0.13
Threonine	0.19	0.16
Selenium	0.1	0.1
Choline chloride, 70%	0.05	0.05
Vitamin E (50 KIU/kg)	0.05	0.05
PellTech	0.4	0.4
Integral	0.05	0.05
Superzyme-CS	0.05	0.05
IntelliBond C (58% Cu)	0.017	0.017
Vitamin and mineral supplement[a]	0.2	0.2

[a] Formulation per kilogram of starter ration: 100 mg zinc oxide, 270 mg iron, 28 mg manganese, 124 mg copper, 0.60 mg iodine, 0.50 mg selenium, 0.16 mg cobalt, 20 mg fluorine, 10 KIU vitamin A, 1.5 KIU vitamin D3, 70 IU vitamin E, 2.0 mg vitamin K, 20 µg vitamin B12, 6.5 mg riboflavin, 30 mg niacin, 1.34 g choline, 24 mg D-pantothenic acid, 2.5 mg pyridoxine, 2.0 mg thiamine, 3.0 mg folic acid, 200 µg biotin.

4.8 – Figures

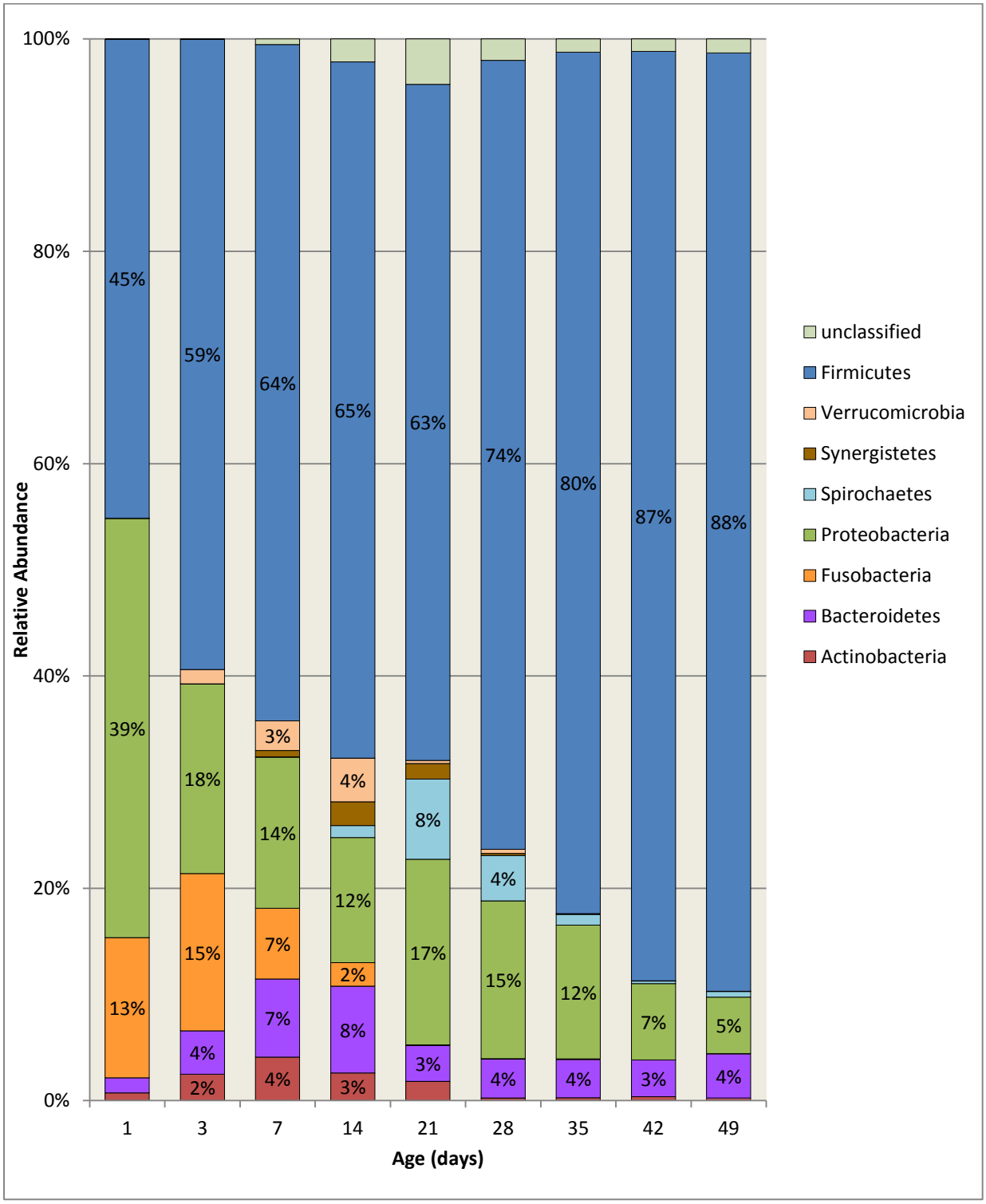


Figure 4.1. Age-associated change in relative abundance of bacterial phyla from the feces of young pigs (n=10).

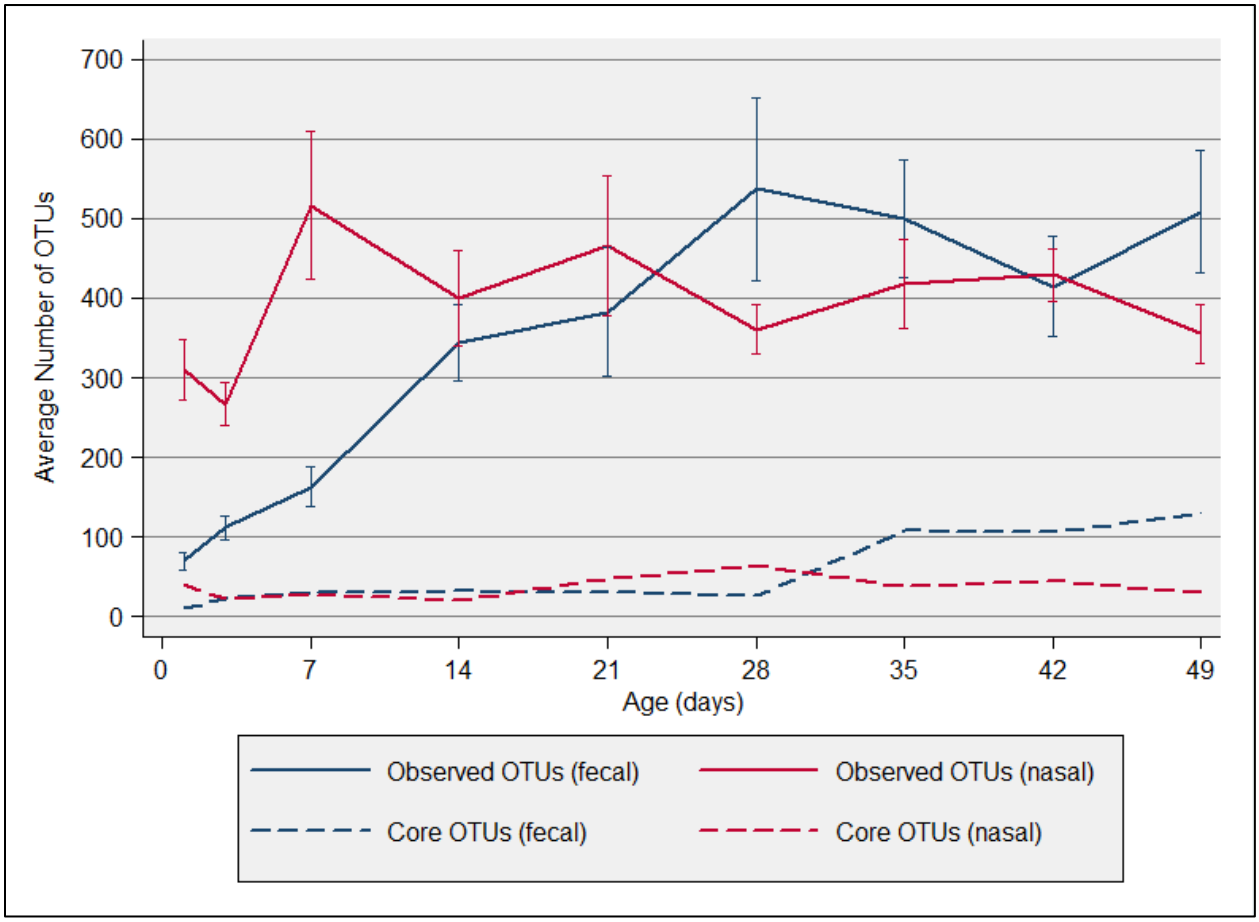


Figure 4.2. Observed and core OTUs in subsampled fecal and nasal samples from young pigs. In this analysis, core OTUs were defined as observed OTUs shared between all pigs at a given time point (no relative abundance threshold).

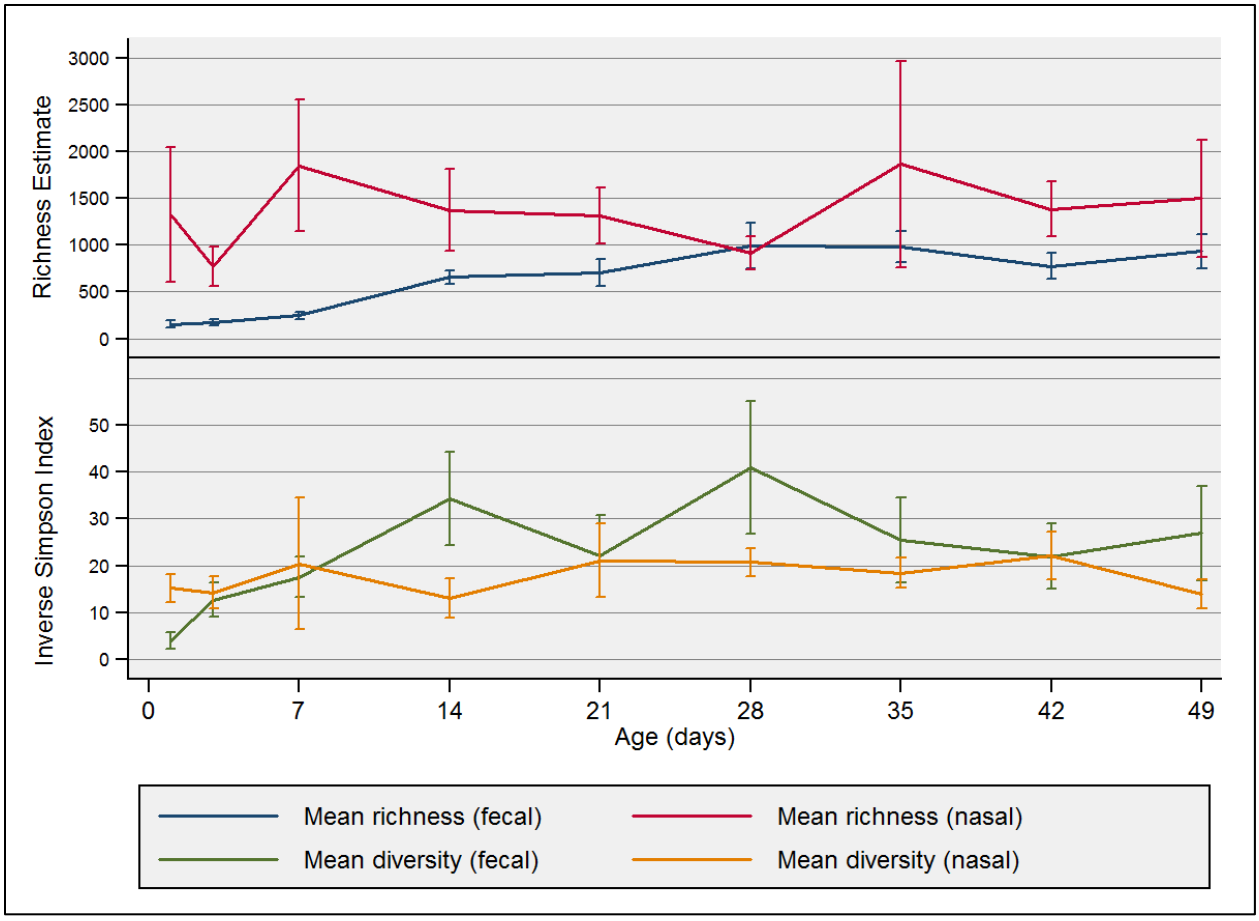


Figure 4.3. Estimated richness (CatchAll) and diversity (Inverse Simpson index) of fecal and nasal samples from pigs.

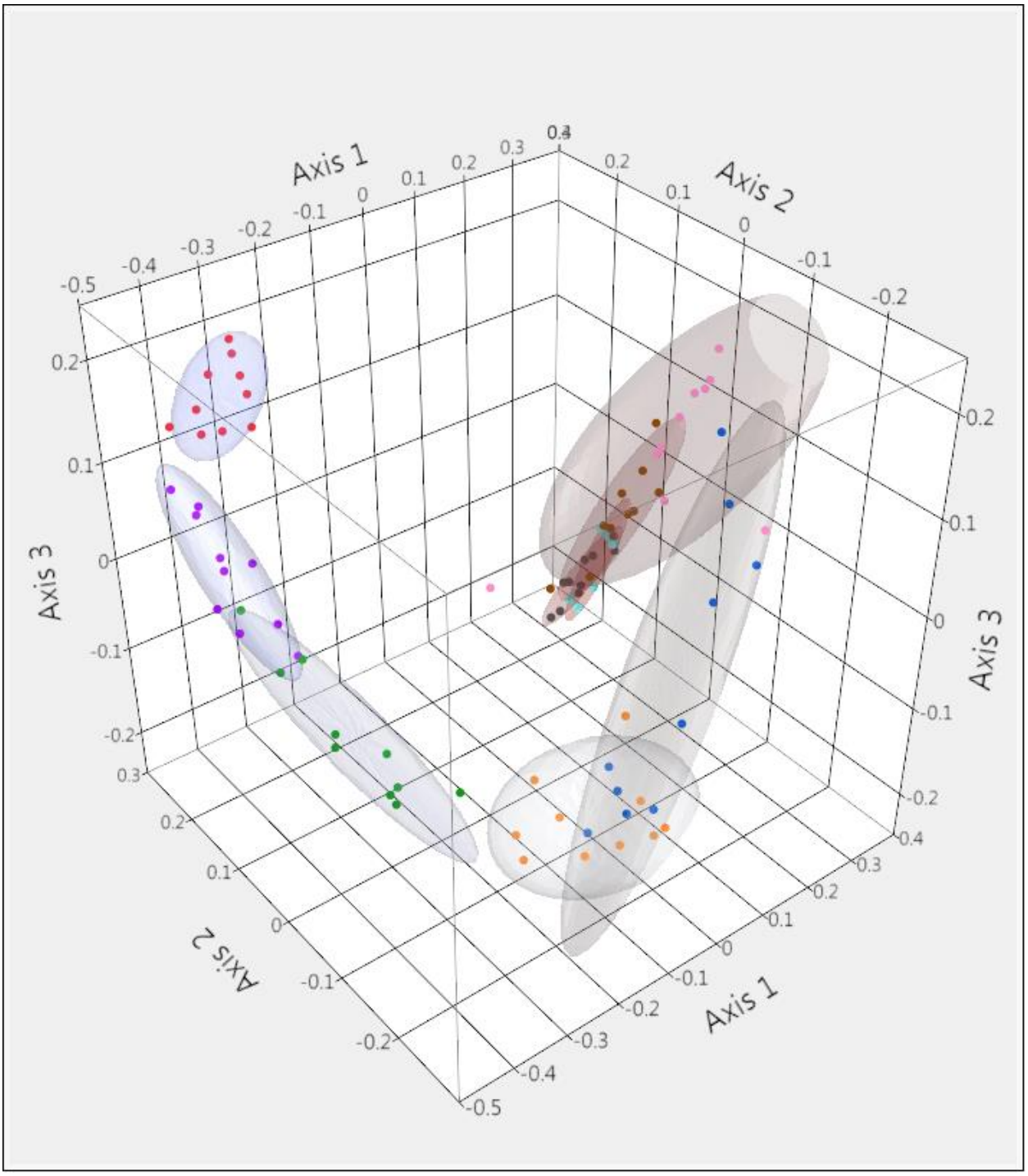


Figure 4.4. Three-dimensional principal coordinates analysis of the community membership of the porcine fecal microbiota (Jaccard Index). Coloured points and ellipses indicate age groups: 1 (red), 3 (violet), 7 (green), 14 (orange), 21 (blue), 28 (pink), 35 (brown), 42 (grey), 49 (indigo) days of age.

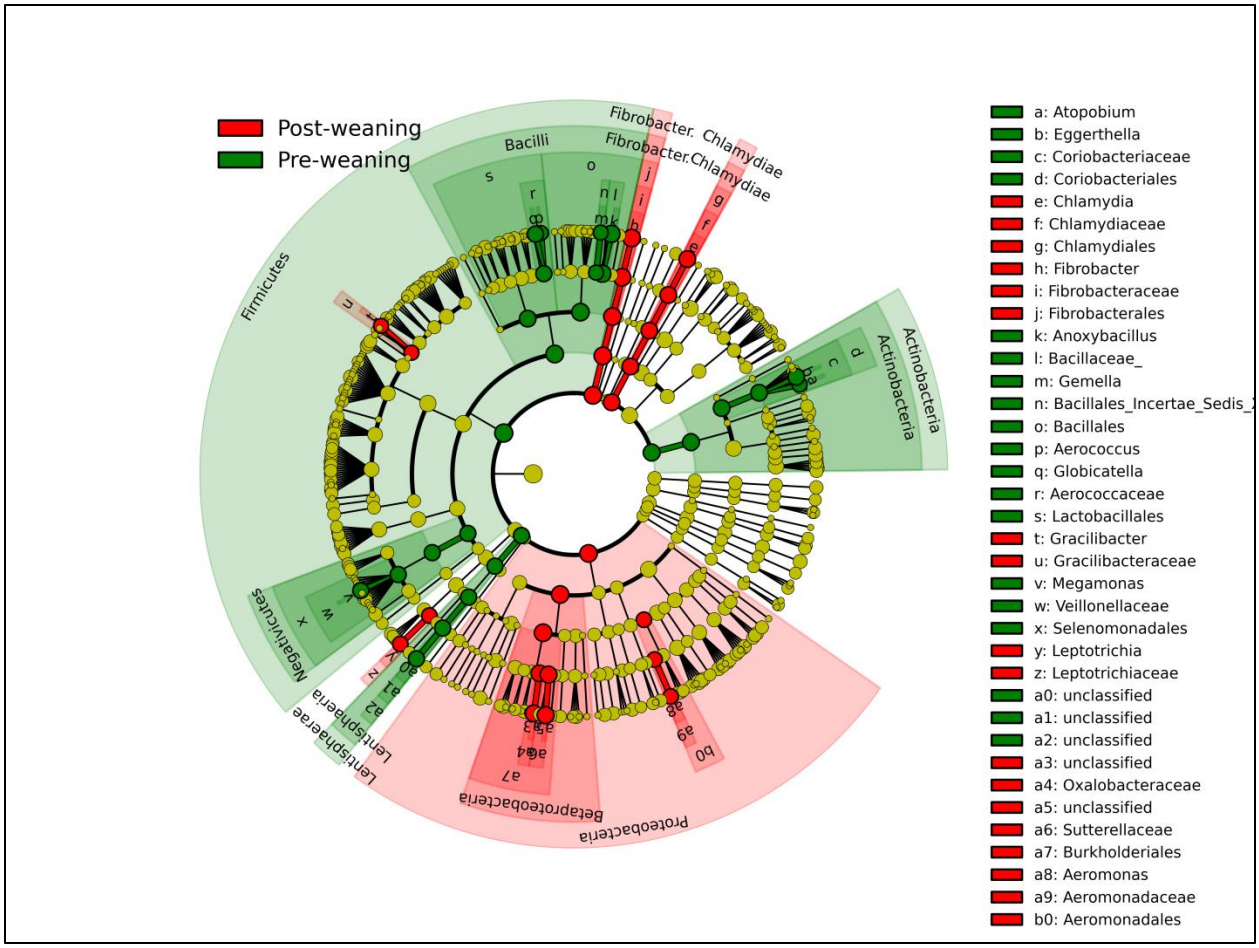


Figure 4.5. Cladogram of bacterial fecal biomarkers associated with phase of production (LEfSe). Samples were grouped into pre-weaning (days 1-21) or post-weaning (days 28-49). This hierarchal tree of taxonomical nodes, where diameter of the nodes indicates relative abundance, shows fecal biomarkers for the pre-weaning and post-weaning phases (LDA log score threshold = 2).

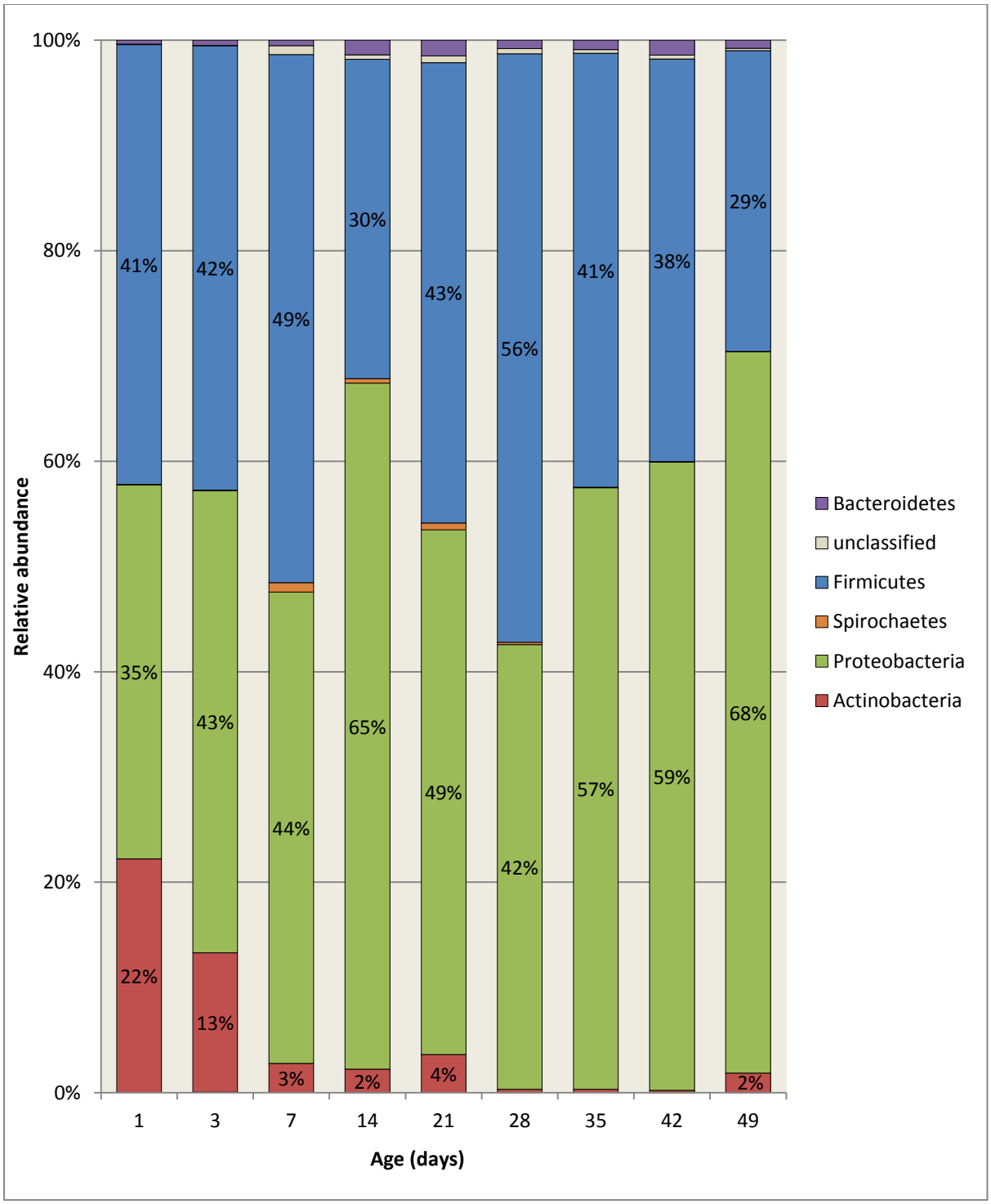


Figure 4.6. Age-associated change in relative abundance of bacterial phyla from the nasal cavity of young pigs (n=10).

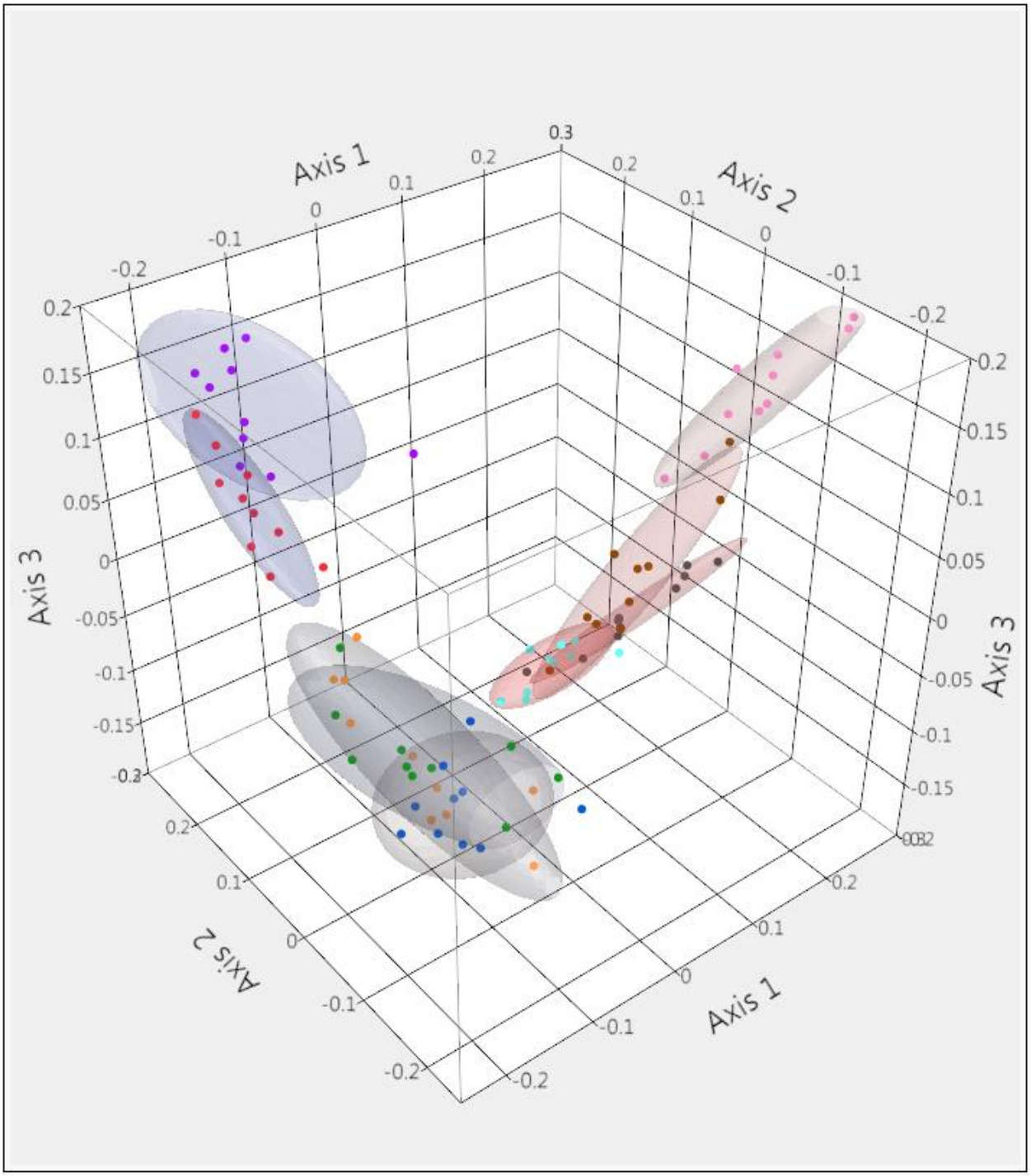


Figure 4.7. Three-dimensional principal coordinates analysis of the community membership of the porcine nasal microbiota (Jaccard Index). Coloured points and ellipses indicate age groups: 1 (red), 3 (violet), 7 (green), 14 (orange), 21 (blue), 28 (pink), 35 (brown), 42 (grey), 49 (indigo) days of age.

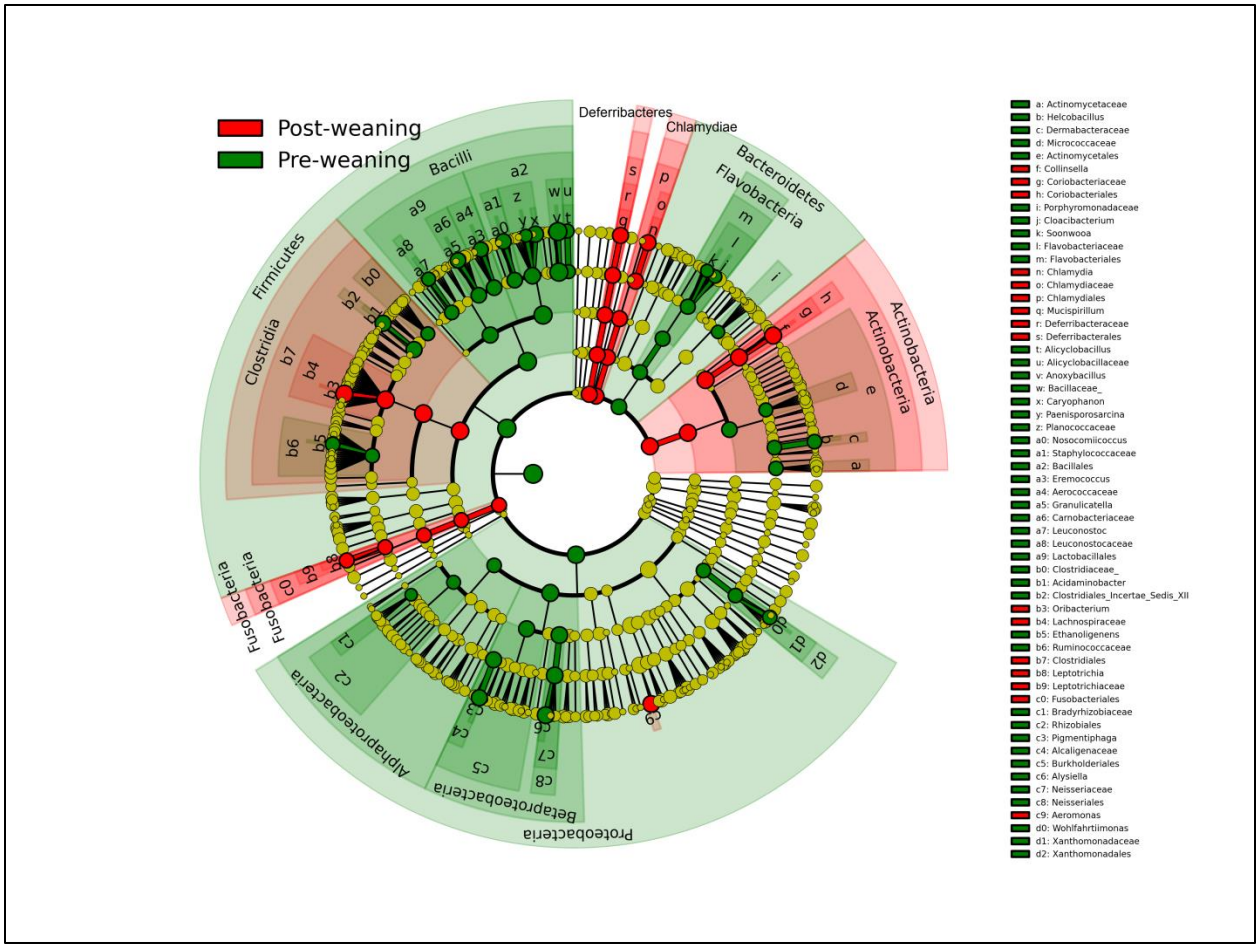
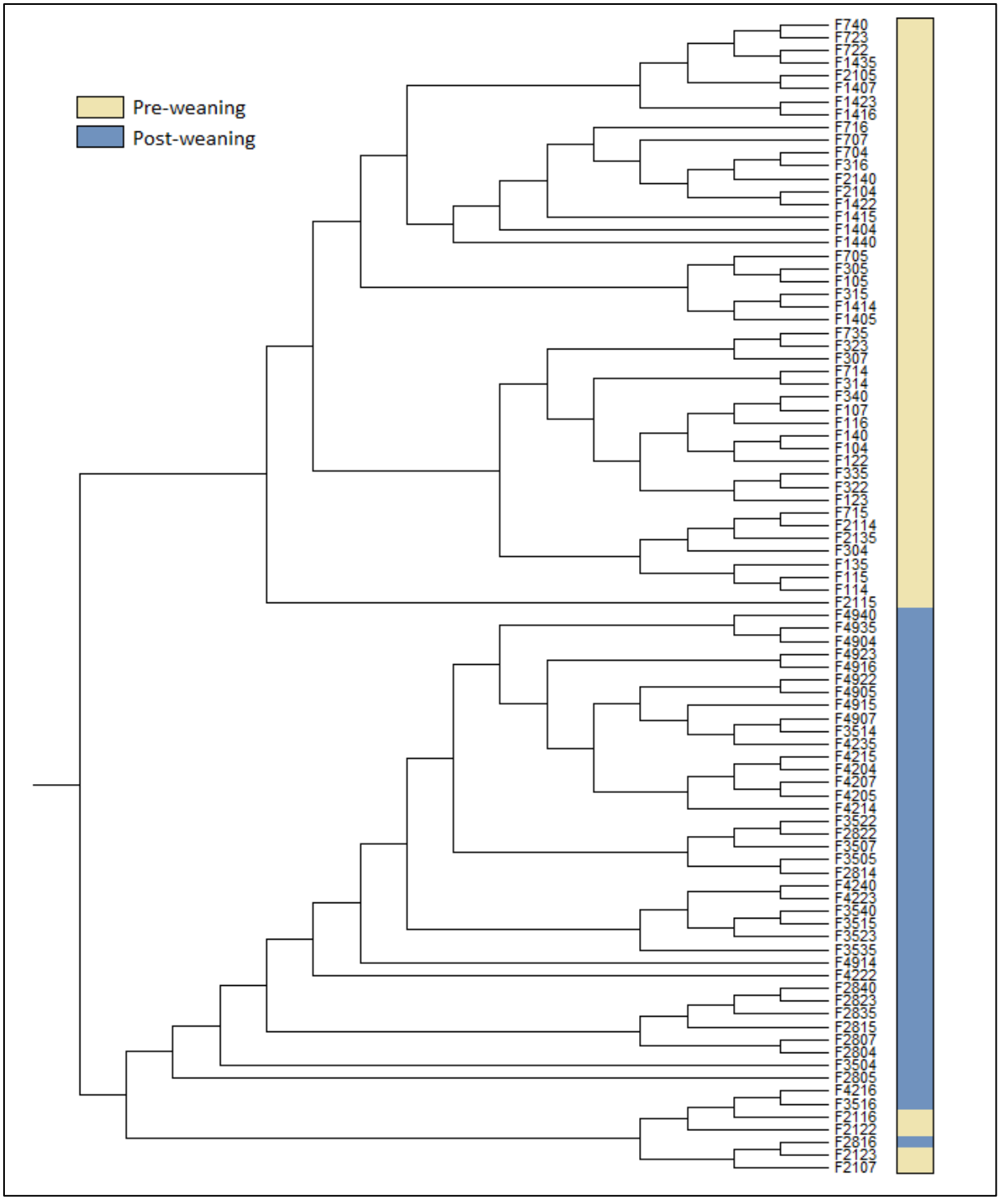
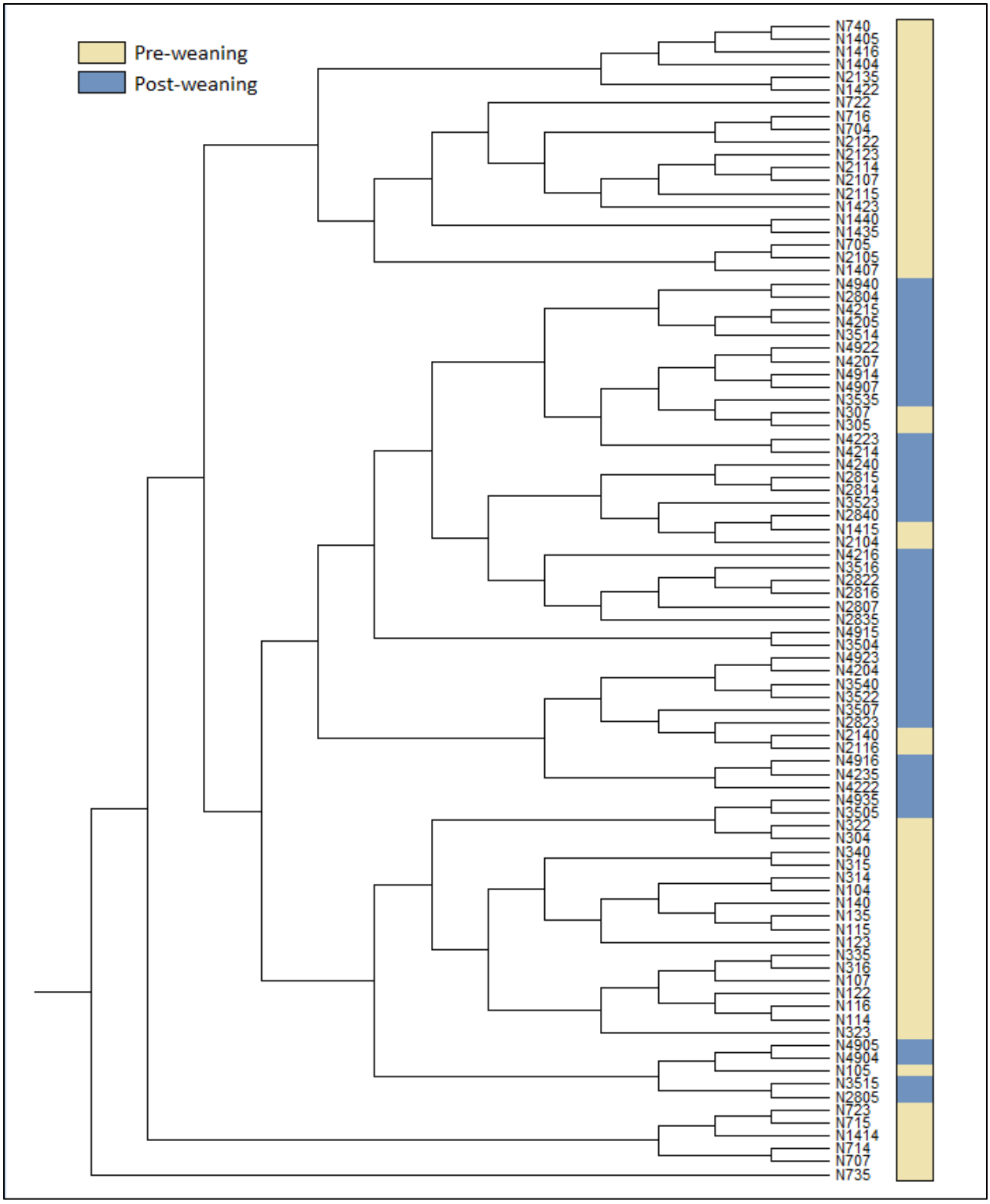


Figure 4.8. Cladogram of bacterial nasal biomarkers associated with phase of production (LEfSe). Samples were grouped into pre-weaning (days 1-21) or post-weaning (days 28-49). This hierarchal tree of taxonomical nodes, where diameter of the nodes indicates relative abundance, shows nasal biomarkers for the pre-weaning and post-weaning phases (LDA log score threshold = 2).



Supplementary Figure 4.1. Dendrogram of the community structure of the porcine fecal microbiota (Yue and Clayton). Sample identification (ABBCC) coded as: A – fecal (F) sample, BB – age of pig, CC – unique pig identifier.



Supplementary Figure 4.2. Dendrogram of the community structure of the porcine nasal microbiota (Yue and Clayton). Sample identification (ABBCC) coded as: A –nasal (N) sample; BB – age of pig; CC – unique pig identifier.

CHAPTER 5: The impact of a therapeutic zinc diet on the developing nasal microbiota of young pigs

5.1 – Abstract

Pigs have a rich and diverse nasal microbiota that develops during early-life. During this development period, the nasal microbiota is more susceptible to insults that could cause long-term changes. One exposure common among young commercial pigs is zinc therapy; yet, there is a lack of research assessing the impact of this therapy on the developing porcine nasal microbiota. To study the effects of this therapy, the nasal microbiota was assessed in 3 week-old pigs just before and 1-week after exposure to either a high zinc diet (HZD: 3,000 mg ZnO/kg feed) or a low zinc diet (LZD: 100 mg ZnO/kg feed) using next-generation sequencing (Illumina MiSeq) of the V4 region of the 16S rRNA gene. After exposure to zinc therapy, the nasal microbiota had an increased richness ($P=0.004$) and an increased relative abundance of uncommon bacterial phyla (Deinococcus-Thermus and Actinobacteria; $P<0.05$), relative to the LZD group. Community membership and structure were also significantly different ($P<0.001$) between the LZD and HZD groups. Lastly, the core microbiota was relatively similar before exposure to each group's respective diets, but differed after exposure with fewer core taxa observed among the HZD group. The presence of more bacterial taxa and fewer core taxa may indicate that a HZD increases the susceptibility of the porcine nasal microbiota to colonization. In conclusion, exposure to zinc therapy can cause a short-term impact to the nasal microbiota of young pigs which may influence susceptibility to foreign bacterial colonization.

5.2 – Background

The microbiota is the totality of microorganisms that populate the body and it plays an important role in the health of the host. One important function is that, based on the bacterial composition of the microbiota, it can protect the host from being colonized with certain pathogenic microorganisms (Pettigrew et al., 2008).

Respiratory diseases are one of the most prevalent causes of morbidity and mortality in pigs, especially in young pigs that are particularly susceptible to such diseases. The porcine nasal microbiota may influence the susceptibility to respiratory diseases, but this relationship is poorly understood and it is unclear how environmental exposures may impact the bacterial ecology and functionality of the nasal microbiota. Previous research has used culture-based techniques to describe the nasal microbiota of young pigs (Woods et al., 1972); however, such studies only describe less than 1% of bacteria that may be present. More recent research has used next-generation sequencing methods to reveal the very rich and diverse nasal microbiota of pigs (Weese et al., 2014; Slifierz et al., 2015a). In young pigs it was found that the nasal cavity may be exposed to over 6,000 bacterial species during the first 7 weeks of life; these bacteria may come from 676 genera and 22 phyla (Slifierz et al., 2015a). Proteobacteria, Firmicutes, and Actinobacteria tend to dominate the early-life nasal microbiota but this trend shifts to overwhelming Proteobacteria dominance in adulthood (Weese et al., 2014; Slifierz et al., 2015a). The nasal microbiota evolves rapidly during early-life but begins to stabilize when the pig is 6-weeks old or at 2-3 weeks post-weaning under antimicrobial-free conditions (Slifierz et al., 2015a).

The incredible richness and diversity of the porcine nasal microbiota may be due in part to the freely exposed snout of the pig. Pigs use their snouts for sniffing, exploring the

environment, and manipulating objects, and so it is common for the snout to be exposed to many surfaces and substances in their environment, including feed, manure, and water (Vaarst et al., 2004). One common substance used in swine feed and excreted in manure is zinc, which has antimicrobial properties when used at high levels (Jacela et al., 2010; Slifierz et al., 2015b; Holman and Chénier, 2015). Pigs only require 50-125 ppm zinc in their diet for normal growth and development, but zinc can also be used therapeutically to control post-weaning colibacilosis at an in-feed concentration of 2,000-3,000 ppm (Jacela et al., 2010). The effects of a high zinc diet on the gut microbiota have been previously researched (Pieper et al., 2012; Starke et al., 2014); yet, it is unknown whether exposure to high levels of zinc in the environment (feed or manure) may affect the porcine nasal microbiota. Hence, the purpose of the present study is to determine whether exposure to a therapeutic zinc diet following weaning impacts the nasal microbiota of nursery pigs.

5.3 – Methods

Experimental design

The use of animals in this study was approved by the Animal Care Committee at the University of Guelph. This experiment was conducted at a 300-sow batch-farrowing swine research facility with no recent history of using therapeutic levels of zinc. Twenty-two Yorkshire-Landrace pigs from 15 different litters were randomly enrolled in the trial at birth and, at weaning (21 days old), randomly assigned to groups that received either a low zinc diet (LZD) of 100 mg ZnO/Kg feed or a high zinc diet (HZD) of 3,000 mg ZnO/kg feed. In the nursey, all of the enrolled pigs were raised in 8 pens (same room) alongside approximately 12 pen-mates. The pigs were not exposed to any other therapeutic or antimicrobial agents during the trial. The interior of the nares were sampled with nasal swabs 1 day before exposure (just prior to weaning)

and 7 days after exposure to their assigned diets. The samples were transported at 4°C and stored at −80°C before DNA extraction was performed.

DNA extraction

Extraction of DNA from nasal samples was completed using a commercial kit and following the manufacturer's protocol for stool DNA extraction for pathogen detection (E.Z.N.A. Stool DNA Kit, Omega Bio-Tek Inc., Doraville, Georgia, USA). The entire tip of the swab was processed through the lysis stage of extraction.

Amplification and sequencing of bacterial 16S rRNA gene

The amplification and sequencing protocol was completed as previously described by Slifierz et al. (2015a) and is briefly summarized here. Previously designed forward (5′-AYTGGGYDTAAAGNG-3′) and reverse (5′-TACNVGGGTATCTAATCC-3′) primers were used to amplify the V4 region of the16S rRNA gene (Caporaso et al., 2010). The 16S primers contained adapter regions (Forward: TCGTCGGCAGCGTCAGATGTGTATAA-GAGACAG, Reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) for annealing to Illumina universal index sequencing adaptors that wereadded in a later PCR.

The amplification of the 16S rRNA V4 region was completed in a 25 µl reaction consisting of 12.5 µl of KAPA 2G Fast HotStart ReadyMix 2X (KapaBiosystems), 9.0 µl of molecular-grade water, 2.5 µl template DNA, and 0.5 µl each of both the forward and reverse 16S rRNA V4 primers (10.0 µM). The reaction conditions for PCR were 94°C for 10 min, and 27 cycles at 94°C for 45 s, 53°C for 60 s, and 72°C for 90 s, followed by a final period of 72°C for 10 min. The product was then purified using AMPure X (Beckman Coulter Inc, Mississauga, Ontario, Canada). The AMPure (20 µl) was mixed with the amplicon (25 µl) and incubated at room temperature for 2 min. After applying a magnetic field, the supernatant was discarded and

the beads were washed twice with 80% ethanol. The beads were then incubated at room temperature for 10 min before eluting with 50 µl of 10 mM Tris buffer.

Illumina universal adapters (Forward: AATGATACGGCGACCACCGAGATCTACAC-index-TCGTCGGCAGCGTC, Reverse: CAAGCAGAAGACGGCATACGAGAT-index-GTCTCGTGGGCTCGG) were then added to the purified 16S rRNA gene product by PCR using a 25 µl reaction consisting of 12.5 µl KAPA 2G Fast HotStart ReadyMix 2X (KapaBiosystems), 8.0 µl of molecular-grade water, 2.5 µl template DNA, and 1.0 µl each of the forward and reverse sample-specific Illumina universal adapters. The PCR conditions were as follows: a single cycle at 94°C for 3 min, 8 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, followed by a final cycle of 72°C for 10 min. The product was purified with AMPure X (Beckman Coulter Inc, Mississauga, Ontario, Canada) as previously described and DNA was eluted into 30 µl of 10 mM Tris buffer. The samples were then quantified by spectrophotometry (Nanodrop, Roche, Mississauga, Canada) and normalized to a final concentration of 2 nM. Sequencing of the library pool was performed using an Illumina MiSeq (San Diego, USA) and 2×250 chemistry at the University of Guelph's Advanced Analysis Centre.

Analysis of sequencing data

The analysis was completed by following the protocol described by Slifierz et al. (2015a) and is briefly summarized here. Sequencing data were analyzed using the mothur software package v.1.33.0 (Schloss et al., 2009). The paired-end reads were aligned and screened to remove sequences with the following irregularities: contiguous sequence lengths >245 bp or <239 bp, ambiguous base calls, stretches of homopolymers >8 bp, and misalignment with the target region. Sequences were then screened for chimeras using the UCHIME tool (Edgar et al., 2011) and sequences belonging to non-bacterial domains, including chloroplasts, mitochondria,

Archaea and Eukaryotes, were removed. Operational taxonomic units (OTUs) were created using a 3.0% dissimilarity threshold and average neighbour algorithm, then taxonomy was assigned using the RDP reference database (http://rdp.cme.msu.edu). The observed relative abundance of taxa was analyzed prior to subsampling and the mean relative abundance of the 12 treated pigs and 10 control pigs was plotted graphically to demonstrate changes in phyla and classes. Random subsampling of 5381 sequences from each individual sample was completed to normalize the sequence count. Community diversity (inverse Simpson index) and richness (CatchAll; Bunge et al., 2012) were computed. The core microbiota (OTUs with >1% relative abundance and shared amongst ≥80% of pigs) was also explored for LZD and HZD groups. The classical Jaccard index was used as an assessment of community membership and the Yue and Clayton measure of dissimilarity was used to as an assessment of community structure. These indices were visualized with a phylogenetic tree (FigTree), and compared between groups using analysis of molecular variance (AMOVA). Non-parametric statistical analysis (Wilcoxon rank-sum test) was performed in STATA 10.0 I/C (Stata Corporation, College Station, TX) to determine differences in taxonomical abundance, richness, and diversity between LZD and HZD groups. The null hypothesis for all statistical tests was rejected at $P<0.05$.

5.4 – Results

Of the 22 pigs enrolled in this study, 10 were weaned onto the LZD and 12 were weaned onto the HZD at 21 days of age. The characteristics of each group are reported in Table 5.1. Demographic characteristics did not differ between the groups. Zinc serum concentrations were not different between the two groups before exposure to their respective diets; however, after exposure the pigs from the HZD group (mean=1.05 µg/ml) had significantly greater (P=0.01)

zinc serum concentrations than pigs from the LZD group (mean=0.84 µg/ml). This indicates adequate exposure to their respective rations.

From the 44 nasal samples collected during the study, a total of 2,753,676 sequences were recovered after removing poor quality and erroneous reads. The median number of recovered sequences, recovered OTUs, richness, and diversity are reported in Table 5.2 for the LZD and HZD groups according to pre-exposure and post-exposure to their respective diets. There were no statistically significant differences between the LZD and HZD groups before exposure to their diets. After exposure to their respective diets, the richness was significantly greater among the HZD compared to the LZD group (P=0.004). The richness and diversity estimates are based on 5,381 subsampled sequences per nasal sample.

Classification of OTUs into bacterial taxa resulted in 22 classified phyla and 41 classes of bacteria identified across all recovered sequences before subsampling. Proteobacteria and Firmicutes were the dominant phyla and together they accounted for >90% relative abundance in both the LZD and HZD groups at each phase of the study. During the study the relative abundances of Proteobacteria and Firmicutes ranged from 42.0 – 58.7% and 33.5 – 55.6%, respectively, and there was no significant difference between the LZD and HZD groups before or after exposure to their diets. There were 5 uncommon phyla identified (Actinobacteria, Bacteroidetes, Spirochaetes, Deinococcus-Thermus, and Verrucomicrobia) that had a relative abundance >0.1% but <5.0% (see Figure 5.1). Statistical testing revealed no significant differences in relative abundance of phyla between the LZD and HZD groups before exposure to their diets. However, after exposure, the relative abundances of both Deinococcus-Thermus and Actinobacteria were greater in the HZD group compared to the LZD group (P<0.05). The remaining 15 phyla had a relatively rare presence within the nasal microbiota (<0.1% relative

abundance) and no significant differences were detected. Similarly with bacterial classes, the only significant differences detected were an increased relative abundance of Deinococcus-Thermus and Actinobacteria among the HZD group post-exposure when compared to the LZD group post-exposure ($P<0.05$).

Phylogenetic trees of the community membership and structure are presented in Figure 5.2 and Figure 5.3, respectively. Pig age (pre-exposure vs. post-exposure) appeared to have the greatest impact on the clustering of community membership and structure, as samples clearly clustered by age. Yet, both community membership and structure of the post-exposure LZD and HZD were significantly different ($P<0.001$).

The core microbiota was defined as being comprised of any OTU found in ≥80% of pigs at a single time-point with a relative abundance greater than 1%. The core microbiota of the nasal samples from pigs in the LZD and HZD groups are described pre- and post-exposure in Table 5.3. Before exposure, both the LZD and HZD groups had similar core microbiotas comprising of OTUs associated with the genera *Megasphaera*, *Selenomonas*, and *Roseburia* (with the exception that the pre-exposure LZD group also had *Haemophilus*). However, after exposure, there was a marked difference in the core microbiota between the LZD and HZD groups; most notable is that the LZD had 6 core OTUs while the HZD only had 1 core OTU (*Lactobacillus* was the single core OTU shared between the groups).

5.5 – Discussion

A therapeutic zinc diet resulted in increased richness, a less established core microbiota, and greater relative abundance of uncommon bacterial phyla and classes. These findings indicate that feeding of this high zinc diet can modify the host's susceptibility to additional bacteria beyond what is normally observed among the nasal microbiota of pigs on a low zinc diet. This

does not necessarily mean that zinc therapy is detrimental to the microbiota of pigs as previous studies have demonstrated that greater colonization with certain bacteria can create competition which limits susceptibility to disease (Pettigrew et al., 2008), and it is also commonly reported that a lack of microbial diversity is associated with disease or a dysbiotic microbiota in certain ecosystems (Mosca et al., 2016). However, any change in a microbiota must lead to consideration of the possible positive and negative impacts.

The finding that zinc therapy can impact the bacterial ecology of the nasal microbiota of pigs was not unexpected. Zinc has antimicrobial properties when used at high levels (Holman and Chénier, 2015), and previous studies have demonstrated that zinc therapy can affect the developing intestinal microbiota of nursery pigs (Pieper et al., 2012; Starke et al., 2014). It has also been reported that certain farm-level parameters, such as diet and antimicrobial exposure, can influence the nasal microbiota of swine (Weese et al., 2014). Hence, the observed impact of zinc therapy on the porcine nasal microbiota is consistent with other findings.

Previous research has established that the microbiota is more variable and unstable during early development than at other stages of life (Lozupone, et al., 2012; Slifierz et al., 2015a), and insults to the early-life microbiota can result in long-term changes (Schokker et al., 2015). This may partly explain why such an immediate and stark impact was observed after only 1 week of exposure to zinc therapy. Furthermore, the means by which the nasal passage becomes exposed to in-feed zinc likely occurs during feeding as it was anecdotally observed during this study that pigs would commonly have feed residue in and around the nares. The the present study also determined that zinc serum levels were elevated in the HZD group, which was expected, but it is unlikely that elevated levels of zinc in the blood would impact the microbiota due to the relatively low serum concentration of zinc. Furthermore, since zinc therapy is also known to

change the gut microbiota (Pieper et al., 2012; Starke et al., 2014) it is also possible that the changes observed to the nasal microbiota may result from exposure to different bacteria that are shed into the environment through the manure.

The observed differences between the core OTUs found in the LZD and HZD groups may be explained by the zinc susceptibility profile of bacterial species from these core genera. Liedtke and Vahjen (2012) found that 8 out of 9 *Lactobacillus* spp. had high resistance to zinc and this may explain the persistence of the *Lactobacillus* genus as a core member of the microbiota among the HZD group in this study. It has also previously been reported that, in general, lactic acid bacteria appear to be more resistant to zinc than strict anaerobic bacteria (Liedtke and Vahjen, 2012), and this may explain the lack of strict anaerobes (*Clostridium* spp. and *Cloacibacillus* spp.) in the core microbiota of the HZD group. Overall, these results, although not conclusive, do suggest that the nasal microbiota may be more susceptible to zinc-resistant bacterial species when pigs are exposed to a therapeutic zinc diet which is unsurprising as zinc is an antimicrobial that can exert selective pressure and exposure to zinc therapy has previously been associated with an increased abundance of zinc-resistant bacterial species in the nasal cavity of pigs, specially *Staphylococcus aureus* (Moodley et al., 2011).

One limitation of this study is that it only investigated the very immediate impact (at 1 week post-exposure) of a high zinc diet on the nasal microbiota; although, Starke et al. (2014) found that when pigs were exposed to a high zinc diet, the most severe effects on the gut microbiota occurred 1 week after exposure. Future studies should determine whether zinc therapy has a long-term or permanent impact on the porcine nasal microbiota. Furthermore, the pigs in this trial were clinically healthy and so these findings have limited extrapolation. The impact of zinc on the nasal microbiota of unhealthy pigs could potentially be different and ought

to be investigated. There are also some technical limitations with next-generation sequencing methods that must be considered, including amplification bias (affects representativeness of taxa) and sequencing errors that could contribute to false OTUs; however, precautions were taken to minimize these occurrences.

In conclusion, zinc therapy was associated with marked changes to the nasal microbiota of 3 week-old pigs, including increased bacterial richness, a significantly greater relative abundance of uncommon taxa, and a less established core microbiota comprised of a very zinc-resistant genus. These results suggest that exposure to a high-dose zinc diet may increase the susceptibility of the nasal microbiota to a greater assortment of bacteria and such conditions may cater favourably to zinc-resistant species, perhaps through selective pressure exerted by zinc exposure. These initial findings warrant further research to determine the full extent to which zinc therapy impacts the nasal microbiota of swine.

5.6 – References

Bunge J, Woodard L, Bohning D, Foster JA, Connolly S, Allen HK. Estimating population diversity with CatchAll. Bioinformatics. 2012;28:1045–7.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335–6.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27: 2194-200.

Holman DB, Chénier MR. Antimicrobial use in swine production and its effect on the swine gut microbiota and antimicrobial resistance. Canadian J Microbiol. 2015;61(11):785-98.

Jacela JY, DeRouchey JM, Tokach MD, Goodband RD, Nelssen JL, Renter DG, Dritz SS. Feed additives for swine: Fact sheets–high dietary levels of copper and zinc for young pigs, and phytase. J Swine Health Prod. 2010;18:132-136.

Liedtke J, Vahjen W. In vitro antibacterial activity of zinc oxide on a broad range of reference strains of intestinal origin. Vet Microbiol. 2012 Nov 9;160(1):251-5.

Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012 Sep 13;489(7415):220-30.

Moodley A, Nielsen SS, Guardabassi L. Effects of tetracycline and zinc on selection of methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type 398 in pigs. Vet Microbiol. 2011 Sep 28;152(3):420-3.

Mosca A, Leclerc M, Hugot JP. Gut microbiota diversity and human diseases: should we reintroduce key predators in our ecosystem? Front Microbiol. 2016;7:455.

Pettigrew MM, Gent JF, Revai K, Patel JA, Chonmaitree T. Microbial interactions during upper respiratory tract infections. Emerg Infect Dis. 2008;14:1584-91.

Pieper R, Vahjen W, Neumann K, Van Kessel AG, Zentek J. Dose-dependent effects of dietary zinc oxide on bacterial communities and metabolic profiles in the ileum of weaned pigs. J Animal Physio Animal Nut. 2012 Oct 1;96(5):825-33.

Schokker D, Zhang J, Vastenhouw SA, Heilig HG, Smidt H, Rebel JMJ, Smits MA. Long-Lasting Effects of Early-Life Antibiotic Treatment and Routine Animal Handling on Gut Microbiota Composition and Immune System in Pigs. PloS One. 2015;10:e0116523.

Slifierz MJ, Friendship RM, Weese JS. Longitudinal study of the early-life fecal and nasal microbiotas of the domestic pig. BMC Microbiol. 2015 Sep 21;15(1):1.

Slifierz MJ, Friendship RM, Weese JS. Methicillin-resistant *Staphylococcus aureus* in commercial swine herds is associated with disinfectant and zinc usage. Appl Environ Microbiol. 2015 Apr 15;81(8):2690-5.

Starke IC, Pieper R, Neumann K, Zentek J, Vahjen W. The impact of high dietary zinc oxide on the development of the intestinal microbiota in weaned piglets. FEMS Microbiol Eco. 2014 Feb 1;87(2):416-27.

Vaarst M, Lund V, Roderick S, Lockeretz W, editors. Animal health and welfare in organic agriculture. CABI; 2004.

Weese JS, Slifierz M, Jalali M, Friendship R. Evaluation of the nasal microbiota in slaughter-age pigs and the impact on nasal methicillin-resistant *Staphylococcus aureus* (MRSA) carriage. BMC Vet Res. 2014;15:69.

Woods GT, Jensen AH, Gossling J, Rhoades HE, Nickelson WF. 1972. The effect of medicated feed on the nasal microflora and weight gain of pigs. Can J Comp Med 36(1):49-54.

5.7 – Tables

Table 5.1. The characteristics of the low zinc diet (LZD) and high zinc diet (HZD) groups.

Group Characteristic	LZD Group (n=10)	HZD Group (n=12)
Demographics		
Males	4 (40%)	8 (67%)
Litter Size	10.20	10.67
Average Sow Parity	2.60	2.83
Average Birth Weight (kg)	1.68	1.61
Zinc serum conc. (µg/ml)		
Pre-exposure (mean)	0.78	0.77
Post-exposure (mean)*	0.84	1.05

* Statistically significant difference ($P<0.05$) between LZD and HZD groups for the parameter.

Table 5.2. The microbiota attributes of the low zinc diet (LZD) and high zinc diet (HZD) groups.

Group Characteristic	LZD Group (n=10)	HZD Group (n=12)
Sequences Recovered, median (range)		
Pre-exposure	54,739 (39,591 – 73,308)	69,587 (5,381 – 119,846)
Post-exposure	54,972 (20,285 – 72,483)	68,780 (31,181 – 108,231)
OTUs Recovered, median (range)		
Pre-exposure	1,020 (784 – 1,711)	1,493 (336 – 2,224)
Post-exposure	728 (409 – 1,050)	1,326 (552 – 2,157)
Richness (sub-sampled)†, median (range)		
Pre-exposure	1,207 (756 – 2,267)	1,913 (370 – 2,961)
Post-exposure*	991 (500 – 1,243)	1,906 (699 – 3,581)
Inverse Simpson (sub-sampled), median (range)		
Pre-exposure	18.5 (14.1 – 53.3)	16.6 (8.3 – 22.4)
Post-exposure	20.4 (15.8 – 27.8)	14.5 (6.1 – 48.0)

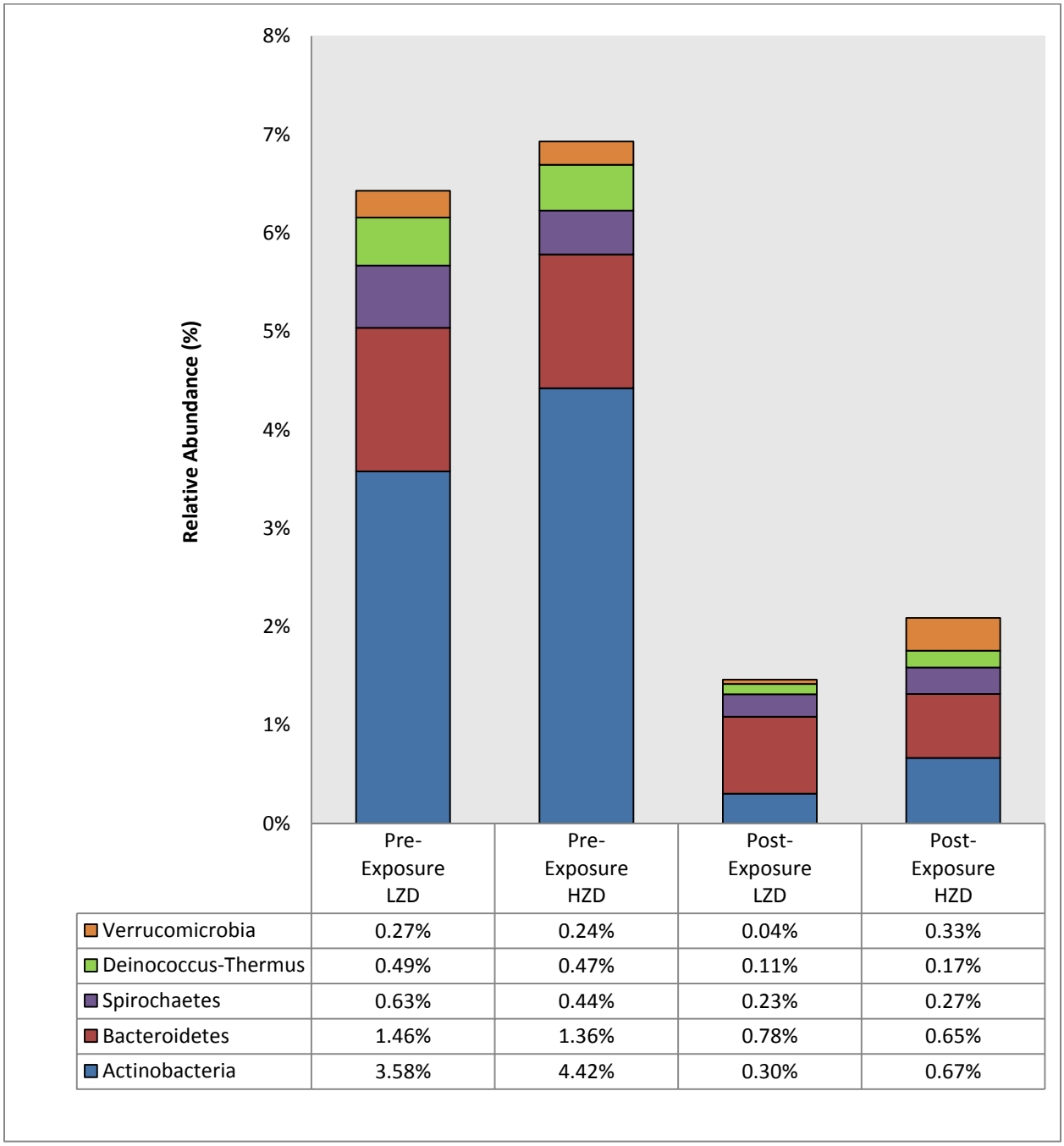
* Statistically significant difference ($P<0.05$) between LZD and HZD groups for the parameter.
† CatchAll (Bunge et al., 2012).
Note: recovered sequences and OTUs exclude erroneous and poor quality reads; richness and diversity were calculated for subsampled sequences.

Table 5.3. The genera of core Operational Taxonomical Units (OTUs) in the nasal cavity of pigs from the low zinc diet (LZD) and high zinc diet (HZD) groups.

Time-point	Core genera of LZD Group	Core genera of HZD Group
Pre-exposure	*Megasphaera* *Selenomonas* *Roseburia* *Haemophilus*	*Megasphaera* *Selenomonas* *Roseburia*
Post-exposure	*Escherichia*/*Shigella* *Lactobacillus* *Clostridium* sensu stricto *Prevotella* *Cloacibacillus* unclassified Firmicutes	*Lactobacillus*

Note: core OTUs were defined as any OTU found in ≥80% of pigs at a single time-point with a relative abundance greater than 1%.

5.8 – Figures



	Pre-Exposure LZD	Pre-Exposure HZD	Post-Exposure LZD	Post-Exposure HZD
Verrucomicrobia	0.27%	0.24%	0.04%	0.33%
Deinococcus-Thermus	0.49%	0.47%	0.11%	0.17%
Spirochaetes	0.63%	0.44%	0.23%	0.27%
Bacteroidetes	1.46%	1.36%	0.78%	0.65%
Actinobacteria	3.58%	4.42%	0.30%	0.67%

Figure 5.1. The relative abundance of uncommon phyla (0.1 – 5.0% relative abundance) of the porcine nasal microbiota in treatment and control groups.

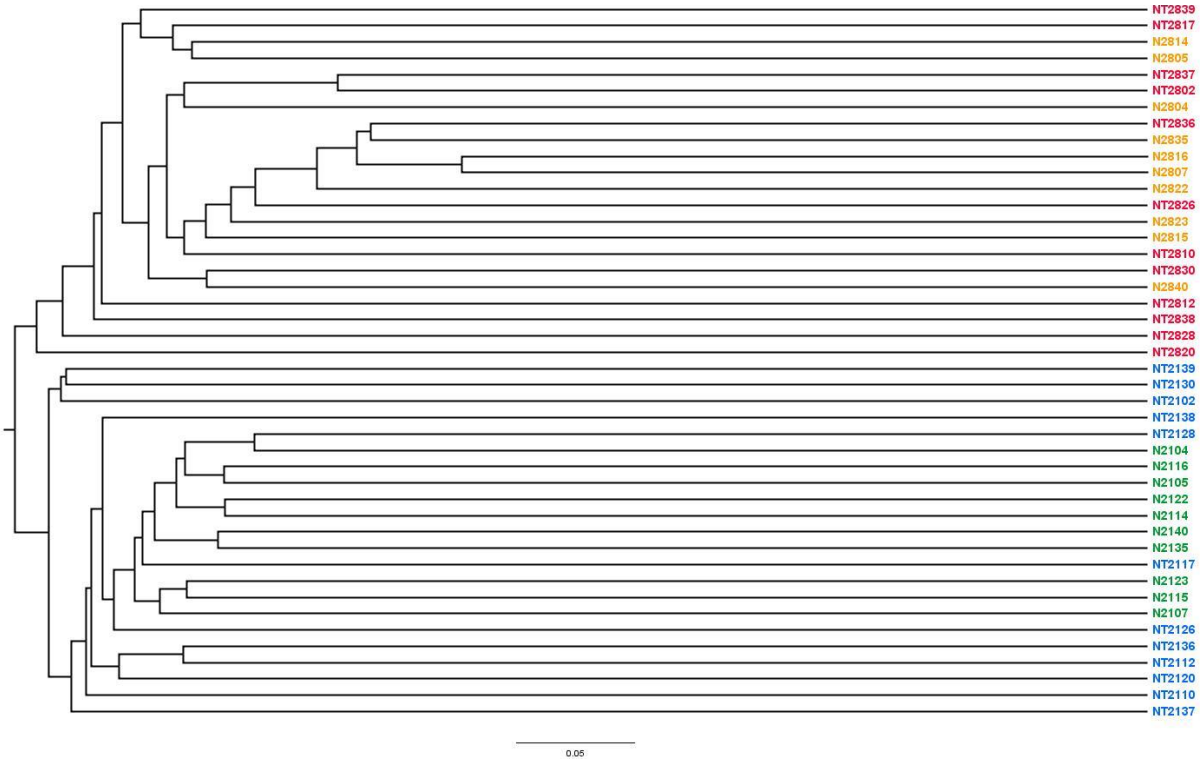


Figure 5.2. Phylogenetic tree of community membership (Jaccard index) of the porcine nasal microbiota for pre-exposure LZD (green), pre-exposure HZD (blue), post-exposure LZD (orange), and post-exposure HZD (red).

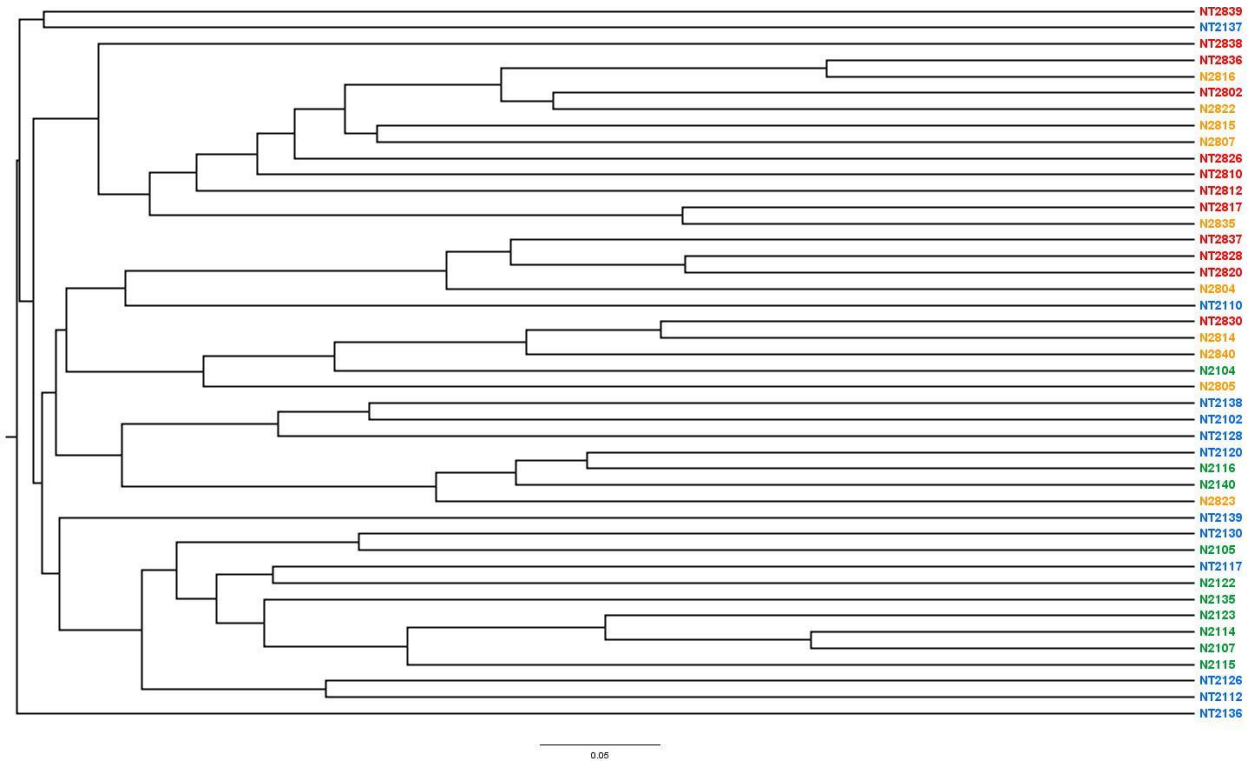


Figure 5.3. Phylogenetic tree of community structure (Yue and Clayton index) of the porcine nasal microbiota for pre-weaning LZD (green), pre-weaning HZD (blue), post-weaning LZD (orange), and post-weaning HZD (red).

CHAPTER 6: Summary and Conclusion

Zinc therapy is commonly used in swine production in Canada but its impact on the bacterial populations that inhabit pigs is poorly understood. The overarching theme of this research was to determine the effects of zinc therapy on swine and their bacterial inhabitants. Specifically, this research project attempted to address two main objectives which are detailed and summarized in the forthcoming sections.

6.1 – First objective and summary of findings

The first objective was to determine whether zinc could select for antimicrobial-resistance given the co-location of the zinc-resistance gene (*czrC*) within a bacterial resistance island in methicillin-resistant *Staphylococcus aureus* (MRSA). A randomized-controlled trial and an epidemiological cohort study were conducted to investigate whether exposure to a high-dose zinc diet was associated with carriage of MRSA among swine herds.

The randomized-controlled trial demonstrated that pigs exposed to a high-dose zinc diet at weaning (21-days-old) had a significantly greater herd-level prevalence of MRSA at 1-week and 2-weeks post-weaning, before decreasing to a herd-level prevalence of 2% at the end of the trial (4-weeks post-weaning). During the post-weaning period, persistent MRSA carriage (that is, testing positive ≥2 times) was observed in 2% (1/49) of control pigs and 22% (11/50) of treated pigs which was statistically significant.

An epidemiological cohort study was conducted to determine whether a similar association between MRSA and zinc therapy could be found under field conditions; in total, 390 pigs from 26 farms were followed from just before weaning to 3-weeks post-weaning. It was found that a therapeutic concentration (2,000 – 3,000 mg zinc/kg feed) was used in 68.2% of

nursery pig herds and that 38.5% of nursery pig herds were positive for MRSA. Multivariate analysis revealed that in-feed concentrations of zinc ($P<0.001$) and frequent disinfection of nursery pens ($P<0.001$) were associated with MRSA carriage among nursery pigs. Twenty-five (62.5%) of the 40 isolates of MRSA (5 collected per farm) carried the zinc-resistance gene, *czrC*, but 90% (36/40) were phenotypically resistant to zinc chloride (MIC >2 mM).

The microbiological and epidemiological evidence found during these investigations supports the hypothesis that a high-dose zinc therapy could lead to co-selection and co-retention of antimicrobial-resistance genes (*mecA*) due to the co-location of the zinc-resistance gene (*czrC*) within the mobile resistance cassette (SCC*mec*) of MRSA (Cavaco et al., 2010). The findings from these two studies are consistent with findings made by other researchers. For example, Moodley and colleagues (2011) found that exposure to zinc was associated with a greater load of MRSA in the nares of pigs, and Amachawadi and colleagues (2015) found that MRSA carriage in swine exhibits a dose-response to in-feed zinc supplementation. Overall, there is sufficient evidence to support the claim that zinc, particularly at high doses, is at least one factor that is affecting the dynamics of MRSA in swine herds.

6.2 – Second objective and summary of findings

The second objective was to further develop the understanding of the early-life microbiota of swine and to determine whether zinc impacts the bacterial ecology of the porcine nasal microbiota. This was investigated through two studies: a longitudinal trial of pigs raised under conventional conditions and an experimental randomized-controlled trial of pigs exposed to either a high-dose zinc diet or a low-dose zinc diet.

A longitudinal study of 10 pigs raised under conventional conditions was conducted to determine the evolution of the porcine fecal and nasal microbiotas from birth to 7-weeks of life (4-weeks post-weaning). It was found that these microbiotas contain a very rich and diverse population of bacteria that undergo a marked change. During this period of study, pigs carried an average of 1,976 and 6,257 species of bacteria in their gastrointestinal and respiratory tracts, respectively. Aging and weaning appeared to be the primary drivers in the development of these microbiotas. The rapidly developing microbiotas appeared to reach a developmental milestone at 2-3 weeks post-weaning (5-6 weeks of age) as a relative degree of stability was evident, such as a decelerating change in relative abundance of taxa, the clustering of community membership and structure, the plateauing of richness and diversity estimates, and the change in core operational taxanomic units.

The experimental trial of pigs exposed to either a high-dose zinc diet (3,000 mg ZnO/kg feed) or a low-dose zinc diet (100 mg ZnO/kg feed) was completed under conventiona pig-rearing conditions to determine how this therapeutic intervention impacts the nasal microbiota of young pigs. It was found that pigs exposed to a high-dose zinc diet had a significantly greater richness of bacteria, had differences in community membership and structure, and had an increased relative abundance of uncommon bacterial taxa within the phyla Deinococcus-Thermus and Actinobacteria. It was also observed that pigs exposed to a high-dose zinc diet had a less diverse core microbiota comprised of only a very zinc-resistant bacterial genus.

Overall, these results demonstrate that the microbiota of swine undergoes a marked and rapid development during the first 7-weeks of life and during this developmental period the microbiota is more suscepbile to insults that can result in long-term changes (Thompson et al., 2008). These results also demonstrate that zinc therapy does impact the porcine nasal microbiota,

but it cannot be stated whether this impact is detrimental or benficial to the host. It does appear that exposure to in-feed zinc therapy may increase susceptibility to additional bacteria and it may also create favourable conditions for highly-zinc resistant bacteria in particular; however, further research is needed to test these hypotheses.

6.3 – Conclusion

This research project was able to provide evidence to support the claim that the use of high-dose zinc therapy in swine production systems could be contributing to the co-selection and co-rention of a genetic cassette (SCC*mec*) of antimicrobial-resistance genes (including *mecA*) in staphylococci, namely livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA). Based on the emerging evidence from this research project, it is also hypothesized that exposure to in-feed zinc therapy may increase susceiptbility of the nasal microbiota to a wider range of bacteria and could create conditions particularly favourable for colonization by highly zinc-resistant bacteria such as LA-MRSA.

These finding are important as it could provide an explanation for the lack of strong evidence for an association between LA-MRSA carriage in pigs and the use of antimicrobials in swine production. Since a high-dose zinc therapy is used as an antimicrobial-alternative, yet still has the potential to select for antimicrobial-resistance, this therapy could confound observational studies that assess the relationship between antimicrobial-useage and the presence of antimicrobial-resistant bacteria such as LA-MRSA. This may also explain the common presence and persistence of LA-MRSA in antimicrobial-free and organic swine herds. While antimicrobial-free pork production, at the surface, appears to be an intrinsictly sound approach to reduce or limit antimicrobial-resistance, there are unintended consequences resulting from

alternative approaches, such as high-dose zinc therapy. Although zinc is not recognized as a classical anti-infective, it still has antimicrobial properties and it can co-select for antimicrobial-resistance.

Further research should monitor zinc-resistance in MRSA isolates from humans and animals to determine whether there are any shifts in antimicrobial phenotypes as regulations and management practices are changed regarding the use of antimicrobials and heavy metals in various swine-producing regions. The hypothesis that zinc therapy may increase the susceptibility of the nasal microbiota to bacterial colonization, particularly zinc-resistant bacteria, requires further study and evidence. It is also recommended that a risk assessment of the use of high-dose zinc therapy in Ontario swine production be completed to determine whether the benefits of this practice outweigh the risks to humans, animals, and the environment.

6.4 – References

Amachawadi RG, Scott HM, Nitikanchana S, Vinasco J, Tokach MD, Dritz SS, Nelssen JL, Goodband RD, Nagaraja TG. Nasal Carriage of mecA-Positive Methicillin-Resistant *Staphylococcus aureus* in Pigs Exhibits Dose–Response to Zinc Supplementation. Foodborne pathogens and disease. 2015 Feb 1;12(2):159-63.

Cavaco LM, Hasman H, Stegger M, Andersen PS, Skov R, Fluit AC, Ito T, Aarestrup FM. Cloning and occurrence of *czrC*, a gene conferring cadmium and zinc resistance in methicillin-resistant *Staphylococcus aureus* CC398 isolates. Antimicrob Agents Chemother. 2010; 54(9):3605-8.

Moodley A, Nielsen SS, Guardabassi L. Effects of tetracycline and zinc on selection of methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type 398 in pigs. Veterinary microbiology. 2011 Sep 28;152(3):420-3.

Thompson CL, Wang B, Holmes AJ. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. ISME J. 2008;2:739-48.