UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Faculty of Veterinary Science

Effect of antimicrobial usage on enteric bacterial populations, with focus on virulence and resistance profiles of *Escherichia coli* in growing pigs

By

Rukayya Hussain Abubakar

Submitted in partial fulfillment of the requirements for the degree of Magister Scientiae (Veterinary Science) in the Faculty of Veterinary Science, University of Pretoria

SUPERVISOR

Professor Folorunso Oludayo Fasina

CO-SUPERVISOR

DR. Evelyn Madoroba

Declaration

I Rukayya Hussain Abubakar declare that this Dissertation, which I hereby submit in accordance with requirements for the degree of Magister Scientiae (Veterinary **S**cience) at the University of Pretoria, is my work carried out under the Supervision of Professor F.O.Fasina and Dr Evelyn Madoroba and has not previously been submitted by me for a degree at any tertiary institution.

Rukayya Hussain Abubakar signature 

Date: 21-05-2018

Acknowledgements

I thank Almighty Allah for giving me good health and the resources to undertake this programme.

I wish to express my sincere appreciation and gratitude to the following people for their contributions in this study:

- My supervisor, Professor Folorunso Oludayo Fasina, for his excellent supervision, patience and support throughout the course of my study.
- DR Evelyn Madoroba, my co-supervisor, for her supervision and invaluable contribution towards the successful completion of this study.
- Professor Don Cowan, our collaborator, for the opportunity to work in his laboratory.
- My gratitude goes to Dr Angel Valverde for his assistance in the lab and excellent supervision of the metagenomics work. My appreciation also goes to Casper Kamuthando for his patience in teaching me how to analyse the sequences.
- My appreciation goes to the OVI staff Keneloe, Elmarie, Aletta, Ayanda, Kuda, Li and Victor for their kind guidance with the laboratory procedures.
- Many thanks go to Dr. Libby Onyeka, Dr. Ibrahim Z. Hassan, Barbra Glover, Zamatunga Khumalo for their assistance and guidance during sample collection and analysis.
- Thanks to Dr David Lazarus, Dr DG Bwala and Dr MM Suleiman for their assistance and words of encouragement.
- Dr Bukola Adenubi and her kids Kunmi, Kitan and Yimika for being a great family away from home.
- My dear friends Mrs Dorcas Tony and Mrs Maimuna Liman for their prayers, friendship and words of encouragement.
- I wish to thank all post graduate students at DVTD and OP residence for being great friends.
- My husband Yahaya Usman for his support, and our lovely daughter Aisha Usman for their understanding, patience, inspiration and unconditional love. You were my true pillars of support through this long and challenging journey.
- Mrs Mistura Mohammed and her kids Adama, Salma, Amina and Iman for their kindness in supporting my girl while i was away.
- My Mother Hajiya Nnagi Layi for her unconditional love and prayers, all my sisters especially Mrs Fatima Adam and brother for their support and prayers.

Dedication

To my lovely daughter, Aisha.

Table of contents

Declaration ii

Acknowledgements iii

Dedication iv

Table of contents v

List of Tables ix

List of Figures x

List of Abbreviations xi

Publications xii

Abstract xiii

Chapter one: xiv

General Introduction xiv

1.1 Introduction 1

1.1.1 Growth and gut development in pigs 1

1.1.2 Pig Management systems 2

1.1.3 Use of Antibiotics in pigs 2

1.1.4 Antibiotic resistant bacteria 3

1.1.5 Mechanism of antibiotic resistance 4

1.1.6 Methods of transfer of antibiotic resistant bacteria from animals to humans 5

1.2 Justification 5

1.3 Hypothesis 6

1.4 Aim and Objectives 6

1.5 References 7

Chapter Two 11

Literature Review 11

Bacteria pathogens of pigs with particular reference to *Escherichia coli*: A systematic review and meta-analysis 12

Preface 12

2.1 Abstract 13

2.2 Introduction 14

2.3 Methodology 14

2.4 Bacterial pathogens of pigs 15
2.4.1 Cutaneous (skin) associated bacteria 15
2.4.1.1 *Staphylococcus* species 15
2.4.1.2 *Treponema* species 15
2.4.2 Reproductive system associated bacteria 15
2.4.2.1 *Leptospira* species 15
2.4.2.2 *Brucella* species 16
2.4.2.3 *Listeria* species 16
2.4.2.4 *Erysipelothrix* species 16
2.4.3 Respiratory System associated bacteria 17
2.4.3.1 *Actinobacillus* species 17
2.4.3.2 *Mycoplasma* species 17
2.4.3.3 *Bordetella* species 18
2.4.3.4 *Pasteurella* species 18
2.4.3.5 *Haemophilus* species 19
2.4.3.6 *Mycobacterium* species 19
2.4.3.7 *Streptococcus* species 19
2.4.4 Digestive system associated bacteria 20
2.4.4.1 *Clostridium* species 20
2.4.4.2 *Salmonella* species 21
2.4.4.3 *Brachyspira* species 21
2.4.4.4 *Campylobacter* species 21
2.4.4.5 *Helicobacter* species 22
2.4.4.6 *Lawsonia* species 22
2.4.4.7 *Yersinia* species 22
2.4.4.8 *Enterococcus* species 23
2.4.4.9 *Bacillus* species 23
2.4.5 Other non-specific bacteria pathogens 23
2.4.5.1 *Actinobaculum* species 23
2.4.5.2 *Chlamydophila* species 23
2.4.6 Miscellaneous bacteria pathogens isolated from pigs 24
2.5 Enteric bacteria in pigs 24
2.6 Escherichia coli 25

2.6.1 Classification of *Escherichia coli* 27
2.6.1.1 Enteropathogenic *E. coli* (EPEC) 27
2.6.1.2 Shiga toxin producing *E. coli* (STEC) 27
2.6.1.3 Extra intestinal pathogenic *E. coli* (ExPEC) 28
2.6.1.4 Enterotoxigenic *E. coli* (ETEC) 28
2.6.2 *E. coli* post-weaning diarrhoea (PWD) 29
2.6.3 Virulence factors of *E. coli* associated with post-weaning diarrhoea 29
2.6.4 Pathogenesis of Post-weaning diarrhoea 30
2.7 Vaccination against pathogenic bacteria 31
2.8 Results of meta-analysis 31
2.9 References 52
Chapter three 70
Antimicrobials usage in pig production: effects on *Escherichia coli* resistance and virulence profiles. 71
Preface 71
3.1 Abstract 71
3.2 Introduction 72
3.3 Materials and Methods 73
3.3.1 Approvals, animal care and welfare 73
3.3.2 Study design 74
3.3.3 Sample collection 77
3.3.4 Classical Microbiological analysis 77
3.3.4.1 *E. coli* Isolation 77
3.3.4.2 Antibiotics resistance testing. 77
3.3.5 Molecular characterization of *E. coli* isolates 78
3.3.5.1 DNA Extraction. 78
3.3.5.2 DNA amplification using polymerase chain reaction (PCR). 78
3.3.5.3 Agarose gel electrophoresis, gel documentation and interpretation of the results 79
3.3.5.4 Statistical analysis 80
3.4 Results 80
3.4.1 Virulence genes and adhesion factors 82
3.4.2 Antibiotic resistance 85

3.4.3 Tetracycline resistance genes 88
3.5 Discussion 94
3.6 References 98
Chapter Four 105
Effect of farm antibiotics usage on fecal microbiome of growing pigs 106
Preface 106
4.1 Abstract 106
4.2 Introduction 107
4.3 Materials and methods 109
4.3.1 Sample collection 109
4.3.2 DNA extraction, PCR amplification and sequencing 110
4.3.3 16S rRNA sequence analysis 110
4.3.4 Statistical Analysis 111
4.4 Results and discussion 111
4.5 References 122
Chapter Five 127
5.1 General discussion conclusion and recommendation. 128
Appendix 131

List of Tables

Table 2.1: Important pathotypes of pathogenic *E. coli* in pigs and associated virulent traits .27
Table 2.2: *Escherichia coli* and its virulence profiles in pigs.....33
Table 2.3: Prevalence of *Escherichia coli* virulence factors in piglets.....40
Table 2.4: *Escherichia coli* virulence factors in piglets based on published information from different countries.....43
Table 2.5: Prevalence of *Escherichia coli* virulence factors in weaners 45
Table 2.6: *Escherichia coli* virulence factors in weaners based on published information from different countries.....47
Table 2.7: Prevalence of *Escherichia coli* virulence factors in porkers 49
Table 2.8: *Escherichia coli* virulence factors in porkers based on published information from different countries.....51
Table 3.1: Disk contents of antibiotics and resistance break points used for disk diffusion testing of the *E. coli* isolates. 80
Table 3.2: Primer sequences and amplicon sizes used for PCR detection of virulence genes, virulence factors and tetracycline resistance genes 81
Table 3.3: Frequency of *E. coli* isolates, virulence genes, adhesion factors and pathotypes from E. coli isolates with virulence genes 84
Table 3.4: Frequency of occurrence of phenotypic Antibiotic resistance during the growing period 87
Table 3.5: Frequency of each of tet genes observed and occurrence of tetracycline resistance genes during the growing period.....89
Table 4.1: Diversity measures during the growing period.....118
Table 4.2: Mean and standard deviation of alpha diversity indices during the growing period. 118

List of Figures

Figure 2.1: Forest plot of *Escherichia coli* prevalence factors in piglets 42
Figure 2.2: Forest plot for prevalence of *Escherichia coli* virulence factors in weaners 46
Figure 2.3: Forest plot for prevalence of *Escherichia coli* virulence factors in porkers 50
Figure 3.1: Standard farrowing pen with creep area, farrowing crate, concrete and vented floor.....76
Figure 3.2: . Frequency of isolation of virulence genes in percentages based on days of sampling.....83
Figure 3.3: Frequency of occurrence of antibiotic resistance for each of the antibiotics tested in this study.....86
Figure 3.4a and b: Frequency of phenotypic combination of resistance among the seven antibiotics tested and genotypic combination of tet genes observed within each of the resistant *E. coli* isolates for both groups. 91
Figure 3.5: Gel picture showing STa, STb and LT bands for *E. coli* isolates that carried virulence genes.....91
Figure 3.6: Gel picture showing Stx1, Stx2 and Stx2e bands for *E. coli* that harbored virulence genes.....92
Figure 3.7:Gel picture showing bands for *E. coli* isolates that harbored EAST1 gene. 92
Figure 3.8:Gel picture showing bands for *E.coli* isolates that carried the F6 gene 93
Figure 3.9: Gel picture showing bands for *E.coli* isolates that harbored AIDA, eae and paa virulence factors.....93
Figure 3.10: Gel picture showing PCR bands of *E. coli* isolates that carried the tet A, B, C and E resistance genes 94
Figure 4.1: Venndiagrams showing bacterial compositional differences between the two groups.....117
Figure 4.2: Relative abundance of the most abundant phyla, classes and genera. 120
Figure 4.3: Anosim plot, showing the dissimilarities in community composition between the two groups and within the age groups 120
Figure 4.4: Ordination plots for the Antibiotics and Non – antibiotics groups 121

List of Abbreviations

AE Attaching and effacing

AIDA Adhesion involved in diffuse adherence

ANOSIM Analysis of Similarity

ATG Antibiotics-treated group

ED Edema disease

EU European Union

LEE Locus of enterocyte effacement

LT Heat labile toxin

MAC Mycobacterium avium-intracellulare complex

NAG Non-antibiotics group

NGS Next generation sequencing

OTU Operational taxonomic units

PCR Polymerase chain reaction

PRDC Porcine respiratory disease complex

ST Heat stable toxin

STEC Shiga like toxin-producing *Escherichia coli*

BLAST Basic Local Allignment Search Tool

DAEC diffusely adherent *E.coli*

EAEC Enteroaggregative *E.coli*

EDTA Ethylenediaminetetraacetic acid

EHEC Enterohaemorrhagic *E.coli*

EPEC Enteropathogenic *E.coli*

ETEC Enterotoxigenic *E. coli*

ExPEC Extra-intestinal pathogenic *E.coli*

IMViC Indole, Methyl red, Voges-Proskauer and Citrate tests

Stx Shiga toxin

TAE Tris-acetate-EDTA

Tet Tetracycline

Publications arising from this work

Published:

Abubakar R H., Madoroba E, Adenubi O , Morar-Leather D, Fasina F. O . 2017. Bacteria pathogens of pigs with particular reference to *Escherichia coli*: A systematic review and meta-analysis. *Journal of Veterinary Medicine and Animal Health*, 9(7): 159-185

Submitted

Abubakar R H., Madoroba E, Fasanmi O.G., Fasina F. O. Antimicrobials usage in pig production: effects on *Escherichia coli* resistance and virulence profiles. *Journal of Infection in Developing Countries*.

Manuscript in preparation

Abubakar R.H., Kamutando C.N., Valverde A., Madoroba E., Cowan D.A., Fasina F.O. Effect of farm antibiotics usage on fecal microbiome of growing pig.

Abstract

Microbial community in the gut of pigs provides a vast and complex microbial network of community diversity important for its health and development. Pathogenic *Escherichia coli* are responsible for acute profuse diarrhoea with resultant high morbidity and mortality. Antibiotics are used as growth promoters and for therapeutic purposes in pigs. Misuse, abuse and overuse of these antibiotics have led to development of resistant bacterial strains. This study reports the effect of antimicrobial usage on frequency in which growing pigs habour ETEC and VTEC virulence genes and compared phenotypic and genotypic antibiotic resistance pattern of *E. coli* and metagenomics analysis of fecal samples collected from; (i) pigs receiving normal farm treatment without antibiotics usage, over a 70 day period, and (ii) pigs allowed treatment with antibiotics and monitored over a 70 day period. Our hypothesis was that the use of antibiotics in commercial pig farms affect gut microbial population. A total of 241 *E.coli* strains were isolated and antibiotics resistance testing through disk diffusion and PCR was conducted. Sequencing was also done using the Miseq Illumina platform. Virulence genes were detected in [24.8% ($Cl_{95\%}$: 18.2-32.7)] of the antibiotic group isolates and [43.5% ($Cl_{95\%}$: 34.5-52.9)] of the non antibiotic group with a significant difference (P=0.002). Phenotypic resistance to oxytetracycline was most common and were significant (P = 0.03) in samples of days 10 (P = 0.02) and 21 (P = *0.01*). Furthermore, [63.9% ($Cl_{95\%}$: 57.6, 69.7)] possesed one or more of the four tested tetracycline resistance genes. Significant statistical difference exists in bacterial structure and composition in the gut of growing pigs $P<0.05$. Firmicutes, Bacteriotedes and Proteobacteria were the three most abundant phyla and composition was statistically significant during the growing period. The study showed that antibiotics usage increases gut bacterial population in growing pigs. Disease causing virulence genes and antibiotics resistance genes may occur even without antibiotics usage in growing pigs and other factors may be involved.

Chapter one:

General Introduction

1.1 Introduction

1.1.1 Growth and gut development in pigs

Pigs are omnivorous in nature consuming food of both plant and animal origin similar to humans (Kantharidis, 2014). The normal gastrointestinal tract (gut, alimentary canal) bacterial flora is established in the newborn animal soon after birth from the environment, and then later influenced by the intake of food (Sørum and Sunde, 2001). Other factors that influence the gut bacterial flora are supplementation of organic acids in the feed of fattening pigs (Swann, 1969); feeding prebiotics to piglets (Janczyk *et al.,* 2010) and antibacterial compounds (Sørum and Sunde, 2001).

The gut of pigs is a complex environment, particularly in the newborns and around the time of weaning (Pluske, Hampson and Williams, 1997). It is a tube like structure with regions performing different functions which provide optimal conditions for the digestion and absorption of ingestas as well as emptying of undigested material (Low, 1990). The size, volume and morphological structure of the gut adapt to the composition of the feeds provided at different ages of the pig life (van Beers-Schreurs *et al.,* 1998; Vente-Spreeuwenberg and Beynen 2003) as a result of digestive, secretory and microbial processes, but in the large intestine, the normal flora is the main contributor to the hydrolytic capacity of the feed (Louis *et al.,* 2007).

Piglets are weaned at approximately 3 weeks of age and are exposed to psychological stressors such as separation from the sows, mixing with unfamiliar littermates and establishment of social hierarchy within the group (Moeser and Blikslager, 2007). Weaned pigs take about 7 days to learn how to eat and take up a level of dry matter (Pluske, Hampson and Williams, 1997). This is due to a significant remodelling of the entire gut, which includes changes in the biochemical, physiological and immunological functions of the gut that occurs during the transition from mother's milk to solid feed after weaning (van Beers-Schreurs *et al.,* 1998; Spreeuwenberg *et al.,* 2001).

Gut health is proposed to consist of three components namely; diet, the mucosa and the normal flora. The mucosa and commensal bacteria interact with each other to form a fragile and dynamic equilibrium resulting in the efficient function of the digestive system and ensuring adequate absorption capacity (Conway, 1994). In this respect, valuable dietary component is necessary to ensure a good gut health with a capacity to stabilize the equilibrium (Knudsen, Hedemann and Lærke, 2012).

1.1.2 Pig Management systems

Management systems in animal production are referred to as a set of different production techniques namely; feeding systems, housing, breeding, nutrition, genetics and general animal health (Whittemore and Kyriazakis, 2008). Some studies showed that a few decades ago, pig production in some parts of Europe was done on small mixed farms usually in combination with crops and dairy cows otherwise known as organic production system (Basset-Mens and VanderWerf 2005). Currently, the intensive production system is preferred with few farmers and consumers still preferring the organic production system (Petit and vanderWerf 2003). In the intensive production system, pigs are raised on slated-floor confined buildings with high animal density while in the organic system, pigs are born and raised outside until weaning and then moved to an open front straw litter building. Pigs are raised at low animal density and feed and housing materials sourced from the farm (Basset-Mens and VanderWerf 2005). Another study on management of pigs in some African villages indicated that pigs are raised outdoors and sometimes confined at night. They scavenge for food during the day and sometimes fed by the farmers 2-3 times a day on local food stuff such as kitchen leftovers, ground maize, dry fish, cassava, sweet potatoes, fruits and vegetables (Mutua *et al.,* 2012)

1.1.3 Use of Antibiotics in pigs

Antibacterials are substances used in the treatment and prevention of bacterial infection. They may either kill or inhibit the growth of bacteria (Darwish *et al.,* 2013). They are used in livestock for bacterial infection and also to prevent transfer of food-borne diseases to the general public (Ungemach *et al.*, 2006). A discovery in the 1950s showed that addition of small quantities of penicillin in the feed of young chickens resulted in noticeable increase in growth and this has led to the use of a variety of chemicals as feed additives (growth promoters) in food animals (Bates, 1997). Some other substances used for this purpose include anabolics, nonspecific chemicals (copper, arsenicals, cobalt), and rumen fermentation modifiers (Bates, 1997).

Antibacterial growth promoters are usually mixed with livestock feed to improve feed conversion efficiency (Reti *et al.,* 2013). They are usually administered in sub-therapeutic doses in the feed to increase animal weight gain per unit of feed consumed (Ørskov and Ørskov, 1992). It has been shown that animals receiving antibacterials as growth promoters in their feed had an estimated growth improvement of between 4 and 8%, whereas feed utilization improved by 2 to 5% (Witte, 1998). Hence, supplementing animal feed with low

doses of antimicrobial agents has become a common practice in modern agriculture worldwide. These products improve feed conversion, animal growth and reduce morbidity and mortality due to clinical and subclinical diseases (Ewing and Cole, 1994).

In South Africa, antibacterials are used largely for the purpose of disease treatments, prophylaxis as well as growth promotion in food animals such as ruminants, poultry and pigs (Eagar, 2008). Generally in pig production, two-thirds (66.7%) of all antibiotics used are for growth promotion; while in poultry, it is about 90% (Aarestrup, 2012). However, adverse effects following the use of antibacterial compounds as feed additives has been a subject of argument for several decades and some studies suggested that antibacterial use as growth promoter should follow specific guidelines to prevent resistant bacteria development (Overland *et al.,* 2000).

Though, it was predicted that the use of antibacterials as growth promoters would have a devastating effect on productivity and economy (Emborg *et al.,* 2001), however, there was no negative effect observed on either the total kilograms of chickens produced per square meter, or the amount of feed consumed following reduction in the use of antibacterials (Emborg *et al.,* 2001). In pigs, reducing antibacterials showed no negative effects on productivity, number of pigs produced per sow, average daily weight gain or the amount of feed needed to produce a kilogram of meat (Aarestrup *et al.,* 2010). Furthermore, study on meat (fresh and retail samples) obtained from cattle dosed with subtherapeutic level of antibacterials as feed additive found high incidence of resistant bacteria (Manie *et al.,* 1999).

1.1.4 Antibiotic resistant bacteria

Antibiotics refer to natural, synthetic or semi-synthetic drugs which prevents the growth of sensitive microorganisms when used at low concentrations. They are commonly administered by the oral route in pigs (Callens *et al.,* 2012; Merle *et al.,* 2012) and oral administration of antibiotics has been shown to increases the risk of antibiotic resistance development in some studies (Taylor *et al.,* 2009; Varga *et al.,* 2009; Lutz *et al.,* 2011; Burow *et al.,* 2014). This could be due to oral administration of drugs frequently carried out in a large group of animals at the same time which may result in inadequate application leading to prolonged period of treatment, and under dosing of the drug favouring the selection of bacterial resistance (Ungemach, *et al*., 2006).

However, resistance to the antibiotics used to treat bacterial infections usually results in adverse effects (Paul *et al.,* 2010). The infections caused by resistant bacteria usually fail to

respond to treatment by the specific antibiotic, consequently resulting in increased economic cost, associated with increased morbidity and mortality (Rice, 2009). Moreso in humans, infections caused by resistant bacteria results in financial burden to healthcare systems, and societies through the worsening or persistence of illness and ensuing in-hospital treatment, with potentially critical health consequences (Capita and Alonso-Calleja, 2013).The European Union (EU) in 1999 restricted antibiotics use as growth promoters and in 2006 banned its use (Avguštin, 2012).

1.1.5 Mechanism of antibiotic resistance

Antibiotic resistance in bacteria develops mainly by the Darwinian process of the survival of the fittest (Rosenblatt-Farrell, 2009). Most antimicrobial-resistant organisms have emerged as a result of genetic changes, obtained through mutation (vertical transfer) or by horizontal transfer from other bacterial organisms (FVE, 2002). Resistance due to mutation occurs as a result of spontaneous changes at a locus on the bacterial chromosome that controls susceptibility to a specific antibiotic (Beinlich, *et al.*, 2001). Horizontal transfer mechanisms occur through conjugation, transduction and transformation. Exchange of conjugative plasmids is the commonest and most effective genetic transfer (Alanis, 2005). However, bacterial resistance genes may also be acquired through spread of transposons or integrons (Capita and Alonso-Calleja, 2013). Horizontal gene transfer mechanism has an important role in enhancing the spread of antibiotic resistance. This spread can occur between strains of the same bacterial species or between different bacterial species (Capita and Alonso-Calleja, 2013). Resistance to antibiotics can be innate; where the bacterial species are not susceptible to a particular antibiotic and the bacterial species are not affected by use or misuse of the antibiotics (Capita and Alonso-Calleja, 2013). When a specific bacterial population in humans, animals or food substance is acted on by a stress factor e.g antibacterial compound, all susceptible bacterium will die, but not those that are resistant by chance. These bacteria will survive and multiply, producing a resistant progeny (World Health Organization, 2002).

Antibacterials fight bacteria through different mechanisms including; damaging or inhibiting the synthesis of bacterial cell walls e.g penicillins; effects on the synthesis of bacterial DNA or RNA e.g quinolones, proteins e.g tetracyclines, or metabolic pathways e.g sulphonamides (Rosenblatt-Farrell, 2009). Bacterial methods for resisting the effects of antibiotics include biofilm formation, changes in surface permeability, enzymatic inactivation of the compound before it reaches its target site, modification or overproduction of the target site, and

acquisition of alternative metabolic pathways to those inhibited by the drug (Institute of Food Technologists, IFT., 2006).

1.1.6 Methods of transfer of antibiotic resistant bacteria from animals to humans

Farmers started the use of antibiotics for livestock in the late 1940s and ever since infections with strains of bacteria resistant to those antibiotics have been noticed in people (Aarestrup, 2000; Aarestrup, *et al.,* 2008; World Health Organization, 2012). The most important reservoir of multidrug resistant enterobacteria is the gut of animals and humans, especially those that have received antibiotic treatments. The most common route of spread of resistant bacteria is through contamination of food, water and the environment (Wellington *et al.,* 2013). Resistant bacteria in animals linger on skin and feces which can easily be transferred into meat, then to the hands of people preparing it, including the kitchen environment (Aarestrup, 2012). Spread of Antibiotic resistance throughout the food chain is basically as a result of the selective pressure exerted by the use of antibiotics in animal production and use of biocides such as disinfectants and feed preservatives (Capita and Alonso-Calleja, 2013).

1.2 Justification

Currently, antibiotic resistance is a global public health challenge and it involves all major microbial pathogens and antimicrobial drugs, which is a problem to both current and future generations (Capita and Alonso-Calleja, 2013). If the rise in antibiotic resistance cannot be reversed, then, a significant rise in incurable infection and death in both developed and developing regions may be observed (Wellington *et al.,* 2013). It has been estimated that the amount of antibiotics consumed by livestock worldwide is about double that used by humans (Aarestrup, 2000; World Health Organization, 2012). Furthermore reports in the United States of America also showed that about 300 milligrams of antibiotics are used to produce every kilogram of meat and eggs (Aarestrup, 2000; FAO, 2010). Also, avoparcin, a growth promoter used in poultry and pig production in Europe has been shown to result in the incidence of vancomycin-resistant enterococci in the normal gut bacteria of these animals (Klare *et al.,* 1995). However, another study on vancomycin resistant enterococci in broilers and pigs showed a statistically significant decrease in resistance of normal flora in broilers but not in pigs after a two and a half year period of ban (Bager *et al.,* 1999). These results underline the importance of having a low antibiotic exposure of animals before they are slaughtered to make sure that as few resistant bacteria as possible are exposed to the meat consumers (Sørum and Sunde, 2001). A study on the enteric bacterial population dynamics in growing pigs will provide clear information on the bacterial population of the pig at different

stages of their life with regards to antibiotics resistance. This will also provide information on whether gut bacterial resistance is transferred to meat during processing and enable informed methods of handling the gut during meat processing to avoid the transmission of the resistant bacteria to the human population.

1.3 Hypothesis

H_0-The pigs gut bacterial flora differs at different growth stages and antibiotics administration influence antibacterial resistance development in growing pigs.

1.4 Aim and Objectives

The overall aim of the study was to monitor pigs from day old until day 70 in order to determine enteric bacteria development and to quantify its population dynamics with specific regards to bacterial resistance following treatments with commonly used antibiotics.

The specific objectives of this study include:

To determine distribution of antibiotics resistance of *Escherichia coli* in a commercial farm setting in growing pigs.

To characterize the virulence profile of *Escherichia coli* in growing pigs.

To monitor the development of enteric bacterial population in growing pigs through metagenomics analysis.

1.5 References

Aarestrup, F. (2012) 'Sustainable farming: Get pigs off antibiotics', *Nature,* 486(7404), pp 465-466.

Aarestrup, F.M., Jensen, V.F., Emborg, H., Jacobsen, E. and Wegener, H.C. (2010) 'Changes in the use of antimicrobials and the effects on productivity of swine farms in Denmark', *American Journal of Veterinary Research,* 71(7), pp. 726-733.

Aarestrup, F.M., Wegener, H.C. and Collignon, P. (2008) 'Resistance in bacteria of the food chain: epidemiology and control strategies', *Epert Review Anti- infective Therapy,* 6 (5), pp. 733-750.

Aarestrup, F.M. (2000) 'Occurrence, selection and spread of resistance to antimicrobial agents used for growth promotion for food animals in Denmark: Chapter 1: Introduction', *Apmis,* 108(S101), pp. 5-6.

Alanis, A.J. (2005) 'Resistance to antibiotics: are we in the post-antibiotic era?', *Archives of Medical Research,* 36(6), pp. 697-705.

Avguštin, J. (2012) 'Animal production systems as a selective environment for antibiotic resistance genes', *Acta agriculturae Slovenica,* 100(1), pp. 7-17.

Bager, F., Aarestrup, F.M., Madsen, M. and Wegener, H.C. (1999) 'Glycopeptide resistance in Enterococcus faecium from broilers and pigs following discontinued use of avoparcin', *Microbial Drug Resistance,* 5(1), pp. 53-56.

Basset-Mens, C. and Van der Werf, Hayo MG (2005) 'Scenario-based environmental assessment of farming systems: the case of pig production in France', *Agriculture, Ecosystems & Environment,* 105(1), pp. 127-144.

Bates, J. (1997) 'Epidemiology of vancomycin-resistant enterococci in the community and the relevance of farm animals to human infection', *Journal of Hospital Infection,* 37(2), pp. 89-101.

Beinlich, K.L., Chuanchuen, R. and Schweizer, H.P. (2001) 'Contribution of multidrug efflux pumps to multiple antibiotic resistance in veterinary clinical isolates of Pseudomonas aeruginosa', *FEMS microbiology letters,* 198(2), pp. 129-134.

Burow, E., Simoneit, C., Tenhagen, B. and Käsbohrer, A. (2014) 'Oral antimicrobials increase antimicrobial resistance in porcine E. coli–A systematic review', *Preventive veterinary medicine,* 113(4), pp. 364-375.

Callens, B., Persoons, D., Maes, D., Laanen, M., Postma, M., Boyen, F., Haesebrouck, F., Butaye, P., Catry, B. and Dewulf, J. (2012) 'Prophylactic and metaphylactic antimicrobial use in Belgian fattening pig herds', *Preventive veterinary medicine,* 106(1), pp. 53-62.

Capita, R. and Alonso-Calleja, C. (2013) 'Antibiotic-resistant bacteria: a challenge for the food industry', *Critical reviews in food science and nutrition,* 53(1), pp. 11-48.

Conway, P. (1994) 'Function and regulation of the gastrointestinal microbiota of the pig. In: Souffrant, W., Hagemeister, H. (Eds.)', *Proceedings of the VIth International Symposium on Digestive Physiology in Pigs,* EAAP Publication no. 80, Dummerstof, pp. 231–240.

Darwish, W.S., Eldaly, E.A., El-Abbasy, M.T., Ikenaka, Y., Nakayama, S. and Ishizuka, M. (2013) 'Antibiotic residues in food: the African scenario', *Japanese Journal of Veterinary Research,* 61(Supplement), pp. S13-S22.

Eagar Anne Hayley (2008) A survey of antimicrobial usage in animals in South Africa with specific reference to food animals*. Msc Pharmacology*.

Emborg, H., Ersbøll, A.K., Heuer, O.E. and Wegener, H.C. (2001) 'The effect of discontinuing the use of antimicrobial growth promoters on the productivity in the Danish broiler production', *Preventive veterinary medicine,* 50(1), pp. 53-70.

Ewing, W.N. and Cole, D.J.A. (1994) The living gut: an introduction to micro-organisms in nutrition*.published by Context, 117 Carrycastle Road, Dungannon Ireland*, pp. 220

FAO (2010) 'Food and Agricultural Commodities Production ', *http:// go.nature.com/hzrvqt FAOSTAT,*

FVE (2002) 'Antibiotic resistance and prudent use of antibiotics in veterinary medicine', *http://www.fve.org/news/publications/pdf/antibioen.pdf,* Federation of Veterinarians of Europe, Brussels. accessed: January 7, 2016.

Institute of Food Technologists, IFT. (2006) 'Antimicrobial resistance: Implications for the Food System', *Compr.Rev.Food Sci.Food Safety,* 5(3):71–137.

Janczyk, P., Pieper, R., Smidt, H. and Souffrant, W.B. (2010) 'Effect of alginate and inulin on intestinal microbial ecology of weanling pigs reared under different husbandry conditions', *FEMS microbiology ecology,* 72(1), pp. 132-142.

Kantharidis, B. (2014) 'pig-and-human-digestive-system', *https://prezi.com/-hqscyhayi5x/,* Accessed 15 January, 2016.

Klare, I., Heier, H., Claus, H., Reissbrodt, R. and Witte, W.v. (1995) 'vanA-mediated high-level glycopeptide resistance in Enterococcus faecium from animal husbandry', *FEMS microbiology letters,* 125(2-3), pp. 165-171.

Knudsen, K.E.B., Hedemann, M.S. and Lærke, H.N. (2012) 'The role of carbohydrates in intestinal health of pigs', *Animal Feed Science and Technology,* 173(1), pp. 41-53.

Louis, P., Scott, K.P., Duncan, S.H. and Flint, H.J. (2007) 'Understanding the effects of diet on bacterial metabolism in the large intestine', *Journal of applied microbiology,* 102(5), pp. 1197-1208.

Low, A. (1990) 'Nutritional regulation of gastric secretion, digestion and emptying', *Nutrition research reviews,* 3(01), pp. 229-252.

Lutz, E.A., McCarty, M.J., Mollenkopf, D.F., Funk, J.A., Gebreyes, W.A. and Wittum, T.E. (2011) 'Ceftiofur use in finishing swine barns and the recovery of fecal Escherichia coli or Salmonella spp. resistant to ceftriaxone', *Foodborne pathogens and disease,* 8(11), pp. 1229-1234.

Manie, T., Brözel, V.S., Veith, W.J. and Gouws, P.A. (1999) 'Antimicrobial resistance of bacterial flora associated with bovine products in South Africa', *Journal of Food Protection,* 62(6), pp. 615-618.

Merle, R., Hajek, P., Käsbohrer, A., Hegger-Gravenhorst, C., Mollenhauer, Y., Robanus, M., Ungemach, F. and Kreienbrock, L. (2012) 'Monitoring of antibiotic consumption in livestock: a German feasibility study', *Preventive veterinary medicine,* 104(1), pp. 34-43.

Moeser, A.J. and Blikslager, A.T. (2007) 'Mechanisms of porcine diarrheal disease', *Journal of the American Veterinary Medical Association,* 231(1), pp. 56-67.

Mutua, F.K., Dewey, C., Arimi, S., Ogara, W., Levy, M. and Schelling, E. (2012) 'A description of local pig feeding systems in village smallholder farms of Western Kenya', *Tropical animal health and production,* 44(6), pp. 1157-1162.

Ørskov, F. and Ørskov, I. (1992) 'Escherichia coli serotyping and disease in man and animals', *Canadian journal of microbiology,* 38(7), pp. 699-704.

Overland, M., Granli, T., Kjos, N., Fjetland, O., Steien, S. and Stokstad, M. (2000) 'Effect of dietary formates on growth performance, carcass traits, sensory quality, intestinal microflora, and stomach alterations in growing-finishing pigs.', *Journal of animal science,* 78(7), pp. 1875-1884.

Paul, M., Shani, V., Muchtar, E., Kariv, G., Robenshtok, E. and Leibovici, L. (2010) 'Systematic review and meta-analysis of the efficacy of appropriate empiric antibiotic therapy for sepsis', *Antimicrobial Agents and Chemotherapy,* 54(11), pp. 4851-4863.

Petit, J. and van der Werf, Hayo MG (2003) 'Perception of the environmental impacts of current and alternative modes of pig production by stakeholder groups', *Journal of environmental management,* 68(4), pp. 377-386.

Pluske, J.R., Hampson, D.J. and Williams, I.H. (1997) 'Factors influencing the structure and function of the small intestine in the weaned pig: a review', *Livestock Production Science,* 51(1), pp. 215-236.

Reti, K.L., Thomas, M.C., Yanke, L.J., Selinger, L.B. and Inglis, G.D. (2013) 'Effect of antimicrobial growth promoter administration on the intestinal microbiota of beef cattle', *Gut pathogens,* 5(1), pp. 1-17.

Rice, L.B. (2009) 'The clinical consequences of antimicrobial resistance', *Current opinion in microbiology,* 12(5), pp. 476-481.

Rosenblatt-Farrell, N. (2009) 'The landscape of antibiotic resistance', *Environmental Health Perspectives,* 117(6), pp. A244-A250.

Sørum, H. and Sunde, M. (2001) 'Resistance to antibiotics in the normal flora of animals', *Veterinary research,* 32(3-4), pp. 227-241.

Spreeuwenberg, M., Verdonk, J., Gaskins, H. and Verstegen, M. (2001) 'Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning', *The Journal of nutrition,* 131(5), pp. 1520-1527.

Swann, M.M. (1969) *Report Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine.* HM Stationery Office.

Taylor, N., Clifton-Hadley, F., Wales, A., Ridley, A. and Davies, R. (2009) 'Farm-level risk factors for fluoroquinolone resistance in E. coli and thermophilic Campylobacter spp. on finisher pig farms', *Epidemiology and infection,* 137(08), pp. 1121-1134.

Ungemach, F.R., Müller-Bahrdt, D. and Abraham, G. (2006) 'Guidelines for prudent use of antimicrobials and their implications on antibiotic usage in veterinary medicine', *International Journal of Medical Microbiology,* 296, pp. 33-38.

van Beers-Schreurs, H.M., Nabuurs, M.J., Vellenga, L., Kalsbeek-van der Valk, H.J., Wensing, T. and Breukink, H.J. (1998) 'Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood', *The Journal of nutrition,* 128(6), pp. 947-953.

Varga, C., Rajić, A., McFall, M.E., Reid-Smith, R.J. and McEwen, S.A. (2009) 'Associations among antimicrobial use and antimicrobial resistance of Salmonella spp. isolates from 60 Alberta finishing swine farms', *Foodborne pathogens and disease,* 6(1), pp. 23-31.

Vente-Spreeuwenberg, M. and Beynen, A. (2003) 'Diet-mediated modulation of small intestinal integrity in weaned piglets', *Weaning the Pig: Concepts and Consequences.Wageningen Academic Publishers, The Netherlands*, pp. 145-198.

Wellington, E.M., Boxall, A.B., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D.L., Lee, N.M. and Otten, W. (2013) 'The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria', *The Lancet infectious diseases,* 13(2), pp. 155-165.

Whittemore, C.T. and Kyriazakis, I. (2008) *Whittemore's science and practice of pig production.* John Wiley & Sons.

Witte, W. (1998) 'Medical consequences of antibiotic use in agriculture', *Science,* 279(5353), pp. 996.

World Health Organization (2012) 'The evolving threat of antimicrobial resistance: options for action. 2012', *published by 20 Avenue Appia, 1211 Geneva 27 Switzerland* pp.119

World Health Organization, W. (2002) 'Antimicrobial resistance. ', *http://www.who.int/mediacentre/factsheets/fs194/en/.*, accessed: January 8, 2016.

Chapter Two

Literature Review

Bacteria pathogens of pigs with particular reference to *Escherichia coli*: A systematic review and meta-analysis

Rukkaya H. Abubakar, Evelyn Madoroba, Olubukola Adenubi, Darshana Morar-Leather, Folorunso O. Fasina

Preface

This chapter provides an extensive literature survey on the bacteria pathogens of pigs grouped by areas of primary lesions with specific reference to *Escherichia coli* and its associated infections. The text has been published: Abubakar R H., Madoroba E, Adenubi O , Morar-Leather D, Fasina F. O . 2017. Bacteria pathogens of pigs with particular reference to *Escherichia coli*: A systematic review and meta-analysis. Journal of Veterinary Medicine and Animal Health. 9(7): 159-185.

2.1 Abstract

Pigs are ungulate animals of the genus *Suis* and family Suidae. They are globally spread but restricted in certain countries due to religious and cultural beliefs. Pork serves as an important source of protein (38% of meat consumed in the world). While pig production remains a profitable enterprise, commercial and particularly the small-scale farmers face huge constraint in this husbandry practice, one of the most important being bacterial infections and its associated morbidity and mortality. In this study, we reviewed the prevalence of bacterial infections in pigs with particular reference to *Escherichia coli*. Literatures were searched on selected veterinary and biological data bases in 2016 with focus on natural infections and isolates from natural infections with epidemiological details. Pathotypes, serotypes and serogroups of *E. coli*, the country of origin, source, growth stage, age of pigs infected, disease outbreak, the number of samples and type of samples, numbers and percentage of positive samples and isolates were used as filters. Pathotypes reported include enterotoxigenic *E.coli* (ETEC) 66.7%, enterotoxigenic *E.coli* and shiga toxigenic *E.coli* (ETEC & STEC) 14.3%, STEC only (7.9%), enterotoxigenic *E.coli*/enteropathogenic *E.coli*/enteroaggregative *E.coli* (ETEC/EPEC/EAE) 31.7%. Others were enterohaemorrhagic *E.coli* (EHEC), diffusely adherent *E.coli* (DAEC) (ETEC, EPEC, STEC) and extra-intestinal pathogenic *E.coli* (ExPEC). Twenty-nine countries with documented records of cases of *E.coli* were included with the USA reporting the highest number followed by China. About 74% of the samples were taken from farms and others were from samples submitted to research laboratories and veterinary faculties for necropsy. Serogroups O141, O149, O139, O138, O8 and O9 were most common. Piglets were most affected (52.3%) followed by weaners (39.6%) and porkers (7.9%) with age ranging from between 1-392 days old. A total of 24,854 isolates were considered and 10477 (42.2%) were positives and the following genes were haboured: STa, STb, LT, stx1, stx2, Stx-2e, F4, F5, F6, F18, F41, AIDA, EAST1, eae, paa and hlyA. The diseases produced by *E. coli* were neonatal diarrhoea, colibacillosis, post-weaning diarrhoea and edema disease. The associated risk factors were poor housing, management and feed changes, extensive use of antibiotics as prophylaxis, overcrowding, and high humidity and temperature changes. India, USA, Japan, Slovakia, Denmark Sweden and Poland were countries with significant reports and high detection of virulence factors (72-100%).

2.2 Introduction

Pigs are ungulate animals of the genus *Suis* and family Suidae. Domesticated pigs originated from the European wild boar *Sus scrofa* and are indigenous to the Eurasian and African continents (Giuffra *et al.,* 2000).The global pig population is estimated to be approximately one billion (Statista, 2016), and although this spread across the world, it may be restricted in certain countries due to religious and cultural beliefs. Pork serves as an important source of protein (38% of meat consumed in the world) and as a means of livelihood especially for women in developing countries (Madzimure *et al.,* 2012). Pigs are also kept for leather, hair, as pets and for use in human research (Gosh, 2014).

While pig production remains a profitable enterprise, commercial and particularly the small-scale farmers face huge constraints in this husbandry practice, one of the most important being bacterial infections and its associated morbidity and mortality. We reviewed the prevalence of bacterial infections in pigs and paid particular attention to *Escherichia coli*, a bacterium that is regularly isolated and can lead to multiple infections in pigs.

2.3 Methodology

The keywords used to gather literature for review include; " *Escherichia coli* or *E. coli*", "pig, swine or porcine", "outbreak", "diarrhoea", "Oedema disease", "post weaning diarrhoea", "colibacillosis" and "prevalence". Literature searches were performed on selected veterinary and biological databases including the CAB Abstract, Medline, Pubmed, Science Direct and Google Scholar between January and November 2016. Particular consideration was given to natural infections and isolates from natural infections with epidemiological details. Pathotypes, serotypes and serogroups of *E. coli*, the country of origin, source, growth stage, age of pigs infected, disease outbreak, the number of samples and type of samples, numbers and percentage of positive samples and isolates were used as filters. All literature considered were in English or where available the English translations were used.

Extracted and compiled published manuscripts from peer reviewed journals were quality-checked and duplicate documents were removed. All remaining documents (n = 61) were filtered, harmonized and coded in a single Microsoft Excel® spreadsheet. The number of events, sample sizes and outcomes were calculated based on the available data. All data was analysed using the Fixed-effect model (precision-based estimates) in the Meta-analyses software on Excel (Neyellof *et al*., 2012). Comparison between individual studies was calculated in WinPepi v11.24 (Abramson, 2011) and presented in percentages with 95%

confidence intervals. Cumulative events with measures of central tendencies were also produced in forest plots.

2.4 Bacterial pathogens of pigs

Based on our evaluation, the bacteria that affect pigs are diverse and vast including but not limited to the following, grouped by areas of primary lesions:

2.4.1 Cutaneous (skin) associated bacteria

2.4.1.1 *Staphylococcus* species

Infection in pigs is caused by *Staphylococcus hyicus* resulting in exudative epidermitis (Greasy pig disease) (Andresen, 1998). *Staphylococcus hyicus* is composed of both non-virulent and virulent strains which produces an exfoliative toxin responsible for skin alteration in exudative epidermitis of pigs (Wegener *et al*., 1993; Andresen *et al.,* 1997). It is characterized by sudden onset of excess sebaceous secretion and exudation from the skin without pruritus leading to dehydration, growth depression and possibly death (Taylor, 2013). Other Staphylococcus species that could be isolated are *S. chromogenes* and *S. Sciuri* (Chen *et al.,* 2007). *Staphylococcus aureus* is another important and common pathogen isolated from swollen ears, umbilical abscesses, subcutaneous abscesses and foot lesions, (Taylor, 2013; De Neeling *et al.,* 2007).

2.4.1.2 *Treponema* species

Treponema species are associated with skin or mucous membrane diseases and regularly cause skin ulcers in pigs (Karlsson, 2014). Three major phylotypes involved in infections include: *Treponema pedis* (the most predominant), *T.parvum* and an undesignated phylotype (Karlsson, 2014). *Treponema pedis* infection can occur as cutaneous spirochaetosis, ear necrosis and spirochaetal granuloma. They have been indicated as secondary bacterial infection in severe and chronic skin lesions of pigs (Taylor, 2013; Karlsson, 2014), such as ulcerative porcine stomatitis (Jensen *et al.,* 2014) and periodic outbreaks of ear necrosis among weaners and gingival infections (Pringle *et al.,* 2009; Karlsson *et al.,* 2013).

2.4.2 Reproductive system associated bacteria

2.4.2.1 *Leptospira* species

Leptospirosis, caused by *Leptospira* species is a disease that occurs worldwide in pigs and infection is more common in animals kept outdoors (Ryley and Simmons, 1954). Route of infection is by ingestion, direct contact and through abrasions, trans-placental transmission or the veneral route (Taylor, 2013). *Leptospira* species are fine, spiral, aerobic, motile, gram-ve

bacteria (spirochete) about 10µm in length and 0.2µm in diameter (Faine, 1994). Approximately 13 serovars of *Leptospira* are involved in infections in pigs and associated primarily with reproductive losses in breeding herds. The organisms persist in the kidneys and genital tracts of carrier pigs and are excreted in urine and genital fluids (Taylor, 2013). Routine vaccination may reduce the effect of the bacteria in a herd (Whyte *et al.,* 1982), but elimination may be a difficult target because pig is a reservoir host for leptospirosis.

2.4.2.2 *Brucella* species

Brucella suis infection in pigs, occurs mainly through the venereal or oral route (Xavier *et al.,* 2010). Infection develops as bacteremia that may persist for as long as 90 days which may lead to localisation in various tissues with resultant stillbirths, abortions, orchitis, lameness, posterior paralysis, spondylitis, occasional metritis and abscess formation. Infertility may occur in both sexes (Xavier *et al.,* 2010; Deyoe, 1967)**.** Other pathogenic species in pigs are *B. abortus* and *B. mellitensis* (Godfroid, 2002). Diagnosis is mainly through the brucellosis card (Rose Bengal) test; but serum agglutination tests or complement fixation tests have also been used (Nicoletti, 2010). Brucellosis in pigs has a global distribution however its prevalence in domestic pigs is low in some countries and is known to have been eradicated in USA (Godfroid, 2002).

2.4.2.3 *Listeria* species

Infection of pigs with *Listeria* species is common and previous studies in Japan, Denmark and Yugoslavia suggested that approximately 10% of pigs at slaughter are infected (Taylor, 2013; Lungu *et al.,* 2010). *Listeria monocytogenes* infection rarely causes disease, but sudden death in piglets, septicaemia and nervous signs have been recorded. Most authors concurred that porcine listeriosis occurs mainly as a septicaemia in piglets less than ten days old (Ladds *et al.,* 1974; Long and Dukes, 1972; Busch *et al.,*1971) and multifocal hepatic necrosis is often the most notable necropsy finding in piglets (Lopez and Bildfell, 1989).Abortions, stillbirths and birth of weak piglets may also occur in sows (Taylor, 2013; Vannier, 1999). *Listeria* infection and carriage in domestic pigs or wild boar is a potential source of infection for man (Taylor, 2013; Borch *et al.,* 1996).

2.4.2.4 *Erysipelothrix* species

Erysipelothrix rhusiopathiae is a small, facultative anaerobic, Gram positive (G+ve) rod that causes a condition known as swine erysipelas (Opriessnig *et al.,* 2010; Wood and Steele, 1994). The disease outbreak may present as acute, sub-acute or chronic. It is characterized by sudden death or fever associated with characteristic diamond skin lesions (Opriessnig *et al.,*

2010). Arthritis, vegetative endocarditis and abortion in pregnant sows may be observed (Schrauwen *et al.,* 1993). Necropsy lesions include enlarged and congested lymph nodes, oedematous and congested lungs, splenomegaly, hepatomegaly, petechial haemorrhages on the kidneys and heart. *Erysipelothrix rhusiopathiae* causes considerable economic losses and remains an animal hygiene problem in swine production areas of the world (Takeshi *et al.,* 1999), and up to 50% of pigs in the world are estimated to harbour the organism in their tonsils and lymphoid organs (Opriessnig *et al.,* 2010).This status results in shedding of the organism in urine, faeces, saliva and nasal secretions (Opriessnig *et al.,* 2004).

2.4.3 Respiratory System associated bacteria

2.4.3.1 *Actinobacillus* species

Actinobacillus suis and *A. equuli* are two important species that may cause fatal septicaemia, endocarditis and arthritis in pigs of 1-6 weeks of age. In older animals, skin lesions and focal necrotising pneumonia, valvular endocarditis, abortion, metritis and polyarthritis may be seen (Radostits *et al.,* 2000; Ramos-Vara *et al.,* 2008). Clinical signs and post-mortem lesions are not specific but suggestive of the disease (Taylor, 2013). Additionally, *Actinobacillus pleuropneumonia* may cause a respiratory infection of weaned, growing and finishing pigs in which there is fibrinous pleurisy and pneumonia with characteristic infarcts in the lungs. This infection is highly contagious, often fatal and progressive weight loss in chronically affected pigs may be observed (Taylor, 2013; Frank *et al.,* 1992).

2.4.3.2 *Mycoplasma* species

Mycoplasma organisms have been isolated from pigs but only four species have repeatedly been associated with clinical disease namely (1) *M. hyorhinis* which cause polyserositis and arthritis in young pigs of about 3-10 weeks; (2) *M. hyosynoviae* causes arthritis in growing pigs weighing between 35kg and 110kg live weight (Taylor, 2013); (3) *M. hyopneumoniae* which is a primary pathogen of enzootic pneumonia, a chronic respiratory disease in pigs, highly prevalent in almost all pig producing areas. It is also considered to be one of the primary agents involved in the porcine respiratory disease complex (PRDC) (Thacker, 2006). The organism is primarily found on the mucosal surface of the trachea, bronchi and bronchioles (Blanchard *et al.,* 1992); and (4) *M.suis* (formerly *Eperythrozoon suis*) infection which affects piglets 0-5 days of age, but weaners, growers and sows may also be infected. Route of infection is by parenteral, transplacental or oral transmission and the clinical disease may manifests in 5-6 days. The acute phase involves fever, anaemia, icterus, unthriftiness, jaundice and poor growth in weaned pigs, and is associated with low morbidity but high

mortality rates (Messick, 2004). The chronic phase results in low reproductive efficiency, growth retardation, abortions, stillbirths and agalactia in sows (Messick, 2004; Heinritzi, 1989). In addition, a study revealed that *M. arthritidis*, a rodent *Mycoplasma* has also been isolated from joints in an outbreak of conjunctivitis, severe polyarthritis and infertility in a boar stud (Binder *et al.,* 1990).

2.4.3.3 *Bordetella* species

Bordetella bronchiseptica is regarded as the aetiologic agent of atrophic rhinitis, colonises the ciliated epithelium of the upper and lower respiratory tract at about one week of age in piglets. It causes rhinitis characterised by sneezing, shortening or twisting of the snout, hypoplasia, mild nasal turbinate atrophy, persistent purulent bronchitis, haemorrhage, pneumonia and impaired growth (Giles, 1992; Duncan *et al.,* 1966; Mazumder *et al.,* 2012). *Bordetella bronchiseptica* importantly predisposes pigs to colonization and disease with other bacteria such as *Pasteurella multocida* and *Haemophilus parasuis* (Brockmeier, 2004; Brockmeier *et al.,* 2001).

2.4.3.4 *Pasteurella* species

Pasteurella multocida is associated with pneumonia and atrophic rhinitis in pigs and can result in important economic losses on large pig farms worldwide (Davies *et al.,* 2003; Dziva *et al.,* 2008). Its strains are grouped into five capsular serogroups; A, B, D, E and F , however only serogroup A,B and D have been recovered from pigs (Davies *et al.,* 2003; Townsend *et al.,* 1998; Tang *et al.,* 2009). Toxigenic *P. multocida* serogroups A and D together with *Bordetella bronchiseptica,* coexist to cause atrophic rhinitis (Davies *et al.,* 2003; Backstrom *et al.,*1988). Toxigenic *P.multocida* infection results in severe sneezing in non-immune piglets. This is later followed by atrophy of the turbinate bones and a distortion of the nasal septum, shortening and twisting of the upper jaw which may be accompanied by reduction in the rate of weight gain (Taylor, 2013). Similarly, pneumonic pasteurellosis is also a condition that results from the colonisation of existing lung lesions with *P. multocida* which may give rise to fever, respiratory distress and death in some cases and it is typically associated with sub-acute or chronic pleuritis. Common route of transmission is by nose-to-nose contact; however, both vertical and horizontal transfer may occur (Taylor, 2013; Davies *et al.,* 2004). *Mannheimia (Pasteurella) haemolytica* has been isolated in piglets from localised areas of fibrinous pleurisy or pleuropneumonia and from outbreaks of diarrhoea in pigs (Taylor, 2013).

2.4.3.5 *Haemophilus* species

Haemophilus parasuis is found in the upper respiratory tract of pigs as a commensal bacterium, but invades and cause severe systemic disease under favourable conditions (Oliveira *et al.,* 2003).It causes Glassers disease and acute septicaemia (Peet *et al.,* 1983; Riley *et al.,* 1977). Glassers disease is an infectious, sometimes fatal polyserositis, polyarthritis and meningitis of young pigs (Amano *et al.,* 1994). Bronchitis and other syndromes may occur in older animals in non-immune herds (Taylor, 2013).Transmission is by direct contact and all age categories of pigs are susceptible to the infection(Oliveira and Pijoan, 2002).Post mortem lesions include serofibrinous or fibrino-purulent exudate on mucosal surfaces, usually in peritoneum, pericardium, pleura or joint surface. In the septicaemic form, petechial and ecchymotic haemorrhages are detected in liver, kidneys and brain (Amano *et al.,* 1994).

2.4.3.6 *Mycobacterium* species

Several species of *Mycobacterium* such as *M. porcinum, M. avium* subsp. *hominisuis*, *M. bovis*, *M. intracellulare, M. fortuitum* and *M. tuberculosis* have been associated with tuberculosis in pigs. *Mycobacterium avium* subsp. *hominisuis* is an opportunistic pathogen, infecting mainly pigs and humans (Mijs *et al.,* 2002; Inderlied *et al.,* 1993; Thorel *et al.,* 2001). A recent study demonstrated cross-reactions between avian and bovine tuberculin in pigs (Agdestein *et al.,* 2011). *Mycobacteria bovis* is the main agent causing tuberculosis in cattle, while *M. tuberculosis* primarily causes tuberculosis in humans. However, they both belong to the *M. tuberculosis* complex (MTC) and can lead to infections in pigs (Komijn *et al.,* 1999; Biet *et al.,* 2005). Tuberculosis is mostly observed in pigs at slaughter when gross lesions are detected primarily through the examination of the lymph nodes of the head and the visceral regions and partial or full carcasses condemnation usually follow, because it is a potential risk to human health.

2.4.3.7 *Streptococcus* species

Streptococcus suis is an encapsulated gram +v ecoccus and occurs singly, in pairs, or occasionally in short chains. It is a normal flora in the upper respiratory tract of pigs, the genital and digestive tracts (Higgins and Gottschalk, 1999). *S. suis* causes septicaemia, arthritis and meningitis in suckling piglets and post-weaning pigs but less commonly in finishing pigs (Taylor, 2013; Gottschalk *et al.,* 2007). The organism is an emerging zoonotic agent responsible for septicaemia which may sometimes be accompanied by septic shock and meningitis in humans (Goyette-Desjardins *et al.,* 2014).

S. porcinus infection also occurs in pigs and is sometimes referred to as streptococcal lymphadenitis or streptococcal abcess. It causes abscess particularly in the cervical lymph nodes (Taylor, 2013). The Lancefield group C and β-haemolytic streptococci are other streptococci infections in pigs that are commonly isolated from the upper respiratory tract, pharynx, retropharyngeal lymph nodes and genital tract of carrier pigs. They are associated with vaginitis in sows and neonatal septicaemia in newborn piglets and may be isolated from arthritis and vegetative endocarditis in older animals and froms epticaemic and pneumonic lesions in older finishing pigs (Taylor, 2013). A presumptive diagnosis of infection in pigs is usually based on clinical signs and macroscopic lesions (Staats *et al.,* 1997).

2.4.4 Digestive system associated bacteria

2.4.4.1 *Clostridium* species

Clostridial pathogens involved in pig infections include *Clostridium perfringens* (type A, C), *Clostridium defficile typhocolitis, Clostridium tetani, Clostridium novyi, Clostridium botulinum, Clostridium septicum* and *Clostridium chauvoei* (Taylor, 2013; Baker et al., 2010). *Clostridium perfringens* type C is a large gram +ve rod, which occasionally forms spores, bears attachment site and produces very potent toxins. The major toxin produced is protease/trypsin-sensitive β toxin which causes fatal necrotic and haemorrhagic enteritis in piglets less than seven days old and may cause chronic infection in older piglets. Clinical signs include profuse, bloody diarrhoea, loss of weight, palour and death within 12-24 hours (Taylor, 2013; Songer and Meer, 1996).

C. perfringens Type A causes a similar syndrome but less severe with the major toxin produced being the α-toxin (Taylor, 2013; Songer and Uzal, 2005).*Clostridium difficile* is toxigenic and produce two major toxins, A and B (Taylor, 2013; Diab *et al.,* 2016). It is a recognized cause of antibiotic-associated diarrhoea and pseudomembranous colitis in humans, domestic and laboratory animals (Songer *et al.,* 2000). Infection in pigs (neonatal enteritis) can be asymptomatic or result in diarrhoea and weight loss which may be chronic in suckling pigs (Taylor, 2013). Other clinical signs such as dyspnoea, mild abdominal distension, and scrotal oedema may be observed with characteristic ulcerative lesions present in the colon at post-mortem (Taylor, 2013).

C. tetani causes tetanus which presents as stiffness and abnormal gait leading to spasm and death. The condition occurs sporadically in young pigs and may be associated with umbilical infections, castration, or ovario hysterectomy (Taylor, 2013; Meseko and Oluwayelu, 2012).

Additionally, *C. novyi* type B causes sudden death in large fattening pigs and sows. Incidence is worldwide and sporadic particularly in swill-fed pigs and older sows (Duran and Walton, 1997).Food-borne botulism is caused by *C.botulinum* through a preformed toxin of this organism in food resulting in a rare, sometimes fatal flaccid paralysis in pigs. The incidence is worldwide but rarely described (Taylor, 2013; Beiers and Simmons, 1967).

2.4.4.2 *Salmonella* species

Salmonellosis in pigs is caused by *Salmonella enterica* serovar Choleraesuis var kunzendorf (Salmon and Smith, 1886; Stevens and Gray, 2013; Pedersen *et al.,* 2015).It is a host specific, facultative, intracellular pathogen that causes paratyphoid (Gray *et al.,* 1996). The infection may result in enteric and fatal systemic disease, however, infected pigs may carry the organism in the tonsils, intestines and the gut-associated lymphoid tissue asymptomatically (Fedorka-Cray et al., 2000; Alban *et al.,* 2012).Whereas transmission is primarily through the faeco-oral route (Stevens and Gray, 2013), some studies have shown that the upper and lower respiratory tract may also serve as routes of infection (Fedorka-Cray *et al.,* 1995).The infections may present in different forms including (1) septicaemic form which is commonest in piglets with up to 100% mortality, (2) the acute enteric form in younger and weaned pigs,(3) the chronic enteric form and (4) the diarrhoeic form, which is usually due to the less invasive serotypes such as *S. typhimurium* (Taylor, 2013).

2.4.4.3 *Brachyspira* species

Brachyspira hyodysenteriae is a large anaerobic spirochaete that causes dysentery-infectious mucohaemorrhagic colitis of pigs (Wills, 2000). It affects pigs during the growth and finishing periods, and is characterised clinically by loss of condition with diarrhoea containing varying amounts of mucus, blood and necrotic material (Burrough, 2016). The bacterium multiplies in the large intestine resulting in superficial mucosa degeneration, inflammation and multifocal points of bleeding along the mucosa. The organism does not infiltrate beyond the intestinal mucosa and results in decreased reabsorption of endogenous secretions from the unaffected small intestine leading to diarrhoea (Kennedy *et al.,* 1988). In instances, a proportion of untreated pigs may die while others may remain stunted (Taylor, 2013). Other *Brachyspira spirochaetes* that may also be involved in diarrhoea are *B.innocens, B. murdochii, B. intermedia* and *B. pilosicoli* (Taylor, 2013).

2.4.4.4 *Campylobacter* species

Campylobacter is a gram -ve, spiral, non-spore forming rod (Penner, 1988; Epps *et al.,* 2013)and pigs are natural reservoirs with a prevalence rate of approximately 50- 100% and

excretion level of about 102 to 107 CFU/g (Jensen *et al.,* 2006; Alter *et al.,* 2005; Nielsen *et al.,* 1997).*Campylobacter* infection causes a mucoid, creamy diarrhoea, which may contain blood in piglets 3 days-3weeks of age (Taylor, 2013). The species associated with disease in pigs include *C.coli* (the most common), *C. jejuni, C. hyointestinalis* and *C. sputorum* (Taylor, 2013; Alter *et al.*, 2005*)*. Other *Campylobacter* species present in the porcine intestine, which may multiply and become associated with enteritis, are *C. hyointestinalis subsp. hyointestinalis, C. hyointestinalis subsp. lawsonii, C. mucosalis, C. hyoilei, C. lari* and *C. lanienae* (Taylor, 2013).

2.4.4.5 *Helicobacter* species

This infection in pigs is caused by *Helicobacter suis*, a gram -ve, spiral-shaped bacterium that is commonly found in the gastric mucosa (Hellemans *et al.,* 2007; Grasso *et al.,* 1996; Park *et al.,* 2004).Piglets and porkers have highest colonisation, found in the pyloric region, however, boars and sows, also have high colonization rates in the fundic region of the gastric mucosa (Hellemans *et al.,* 2007).The clinical infection by this organism is rare, and the main evidence for the pathogenicity of *H. suis* was from experimental studies that showed clear association between *H. suis* infection and the development of gastritis as well as a decrease in daily weight gain (De Bruyne *et al.,* 2012).

2.4.4.6 *Lawsonia* species

This organism, *Lawsonia intracellularis* is an obligate, intracellular, gram -ve, small, rod-shaped, intestinal, bacterium;it is the cause of proliferative enteropathy in pigs (Guedes and Gebhart, 2003), a frequent diarrhoeal disease of piglets and weaners characterised by hyperplasia and inflammation of the ileum and colon (Smith and McOrist, 1997). Study has suggested that it infect mitotically the active epithelial cells of the intestinal crypts, which later multiply and spread in the cells as they divide (Boutrup *et al.,* 2010). The condition is often mild and self-limiting but sometimes may result in necrotic enteritis, regional ileitis and proliferative haemorrhagic enteropathy. Affected pigs appear pale, may be stunted and may die suddenly with clotted blood in the lumen of the small intestine (Taylor, 2013; Guedes and Gebhart, 2003).

2.4.4.7 *Yersinia* species

Yersinia species are gram -ve bacilli and the species associated with pig infection include (1) *Yersinia enterocolitica,* which easily colonise the gut of neonate piglets and subsequently become healthy carriers (Skjerve *et al.,* 1998), it is capable of causing enteritis and typhilocolitis in weaned pigs and abortion in sows (Bhaduri *et al.,* 2005); (2) *Yersinia*

pseudotuberculosis which is also carried normally as gut resident (Taylor, 2013; Laukkanen, 2010). Acute cases are characterised by enteritis, lymphadenitis and splenomegaly while chronic cases result in granulomatous nodules and localised abscesses affecting various organs, usually the liver and lungs (Brugmann *et al.,* 2001). About 35 to 70% of herds and 4.5 to 100% of individual pigs carry *Yersinia* species asymptomatically (Bhaduri *et al.,* 2005).

2.4.4.8 *Enterococcus* species

Enterococcus durans (Lancefield Group D) is a motile gram +ve cocci that has been isolated from the intestines and faeces of 3-5 day old piglets, usually as commensals but may sometimes be associated with diarrhoea (Taylor, 2013; Cheon and Chae, 1996). Enterotoxins and mucosal damage have been identified with the diarrhoea caused by *E. durans*, however decreased activity of digestive enzymes at the mucosal brush borders have suggested that the entire pathogenesis of diarrhoea due to *E. durans* has not been completely understood (Cheon and Chae, 1996; Tzipori *et al.,* 1984).

2.4.4.9 *Bacillus* species

Bacillus anthracis causes anthrax but this is rare in pigs. Affected animals may die suddenly, pass bloody faeces or die after swelling of the neck. Route of entry is mainly by ingestion of contaminated feed (Taylor, 2013).

2.4.5 Other non-specific bacteria pathogens

2.4.5.1 *Actinobaculum* species

Actinomyces suis reclassified as *Actinobaculum suis* is associated with urinary tract infections in pigs (Lawson *et al.,* 1997; Woldemeskel *et al.,* 2002). It is linked with cystitis-pyelonephritis complex, a syndrome in which a small group of sows or gilts pass bloody purulent urine, often soon after service. They rapidly lose condition and sudden death may supervene (Taylor, 2013).

2.4.5.2 *Chlamydophila* species

Chlamydophila pathogens in pigs are *Chlamydia suis, Chlamydophila pecorum, Chlamydophila psittaci* and *Chlamydophila abortus*. The infections from this organism may results in multiple lesions including conjunctivitis, enteritis, pneumonia, pleurisy, pericarditis, polyarthritis, orchitis, infertility, abortion and birth of weak piglets (Taylor, 2013; Szeredi *et al.,* 1996; Jiang *et al.,* 2013).

2.4.6 Miscellaneous bacteria pathogens isolated from pigs

- Since 1978, *Arcobacter* species have been associated with reproductive disorders, but excretion by clinically healthy pigs has been frequently reported as well. Information on *Arcobacter* colonization of the porcine gastrointestinal tract is lacking to date (De Smet *et al.,* 2012).
- *Aeromonas hydrophila* has been isolated from enteritis, urine infections and lymphnodes (Igbinosa *et al.,* 2016; Gray and Stickler, 1989).
- *Acinetobacter calcoaceticans, Trueperella (Arcanobacterium) pyogenes, Bacteroides fragilis* are found in the large intestine and have been isolated from diarrhoea in piglets both before and after weaning (Taylor, 2013; Hijazin *et al.,* 2012; Myers and Shoop, 1987).
- *Flavo bacterium*, a ciliated bacillus has been identified in the trachea of pigs. It has been recorded in cases of pneumonia and has been associated with lesions of active tracheitis (Nietfeld *et al.,* 1995).
- *Corynebacterium pseudotuberculosis* has been recovered from the vagina and prepuce of healthy swine and from mandibular abscess of black Alentejano pigs (Kudo and Yanagawa, 1987; Oliveira *et al.,* 2014) while *Corynebacterium ulcerance* was recovered from a case of caseous lymphadenitis in Germany(Contzen *et al.,* 2011).
- *Coxiella burneti* antibodies have been demonstrated in pigs (Taylor, 2013).
- *Klebsiella* species sometimes seen in chronic respiratory tract diseases, enteritis and mastitis (Došen *et al.,* 2007; Ross *et al.,* 1975; Wilcock, 1979).
- *Legionella pneumophila* has been demonstrated in the sera of pneumonic pigs in the UK (Taylor, 2013).
- *Burkholderia pseudomallei* is the cause of meliodosis in pigs in tropical and subtropical regions (Omar *et al.,* 1962; Rampling, 1964).
- *Rhodococcus equi* usually present in granulomatous lesions in submandibular lymphnodes (Witkowski *et al.,* 2016; Rzewuska *et al.,* 2014).

2.5 Enteric bacteria in pigs

The pig gastrointestinal tract has a complex and dynamic microbial ecosystem, the composition of which differs between individuals, region of the gastrointestinal tract, as well as age of the animal (Konstantinov *et al.,* 2004). This microbial flora has an important role as one of the major defense mechanisms of the animal, mainly through competition for nutrients and attachment sites and stimulation of cross-reactive antibodies, which prepares the immune

system in defense against pathogenic microbes (Tancrede, 1992). The large intestine contains most of the microbial flora (over 400 species) (Sørum and Sunde, 2001) and consist of (a) strict G+ve anaerobes such as *Bacteroides* species, *Fusobacterium* species, *Clostridium* species and *Peptostreptococcus* species; and (b) facultative anaerobes such as *Escherichia coli, Klebsiella* species, *Enterobacter* species, *Streptococci* species, *Lactobacilli* species, and *Enterococcus* species (Sørum and Sunde, 2001; Jensen, 2001). Factors which can cause microbial flora changes in the pig gastrointestinal tract include psychological and behavioural stressors, environment, weaning, age, feeding systems and the pigs genotype (Burrin and Stoll, 2003).

Global pig production is most frequently and economically affected by enteric bacterial infections (Moxley and Duhamel, 1999). Common clinical signs found include diarrhoea, reduced growth rate, weight loss and death (Moxley and Duhamel, 1999). Some changes found in the intestines of pigs with enteric bacterial infections include: attaching and effacing lesions, in enteropathogenic *E. coli* and *Brachyspira pilosicoli* infection, inflammation with *Salmonella enterica* and necrotizing and haemorrhagic lesions with certain *Clostridium perfringens* (Moxley and Duhamel, 1999).

The *lactobacillus* species dominates the normal bacterial flora in pigs and produces lactic acid as an essential metabolic end-product. The concentration of lactic acid increases several-fold within the first few days post-weaning and results in decreased pH of the gut which eliminates other pathogenic enterobacteria (Janczyk *et al.,* 2007; Pieper *et al.,* 2008). This group of microorganisms is generally considered beneficial as their attachment to the mucosa may protect the animals from gut infection (Houdijk *et al.,* 2002). Furthermore, cultivation-based studies have shown that lactic acid bacteria, *Enterobacteria* and *Streptococci* were the most important first colonisers of the pig intestine (Stewart, 1997). Similarly, 16S rRNA gene clone analysis indicated that ileal samples of two-day old piglets harboured a group of *E. coli, Shigella flexneri, Lactobacillus sobrius, L. reuteri* and *L. acidophilus* related sequences (Konstantinov *et al.,* 2006).

2.6 Escherichia coli

Escherichia coli strains in pigs forms part of the normal faecal flora. However, when they acquire virulent genes they are able to cause disease (Taylor, 2013).These coliform bacteria are commonly used as representatives of the enterobacteria from faecal samples in culture based studies of the intestinal bacterial flora, as they are the major facultative anaerobic

bacteria in the intestinal tract of most animal species (Dubreuil, 2012). *Escherichia coli* are gram -ve rods, flagellated with variable length and diameter of about 1μm.On culture, colonies grow on solid media within 24 hours after incubation and may be smooth, rough or mucoid (Fairbrother and Gyles, 2012). Major characteristics associated with pathogenic *E.coli* infections are proteins such as fimbriae and production of enterotoxins usually by the enterotoxigenic *E. coli* (ETEC) and Shiga toxin by Shigatoxigenic *E. coli* (STEC). Other toxins previously described are EAST1, cytotoxins, cytolethal distending toxin, hemolysin; outer membrane proteins (intimin) and adhesin involved in diffuse adherence (Taylor, 2013). In addition,a study has shown that F18 was the main colonisation factor for STEC and ETEC with F18ab and F18ac as subgroups (Cheng *et al.,* 2005).

Escherichia coli strains have been identified as important causes of several diseases in pigs worldwide including neonatal septicaemia, neonatal diarrhoea, post-weaning diarrhoea, oedema disease (bowel oedema or gut oedema), cystitis, septicaemia, polyserositis, coliform mastitis and urinary tract infections. They can also colonise existing lesions elsewhere in the body (Taylor, 2013; Fairbrother and Gyles, 2012). Post-weaning diarrhoea (post-weaning enteric colibacillosis) and oedema disease have a more significant impact in the porcine industry because they result in high economic losses due to high morbidity and mortality, decrease weight gain, the cost of treatments, vaccination and feed supplementation (Fairbrother and Gyles, 2012).The *E. coli* infections occur at different ages in the pigs. Colisepticaemia occurs in 0-4 days old piglets and may be associated with diarrhoea.Enteritis (enteric colibacillosis) which is also associated with diarrhoea, occurs at three main periods in the pigs life; neonatal diarrhoea occurs at 0-4 days of age, neonatal-weaning diarrhoea at 4 days to 3-4 weeks and post weaning diarrhoea is usually associated with weaning, oedema disease occurs in recently weaned pigs while mastitis and cystitis occur in adult sows (Taylor, 2013).

Outbreaks of *E.coli* diarrhoea have increased worldwide with post-weaning diarrhoea being the most common where F4 and F18 are usually the associated adhesion factors (Fairbrother and Gyles, 2006). This could be due to the emergence of more virulent *E. coli* clones, a benign commensal of the gut microflora which multiply rapidly and cause disease through colonisation of the intestinal mucosa or changes in the management of pigs (Fasina *et al.,* 2015). Furthermore, a potentially beneficial method of feeding behaviour and maintaining gastrointestinal health in pigs is through feeding weaners with liquid feed or fermented liquid feed, in contrast to dry feed, as it is considered a possible feeding strategy to maintain a high

and regular feed and water intake of weaners (Canibe and Jensen, 2012). Avoiding a drastic decrease in feed and water intake after weaning is believed to ameliorate the post-weaning lag period in piglets which may predispose them to *E. coli* infections (Canibe and Jensen, 2012).

2.6.1 Classification of *Escherichia coli*

The best approach to classify *E. coli* is by serotyping in association with virulent strains. However, only a small percentage of the organisms are typeable based on the O, K, H and F antigens, and only about 175O, 80K, 56H and over 20F antigens have been officially recognised to date based on proven or suspected pathogenicity of *E. coli* isolates (Fairbrother and Gyles, 2012). Pathotype is the term used to classify *E. coli* by their virulence mechanisms. The broad classes identified include, Shiga toxin producing *E. coli* (STEC), enterohaemorrhagic *E.coli* (EHEC), enteropathogenic *E.coli* (EPEC) and extraintestinal pathogenic *E.coli* (ExPEC) (Fairbrother and Gyles, 2012) (Table 2.1).

Table 0.1: Important pathotypes of pathogenic *E. coli* in pigs and associated virulent traits

Pathotype	Adhesins	Toxins
ETEC	F5(K99), F6 (987P), F41	STa
	F4(K88)	STa, STb, LT, EAST-1, α-hemolysin
	F4(K88), AIDA,	STa, STb, LT, EAST-1, α-hemolysin
	F18, AIDA	STa, STb, LT, Stx(VT), EAST-1, α-hemolysin
EPEC	Eae (intimin)	
STEC (VTEC)	F18, AIDA	Stx2e,(VT2e), EAST-1, α-hemolysin
	Eae (intimin)	Stx1 and/or Stx2
ExPEC	P,S	CNF
	P,S	CNF

2.6.1.1 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* is a pathotype found in post weaning diarrhoea of pigs. This bacterium possesses a complex secretion system that injects over 20 effector proteins into the host enterocyte. This allows intimate adherence of the bacteria into the pigs intestinal epithelium to develop a characteristic "attaching and effacing" (AE) lesion. The EPEC together with other *E. coli* pathotypes that result in AE are collectively known as attaching and effacing *E. coli* (AEEC) (Zhu *et al.,* 1994).

2.6.1.2 Shiga toxin producing *E. coli* (STEC)

Shiga toxin producing *E. coli* produce a family of cytotoxins known as Shiga toxin (Stx) or verotoxin (VT). Many STEC are not pathogenic in the intestinal flora but when they possess additional virulence traits, they become highly pathogenic (Fairbrother and Gyles, 2012). In pigs, the most pathogenic STEC are those that cause oedema disease known as oedema

desease *E. coli* (EDEC). EDEC produces stx2e and F18ab or F18 ac (DebRoy *et al.,* 2009). Another subgroup of STEC is the enterohaemorrhagic *E. coli* (EHEC) which also possess the eae and the same secretion system as EPEC (Fairbrother and Gyles, 2012). However, production of Shiga toxins alone may not be sufficient for *E. coli* O157:H7 pathogenicity (Mead and Griffin, 1998). Other virulence factors such as the intimin protein (involved in the attachment of the *E. coli* O157 to enterocytes), the presence of a plasmid encoded hemolysin, or both, are important in the pathophysiology of haemorrhagic disease (Mead and Griffin, 1998).

2.6.1.3 Extra intestinal pathogenic *E. coli* (ExPEC)

Extra intestinal pathogenic *E. coli* are a group of heterogeneous *E.coli* in the intestinal tract of pigs that can invade other systems to cause bacteraemia resulting in septicaemia or localised infections such as meningitis and arthritis (Fairbrother and Ngeleka, 1994). The ExPEC possess lipopolysaccharides which protect the bacteria from being killed by serum complement and phagocytes (Fairbrother and Gyles, 2012).

2.6.1.4 Enterotoxigenic *E. coli* (ETEC)

The ETEC pathotype is the most important among the pathogenic *E. coli* producing one or more enterotoxins that induce secretory diarrhoea in pigs (Fairbrother and Gyles, 2006). This pathotype produces two major enterotoxins; heat stable toxin (ST) and heat labile toxin (LT) which are both further subdivided into STa, STb, LTI and LTII respectively (Evans *et al.,* 1972; Czirók *et al.,* 1992).The ETEC that causes neonatal diarrhoea produces only STa and possess one or more fimbriae F4 (K88), F5 (K99), F6 (987P) and F41 (Fairbrother and Gyles, 2012). Similarly, ETEC that causes post-weaning diarrhoea produces STa, STb, LT, and enteroaggregative heat stable enterotoxin (EAST-1) (Zhang *et al.,* 2007) while ETEC isolates that produces STb or STb: EAST-1 from weaned pigs may also produce an adhesion involved in diffuse adherence (AIDA-I) (Mainil *et al.,* 2002; Ngeleka *et al.,* 2003; Niewerth *et al.,* 2001).

Enterotoxigenic *E. coli* causes an estimated 840 million gastrointestinal infections and about 380,000 deaths worldwide each year in pigs (Gupta *et al.,* 2008), leading to substantial economic losses for swine producers worldwide (Nagy and Fekete, 2005). The bacteria adhere to and colonize the intestinal mucosa of the small intestine (jejunum, ileum and to a lesser extent, the duodenum) (Arbuckle, 1970; Cox and Houvenaghel, 1993). They also adhere to enterocytes using surface fimbriae (pili) that adhere to specific receptors on enterocytes, without inducing morphological lesions but elaborate enterotoxins that act

locally on enterocytes, leading to fluid secretion resulting in the exacerbation of the diarrhoeal illness in pigs (Verbrugghe *et al.,* 2015). A very important illness induced by ETEC toxins is post-weaning diarrhoea in piglets (Verbrugghe *et al.,* 2015).

2.6.2 *E. coli* post-weaning diarrhoea (PWD)

Post-weaning diarrhoea, also known as post-weaning enteric colibacillosis, is an important cause of death in weaned pigs worldwide. Infection usually occurs during the first weekpost weaning and often results in decreased weight gain (Taylor, 2013). Several factors, such as the stress of weaning, lack of antibodies originating from the sow's milk and dietary changes, contribute to the severity of the disease, manifesting as sudden death or severe diarrhea (Fairbrother, 1999; Amezcua *et al.,* 2002; Maynard *et al.,* 2003). Most outbreaks have occurred in early-weaned piglets although traditional herds are being increasingly affected (Fairbrother, 1999; Amezcua *et al.,* 2002; Maynard *et al.,* 2003).

2.6.3 Virulence factors of *E. coli* associated with post-weaning diarrhoea

Post-weaning diarrhea is caused primarily by ETEC, a pathotype that is characterized by the production of adhesins and alpha-hemolysin which produce colonies with clear zones of haemolysis on blood agar. Several studies have shown that *E. coli* isolated from weaned pigs with diarrhoea were haemolytic (Frydendahl *et al.,* 2003; Chen *et al.,* 2004). Alpha-hemolysin, an approximately 110 kDa pore-forming cytolysin, belongs to the RTX family of toxins. The hlyA gene that encodes the hemolysin is part of an operon that is found on plasmids in ETEC. It is a potent cytotoxin that can damage a variety of cells (Frydendahl, 2002). Serological typing has been expanded to include fimbrial antigens, which are virulence factors, as well as O and H antigens which are virulence markers (Chen *et al.,* 2004). Some strains of ETEC that cause PWD possess additional genes that encode Shiga toxin 2e (Stx2e), allowing them to cause edema disease (ED). The ETEC strains that produce Stx (VT) are appropriately called ETEC/STEC or ETEC/VTEC (Nagy and Fekete, 1999).

Enteropathogenic *E. coli* have also been implicated in PWD (Zhu *et al.,* 1994; Zhu *et al.*, 2010; Janke *et al.,* 1989; An *et al.,* 2000). Identification of porcine EPEC (PEPEC) is challenging and veterinary diagnostic laboratories do not routinely seek to identify this pathotype of *E. coli* (Fairbrother, 1999). The eae (*E. coli* AE) gene is a marker for PEPEC, but some eae-positive porcine *E. coli* isolates may be non-pathogenic. O45 serogroup has been shown to possess genes of the locus of enterocyte effacement (LEE), a locus well established to confer ability for AE lesions (Zhu *et al.,* 1994; An *et al.,* 2000; Helie *et al.,*

1991). Immunity to one strain of pathogenic *E. coli* does not essentially protect from others, and successive strains can pass through herds (Bertschinger, 1999).

2.6.4 Pathogenesis of Post-weaning diarrhoea

Post-weaning diarrhoea is an enteric disease in pigs localised in the small intestine, where digesta flows quickly. The EPEC that causes this condition attaches to the enterocytes lining of the villi or to the mucus covering the villi with the fimbriae or pili, which prevents the bacteria from being flushed to the large intestine. Thereafter, the enterotoxigenic *E.coli* which have colonised the small intestine incites hypersecretory diarrhoea through the release of distinct enterotoxins such as the LT and ST. The LT induces secretion of chloride ions, sodium ions, bicarbonate ions and water into the lumen by binding irreversibly to the mucosal cells and activating the adenylcyclase cyclic AMP system (Thiagarajah and Verkman, 2003; de Haan and Hirst, 2004, Fairbrother *et al*., 2005) while the ST (STa and STb) inhibits the absorption of sodium and chloride ions from the lumen into the epithelial cell via the guanylcyclase–cyclic GMP system, both resulting in fluid retention. Intestinal colonisation and diarrhoea typically last for about 4 to 14 days, with the organism being spread between animals by the faeco-oral route and aerosols (Bertschinger, 1999). Pigs displaying PWD harbour massive numbers of haemolytic *E. coli* in the jejunum, whilst there is minimal change in numbers of other bacteria (Smith and Jones, 1963). It is common for EHEC to appear in the faeces of pigs in increased numbers in the first week after weaning in both healthy and diarrhoeic pigs, although the numbers are higher in diarrhoeic pigs (Kenworthy and Crabb, 1963; Hampson *et al.,* 1985).

The act of weaning is an essential precipitating factor for PWD, regardless of the age at weaning. All of the factors involved with weaning create an environment suitable for the proliferation of *E. coli* in the small intestine. Slower gut transit time and gut stasis immediately after weaning allow bacteria the opportunity to attach and time to multiply (Pluske *et al.,* 2002). An inability of piglets to adequately thermoregulate, combined with sub-standard weaning accommodation, may result in cold stress. This alters intestinal motility and is thought to be a major factor in the pathogenesis of PWD (Wathes *et al.,* 1989). Other factors include social stresses from mixing, fighting and crowding which trigger cortisol release, most likely increasing transit time and depressing the immune response to enhance bacterial infection; moving to a new pen increases the chance of exposure to microbes residing in fresh or dry matter in the environment; the presence of other pathogens such as rotavirus in the environment thereby increasing the likelihood and severity of disease (Lecce

et al., 1983).Poor hygiene will also increase the pathogenic *E.coli* load delivered to the small intestine because of faeco-oral cycling (Madec *et al.,* 1998).

2.7 Vaccination against pathogenic bacteria

Vaccination against pathogenic bacteria has become necessary as an alternative control measure due to the development of different serotypes of bacteria and bacterial resistance to a wide range of commonly used antibiotics (Fairbrother *et al.,* 2005). Frequently used vaccines against bacterial diseases in swine contain whole-cell killed micro-organisms, purified microbial components, or recombinant proteins (Haesebrouck *et al.,* 2004).Vaccination against bacteria pathogens in pigs is directed towards either the extracellular bacteria or the exotoxin produced by the bacteria (Haesebrouck *et al.,* 2004).

Exotoxins are produced within the bacterial cytoplasm. Some are excreted through the living cell wall, while others are released only by lysis of bacteria. In diseases caused by exotoxigenic bacteria, antibodies neutralizing that toxin play an important role in protection of the host against disease provided they are able to prevent binding of the exotoxin to its receptor on the host cell. Vaccines containing the inactivated toxin (toxoid) or a non-toxic but antigenic recombinant protein derived from the exotoxin can be expected to provide protection against disease. Antibodies generally mediate protection against the surface antigens and certain secreted antigens of extracellular bacteria. Cellular immunity may also play a role (Haesebrouck *et al.,* 2004).

Oral immunization of piglets with live avirulent strains of bacteria is a new vaccination strategy for bacterial diseases. An example is the administration of avirulent *E. coli* carrying the fimbrial adhesins or oral administration of purified F4 (K88) fimbriae. Other approaches to control bacterial diseases include supplementation of the feed with egg yolk, antibodies from chickens immunized with F4 or F18 adhesins, breeding of F18 and F4 resistant animals, supplementation with zinc and/or spray-dried plasma, dietary acidification, phage therapy, or the use of probiotics. However, to date, no single strategy has proven to be totally effective (Fairbrother 2005).

2.8 Results of meta-analysis

In the present review, 29 countries with documented records of cases of *E.coli* were included with the USA having the highest number of references followed by China. About 74% of the samples were taken from farms and others were from samples submitted to research laboratories and veterinary faculties for necropsy. In general seven pathotypes were reported

and 66.7% of the pathotypes identified were ETEC, 14.3% were ETEC and STEC, 7.9% were STEC, 31.7% were classified as ETEC/EPEC/EAEC, others were EHEC and DAEC (Table 2.2).Several serogroups were identified and the commonest were O141, O149, O139, O138, O8 and O9. 33.3% of samples collected were faecal swabs or faeces, 14.2% were intestinal segments, 17.4% were intestinal segments, feaces or rectal swabs and other organs, 6.3% were intestinal segments and other organs, 7.9% were lab isolates (Table 2.2). Piglets were 52.3%, 7.9% were porkers, 39.6% were weaners and all pigs were between 1-392 days old. A total of 24,854 isolates were considered and 10477 were recorded as positives, the gene looked out for were STa, STb, LT, stx1, stx2, stx-2e F4, F5, F6, F18, F41, AIDA, EAST1, eae, paa, hlyA (Table 2.2). The diseases examined were diarrhoea in form of neonatal diarrhoea, colibacillosis, PWD and oedema disease. Some of the associated risk factors identified were poor housing, management and feed changes, extensive use of antibiotics as prophylaxis, overcrowding, high humidity and temperature changes (Table 2.2)

This study showed that India, USA, Japan, Slovakia and Denmark were the countries with the highest detection of virulence factors in piglets (100%; n = 3, 55, 42, 92 and 191 respectively), followed by Sweden (74%; n = 856) and Poland (72%; n =1125) (Table 2.4). For all the cases of virulence in piglets an overall prevalence of 57.93% (CI_{95}: 57.0-58.8) was estimated (n = 12970) (Table 2.3, figure 2.1)

Similarly, South Africa, Cuba, Poland, Denmark, had the highest cases of virulence factor detection in *E.coli* in weaners (100%; n =2, 36, 46, and 240 respectively), followed by Canada (74.1% n = 135), The least detection was in found in China (6.5% n=324) (Table 2.6) The overall prevalence of virulence factor detection in weaners was 57.9% (CI_{95}: 56.99-58.83; n = 8058) (Table 2.5, Figure 2.2)

Furthermore, in porkers the overall prevalence of E.coli virulence factor detection was 36.45% (CI_{95}: 35.73- 37.57) (Table 2.7). The highest prevalence was found in the USA (70.5%; n = 687) and the lowest was in Norway (0.15% n= 1976) (Table 2.8, figure 2.3)

Table 0.2: *Escherichia coli* and its virulence profiles in pigs

Country	Source	Sample	Pathotype	Serotype	Age(days)	Virulence factor	Summary of result	Reference
South Africa	Farm	Faeces and intestinal tissues	ETEC		35	EAST 1	*E.coli* associated endotoxaemia.	(Fasina*et al.*,2015)
China	lab samples	Bacterial isolates	ETEC and VTEC			F18+	F18+ is the main colonization factor for VTEC and ETEC.	(Cheng *et al.*, 2005)
Norway	Abbatior	intestinal content	STEC	O157: H7		stx2, eae, *fli*C-H7	Prevalence of *E.coli* O157:H7 in pigs is low in Norway	(Johnsen *et al.*, 2001)
US	Herd	Isolates	ETEC		14	K88, K99, 987P, ST, LT	ETEC produces k88,K99, 987P, LT and ST.	(Moon *et al.*, 1980)
US	Slaughter facility	Colon(faeces)	STEC	O157: H7		stx1, stx2, eae, hly	Pigs in the US can habour*E.coli* O157:H7.	(Feder *et al.*, 2003)
Denmark	Lab samples	intestinal content	PEC	O8, O45, O138, O139, O141, O147, O149, O157	22	F4, F18, STa, STb, LT,ESAT1, VT2e	VTEC and ETEC in PWD and ED belong to limited serogroups and are haemolytic.	(Frydendahl, 2002)
US	Farm	faeces	STEC		140	stx1,stx2	The incidence of STEC in swine varies.	(Fratamico *et al.*, 2004)
Canada	Farm	rectal swab	ETEC	O149	35	Sta, STb, LT, Vtx, F18 F4	PWECD is an economically important disease in pigs.	(Amezcua *et al.*, 2002)
US	Farm	Faecal swabs, faeces or intestinal content	ETEC		35	K88, K99, STa, STb, LT, F18, F41, stx2e, EAST1, AIDA-1, paa, eae	Broad arrays of virulence genes are associated with PWD in pigs.	(Zhang *et al.*, 2007)
China	Field isolates	Faecal swabs		O8, O9, O11, O20, O32, O91, O93, O101, O107, O115, O116 and O131	49	F4, F5, F6, F18, F41 STI, STII, stx2e	Pigs with PWD have*E.coli* enterotoxins and shiga toxin 2 variant.	(Chen *et al.*, 2004)
Mexico	Farm	faeces swabs			11& 28	LT, STa, STb, Stx1, Stx2 and EAST 1, F4, F5, F6, F17, F18 and F41	There are a there wide variety of virulence genes associated with diarrhoea in piglets.	(Toledo *et al.*, 2012)
Switzerland	Farm	intestinal content	ETEC and	O139, O141 and O149	56	F107 SLT-IIv, LTI, STIa, STII	F107 are a major colonisation factor in O139: K12 and O141:	(Imberechts *et al.*, 1994)

			VTEC				K85ab *E.coli* serogroups.	
Japan	Necropsy specimen	intestinal content and rectal swab	ETEC	O149, O157, O141, O8	26	Sta, LT, K88, K99, 987P, F41, 4F	There are clear differences in strains of ND and PWD in terms of ETEC strain, enterotoxin type and adhesins.	(Nakazawa *et al.,* 1987)
Belgium	Farm	Serum	ETEC		392	F4	F4+ ETEC is highly prevalent and widely spread in non-vaccinated pig breeding farms in Belgium.	(Van den Broeck*et al.,*1999)
India		Faeces	STEC and EPEC	O9, O20, O24, O59, O60, O85, O100, O103, O112, O113, O116, O118, O119, O123, O137 and O152	40	stx1, stx2, eaeA, hlyA	STEC and EPEC are associated with diarrhoea in piglets and infants.	(Begum *et al.,* 2014)
Cuba	Farm	Fecal Isolates	EPEC	O141 and O157	30	STb, STa, VT2e, LT, F18, F6	ETEC and VTEC isolates from diarrhoic pigs belong to restricted number of serogroups and serotypes.	(Blanco *et al.,* 2006)
India	Farm	Liver, Lung, Intestine (necropsy)	STEC			stx2, eae	eae and stx genes are the prime causes of oedema disease in pigs.	(Barman *et al.,* 2008)
Germany	Field strains	Isolates	STEC and ETEC			F18ac, F18ab	The F18 fimbrial subtypes are significantly associated with pathovars of *E. coli* strains.	(Barth *et al.,* 2011)
Denmark	Institute	Faeces	ETEC	O149, O138,	21		Diarrhoea occurrence is associated with faecal shedding of haemolytic *E. coli*.	(Carstensen *et al.,* 2005)
Vietnam	Farm	Faeces, intestinal content	ETEC	O149 and O8	16	F4, F5, Sta,STb,LT	A large number of ETEC isolates belong to O8 serogroup, producing Sta, STb and LT but lacked fimbriae genes.	(Do *et al.,* 2006)
Canada	Farm	Lab isolates	EPEC	O8, O116, O147, O138 and O45, O4 and O98	29		82% of *E. coli* isolates were enteropathogenic by the ligated intestine test in pigs.	(Gyles *et al.,* 1971)
Uganda	Farm	Faeces	ETEC and VTEC			F18+, F4, LT, STa, STb and Stx2e	ETEC and VTEC infections are common in central uganda but clinical cases are masked by management practices.	(Okello *et al.,* 2015)
China	Farm	Liver, spleen,		O107, O101, O9, O60, O26.			O107, O101, O9, O26, and O60	(Chen *et al.,*

		kidney, heart, mesenteric lymph node					are the dominant serotypes in southern china.	2013)
Korea	Farm	ileal contents	EAEC, ETEC		14	EAST 1, F4, F5, F6, F41, STa, STb, LT.	EAST1 is prevalent in *E. coli* and it is a virulence determinant in the pathogenesis of enteric colibacillosis of pre-weaned pigs.	(Choi *et al*., 2001b)
US	Research and diagnostic lab	Lab specimens	ETEC	O8, O9, O20, O101, O141, O149, O157, O138, O139.		K88, LT, Stx2e; F18, STa, and STb	Loss of virulence genes is not uncommon in ETEC.	(Francis, 2002)
Spain	Farm	Isolates	ETEC	08, 09, 020, 0101, 0141, 0149	15	F6 (987P), F5 (K99), F4 (K88) and F41	F6 fimbriae were found in higher rates than F5, F4, and F41 in younger and older piglets with ETEC strains.	(Garabal *et al.,* 1997)
US	Farm	Isolates	STEC and ETEC	O138, O139, O141 and O147		f18, sta, stb, and stx2	Serogroup O147 may be a common serotype of oedema disease-causing *E. coli* in the United States.	(Helgerson *et al.,* 2006)
England	Farm	Intestinal content	ETEC	O149	28	LT	Weaning at three weeks of age did not precipitate a profound change in the *E. coli* intestinal flora in the post-weaning period.	(Hinton *et al.,* 1985)
Bulgeria	Farm	faeces and intestinal content	EHEC; VTEC		52	F18, F4	In the 2–3 post-weaning weeks, toxin producing *E. coli* that possesses the adhesion factor F4 is responsible for diarrhoea.	(Lyutskanov, 2011)
Zimbabwe	Farm	Faecal swabs	ETEC , VTEC		13	STa, STb, LT, Stx-2e F4, F18, F5 and F41, and F6	Vaccination or vaccinedevelopement based on F4, F5, F6, F18, and F41 antigens continues to be appropriate for ETECinfections.	(Madoroba *et al.,* 2009)
Canada	Farm	Fecal swabs, faeces or intestinal content	ETEC and EPEC	O7, O157, O149, O26, O69, O139, O141, O103, O108, O109,O119, O20, O21, O22	29	EAST1, AIDA-I, F4, F5, F6, F18, LT,STb,STa, EAE, Paa	AIDA-I/STbwasdominant and EAST-I may not be an important marker for diarrhoea in pigs.	(Ngeleka *et al.,* 2003)
Hungary	Farm	Intestine		O8, O138, 0139, 0141, 0147, O149, and 0157	49	K88, STa, STb, LT and VT	73% of K88 isolates had the capability to produce	(Nagy *et al*., 1990)

							enterotoxins or VT which could contribute to post-weaning diarrhoea in pigs.	
Japan	Farm	Faece, cerebrum, liver, lung, heart blood, kidney, intestinal content, mesenteric lymph node	ETEC	O149: H-, O149: H10, O15:H9, O111:H-, OR: H6	19	STa, LT, K88,	Growth of ETEC was not active in healthy weaning pigs but infection with PRRS virus results in ETEC systemically with per acute death.	(Nakamine *et al.,* 1998)
Germany	Farm			O138, O139, O139, O141, O141, O147 andO149		stx2e, fedA, orfA,orfB, AIDA	Porcine *E.coli* strains are a major reservoir for AIDA genes.	(Niewerth *et al.,* 2001)
Canada	Animal health laboratory	Isolates	ETEC	O149		estA(STa), STb, LT, astA(EAST1), K88ac	Recent O149 ETEC associated with PWD in pigs in Ontario are different from the old O149 ETEC, and the newisolates has estAgene.	(Noamani *et al.,* 2003)
Vietnam	Farm	small intestine, mesenteric lymph node, liver, lung, and spleen		O139, O141, O138, and O149	28	VT2e+, fedA(F18), AIDA, Sta, STb	0139:K82+/VT2e+/F18+/AIDA+ seropathotype was predominant and antibiotic resistances is widely distributed in *E. coli* causing ED in northern Vietnam.	(Oanh *et al.,* 2010)
Poland	Farm	Faeces or rectal swabs		O139, O141 and O138	35	Stx2e, F18, STI, STII.	Molecular characterization of *E. coli* using the RAPD polymorphism analysis is a quick and convenient method to differentiate *E. coli* bacteria of the same and different serogroups.	(Osek, 2000)
China	Farm	rectal swab	ETEC	O80, O141, O139, O6, O9, O20, O101, O93, O138, O147, O157, O38 and O45.	26	K88, K99, F41, F18, 987P, STa, STb, LT, Stx2e	Novel serogroups O80, O6 and O38 of *E.coli* in pigs were identified in western China.	(Qi *et al.,* 2012)
south Africa	Research council	rectal swab	ETEC, STEC,		73	STa, STb, LT, Stx2e, EAST-	PAA and AIDA-1 are important in South African pigs.	(Mohlatlole *et al.,* 2013)

			EAEC			1, PAA, AIDA-I, EAE		
Korea	Farm	caecal and ileal contents	ETEC, STEC, DAEC			AIDA1, F18ab, Stx2e, Sta, STb, EAST1	AIDA gene is not restricted to DAEC strains.	(Ha *et al.*,2003)
Brazil	Farm	swabs				F4, F5, F6, F18, F41, STa, STb, LT and STx2e	*E. coli* strains isolated from pigs with diarrhoea possessed the genes for LT or/and ST enterotoxins.	(Vidotto *et al.*, 2009)
Slovakia	Farm	Rectal swab and intestinal content	ETEC	O8, O54, O84, O101, O141, O141, O147, O149, O163, O2, O15, O84 andO157.	14	LT, Sta, STb, Stx1, Stx2, F4, F18, F6, F5, F41, F17, eae, EAST1	There is high prevalence of ETEC that possess LT and STb genes and the F4 colonization factor in piglets with diarrhoea in Slovakia.	(Vu-Khac *et al.*, 2007)
China	Farm	Faeces	ETEC			EAST1, irp2, paa, STb, AIDA-I, LT-I, ler, hlyA, K88, eae, STa, sepA, F18, afaD, afaE, K99 and Stx2e	Relatively few isolates from the study express K88, K99, LT-I or STa, but EAST1, irp2, AIDA-I, paa and STb were frequent in *E. coli* strains in suckling pigs with diarrhoea in China.	(Liu *et al.*, 2014)
Slovakia	Farm	Intestinal content	ETEC		21	F4, F5, F6, F18, F41, STa, STb, LT, STx2e, EAST1	There is a wide distribution of the astA gene among *E. coli* strains isolated from diarrhoeic piglets in Slovakia and a strong association of the astA gene with F4-positive strains.	(Vu-Khac *et al.*, 2004)
Czech Republic	Farm	Rectal swab and intestinal content	ETEC and STEC	O149		LT+, STa+, K88, F18, EAST-1, paa.	There is a significantly higher prevalence of astA positive *E. coli* isolates among apparently healthy piglets in comparison with diarrheic piglets.	(Zajacova et al., 2012)
China	Farm	Rectal swab	ETEC and STEC	O141, O9, O32, O2, O116, O107, O147, O139, O91, O45, O98.	49	AIDA-I, Sta, Stb, Lt, Stx2e, EAST1, F5, F6, F18, F41	AIDA-I represents an occasional virulence factor for PWD and ED in pigs and has the potential to transfer between porcine and human *E. coli*.	(Zhao *et al.*, 2009)

Spain	Farm	Reactal swab	ETEC andVTEC		15	STa, LT, VT, CNF1, CNF2, α-hemolysin.	Majority of piglets in this study that produced verotoxin also produced STa enterotoxin but CNF1 was produced from only 1.5% of sick piglets.	(Garabal *et al.,* 1995)
Korea	Pathology department	Ileal and cecal contents	ETEC, STEC			EAST1, STa, STb, LT, Stx2e, F4, F5, F6, F18, F41.	*E.coli* carries east1 gene in high prevalence in weaned pigs with diarrhoea and/or edema disease.	(Choi *et al.,* 2001a)
Korea	Farm	Jejunal, ileal and caecal contents	ETEC, STEC			STa, STb, LT, Stx2e, F4, F5, F6, F18, F41	Genes for F18 and Stx2e are prevalent among *E coli* isolated from post-weaning pigs with diarrhoea or oedema disease.	(Kwon *et al.,* 2002)
Denmark	national vet laboratory	Intestinal content and faeces		O149, O139, O138,O101 O8, O64, O147, O141 and O157	4,15,36 & 56	STa, STb, LT, VT, K88, 987P , F107, K99 and F41	Results correlation between genotypic and phenotypic methods was 97.7-100%. VT and F107 genes were more frequent in post-weaning than in neonatal *E.coli* strains.	(Ojeniyi et al., 1994)
Canada	vet medicine faculty	intestinal content	ETEC	O8,O9, O101, O9, O20,O64,O10, O157, O147,O149, O115, O138,O139,O141,O45,O26, O119,O15 and O108	31	F4, F5, F6, F41, STa, STb, LT and VT	The most important pathotypes among enterotoxigenic isolates in this study were F4:LT:STb, F5:STa, STb, F5:F41:STa, F4:STb, F6, STa, and LT.	(Harel *et al.,* 1991)
Brazil	Farm	Faeces	ETEC, STEC		11	LT-I, STa, SLT-I, SLT-II, SLTIIv, F18ac.	eaeA gene and intimin production and/or Shiga-like toxins may be an important cause of diarrhoea among piglets.	(Martins *et al.,* 2000)
Poland	Farm	Faeces or rectal swabs			35	LTI, STI, Stx2e, F4 F5 F6 F17 F18 and F41	Low prevalence of fimbria-positive *E. coli* strains isolated from pigs with PWD was found in this study.	(Osek, 1999)
Poland	Farm	Rectal swabs	ETEC	O1, O8, O9, O66, O138, O141, O147 and O149	35	EAST1-1, F4, LTI STI and STII	EAST1 gene is widely distributed among *E. coli* strains isolated from piglets with post-weaning diarrhoea.	(Osek, 2003)
Poland	Farm	Faecal swabs	ETEC		5& 42	Sta, LT, STb, K88, K99, 987P, F1	Enterotoxigenic (LT and STb) *E.coli* from suckling and weaned piglets with diarrhoea were	(Osek and Truszczyński, 1992)

							90.5% and 69.1% respectively and 18.5% of strains from healthy piglets were STa.	
Poland	Farm	Feaces		O157, O149, O66, O138, O139 and O141		F4, fedA, eltI, estI, estII, stx1, stx2e	*E. coli* from pigs with post-weaningdiarrhoea (13 out of 21 isolates) or from oedema disease (16 out of 19 strains) are able to produce F18 fimbriae.	(Osek *et al.,* 1999)
Argentina	Farm	Rectal swabs spleen, kidney and liver	ETEC and VTEC	O8, O9, O64, O101, O138, O139, O149 and O162		STIa, STb, LTI, VT2e, VT1, VT2all	ETEC strains predominate in the group of animals with diarrhoea, STIa prevails in ETEC from pigs with diarrhoea, O64 prevails among ETEC and O138 prevails for ETEC/VTEC strains.	(Parma *et al.,* 2000)
Sweden	Farm	faeces and intestinal content	ETEC	O149, O101, O9, O20, and O8,	25	LT, ST, K88, K99, 987P, F41	Frequency of O149 has been reduced, while that of O101 has increased to the same level as that of O149 in sweden.	(Soderlind et al., 1988)
Indonesia	Farm	Rectal swab		O20, O9 and O141	14	987P, ST, K88, K99.	Mixed infections with *E. coli* bearing different fimbrial antigens occur both within a group of piglets and in a single piglet.	(Hirst and Patten, 1991)
Slovakia	Farm	intestinal content and rectal swab	ETEC		15	F4, F5, F6 and F41	The frequency of occurrence of individual types of adherence antigens is related to geographical location.	(Vu-Khac *et al.,* 2004)
Brazil				O139, O8, O9, O15, O20, O82, O101, O110 and O153		Stx2e, F18ab, STI, STII, LTI	Enterotoxin genes detected in high frequency are responsible for diarrhoea seen in pigs with oedema disease,	(da Silva *et al.,* 2001)

Table 0.3: Prevalence of *Escherichia coli* virulence factors in piglets

Serial number	Study	Events	Sample Size	Outcome	SE	CI lower	CI upper	Forest Plot ID	Rate
1	US (herd)	108	111	0.972973	0.093624368	0.789469	1.156477	32	97.2973
2	Denmark (Diagnostic samples)	219	563	0.388988	0.026285344	0.337468	0.440507	31	38.89876
3	Mexico (Farm)	116	503	0.230616	0.021412186	0.188648	0.272584	30	23.06163
4	Japan (Necropsy samples)	214	567	0.377425	0.025800245	0.326857	0.427994	29	37.7425
5	India (Farm)	3	3	1	0.577350269	0.131607	2.131607	28	100
6	Denmark (Research institute)	45	90	0.5	0.074535599	0.35391	0.64609	27	50
7	Vietnam (Farm)	126	200	0.63	0.056124861	0.519995	0.740005	26	63
8	Canada (Farm)	164	200	0.82	0.064031242	0.694499	0.945501	25	82
9	Korea (Farm)	164	720	0.227778	0.017786456	0.192916	0.262639	24	22.77778
10	US (Research institute)	330	660	0.5	0.027524094	0.446053	0.553947	23	50
11	Spain (Farm)	36	69	0.521739	0.086956522	0.351304	0.692174	22	52.17391
12	US (Farm)	55	55	1	0.134839972	0.735714	1.264286	21	100
13	England (Farm)	14	51	0.27451	0.073365831	0.130713	0.418307	20	27.45098
14	Zimbabwe (Farm)	63	1984	0.031754	0.004000632	0.023913	0.039595	19	3.175403
15	Japan (Farm)	42	42	1	0.15430335	0.697565	1.302435	18	100
16	Germany (Farm)	27	104	0.259615	0.049963004	0.161688	0.357543	17	25.96154
17	Vietnam (Farm)	69	92	0.75	0.09028939	0.573033	0.926967	16	75
18	China (Farm)	112	208	0.538462	0.050879833	0.438737	0.638186	15	53.84615
19	Brazil (Farm)	92	100	0.92	0.09591663	0.732003	1.107997	14	92
20	Slovakia (Farm)	174	220	0.790909	0.059958663	0.67339	0.908428	13	79.09091
21	China (Farm)	206	381	0.540682	0.037671129	0.466847	0.614518	12	54.06824
22	Slovakia (Farm)	92	92	1	0.104257207	0.795656	1.204344	11	100
23	Czech Republic (Farm)	277	800	0.34625	0.020804146	0.305474	0.387026	10	34.625
24	Spain (Farm)	280	1334	0.209895	0.012543629	0.18531	0.234481	9	20.98951
25	Denmark (Research Laboratory)	191	191	1	0.072357461	0.858179	1.141821	7	100
26	Denmark (Research Laboratory)	28	194	0.14433	0.027275787	0.090869	0.19779	6	14.43299

27	Brazil (Farm)	45	70	0.642857	0.095831485	0.455027	0.830687	5	64.28571
28	Poland (Farm)	819	1125	0.728	0.025438379	0.678141	0.777859	4	72.8
29	Argentina (Farm)	70	223	0.313901	0.037518387	0.240365	0.387437	3	31.39013
30	Sweden (Farm)	634	856	0.740654	0.029415136	0.683001	0.798308	2	74.06542
31	Indonesia (Farm)	484	858	0.564103	0.025641026	0.513846	0.614359	3	56.41026
32	Slovakia (Farm)	74	160	0.4625	0.053764533	0.357122	0.567878	4	46.25
33	Brazil (Farm)	99	144	0.6875	0.06909635	0.552071	0.822929	1	68.75
				0.579256	0.0047	0.570044	0.588468	Central Tendency	57.92558

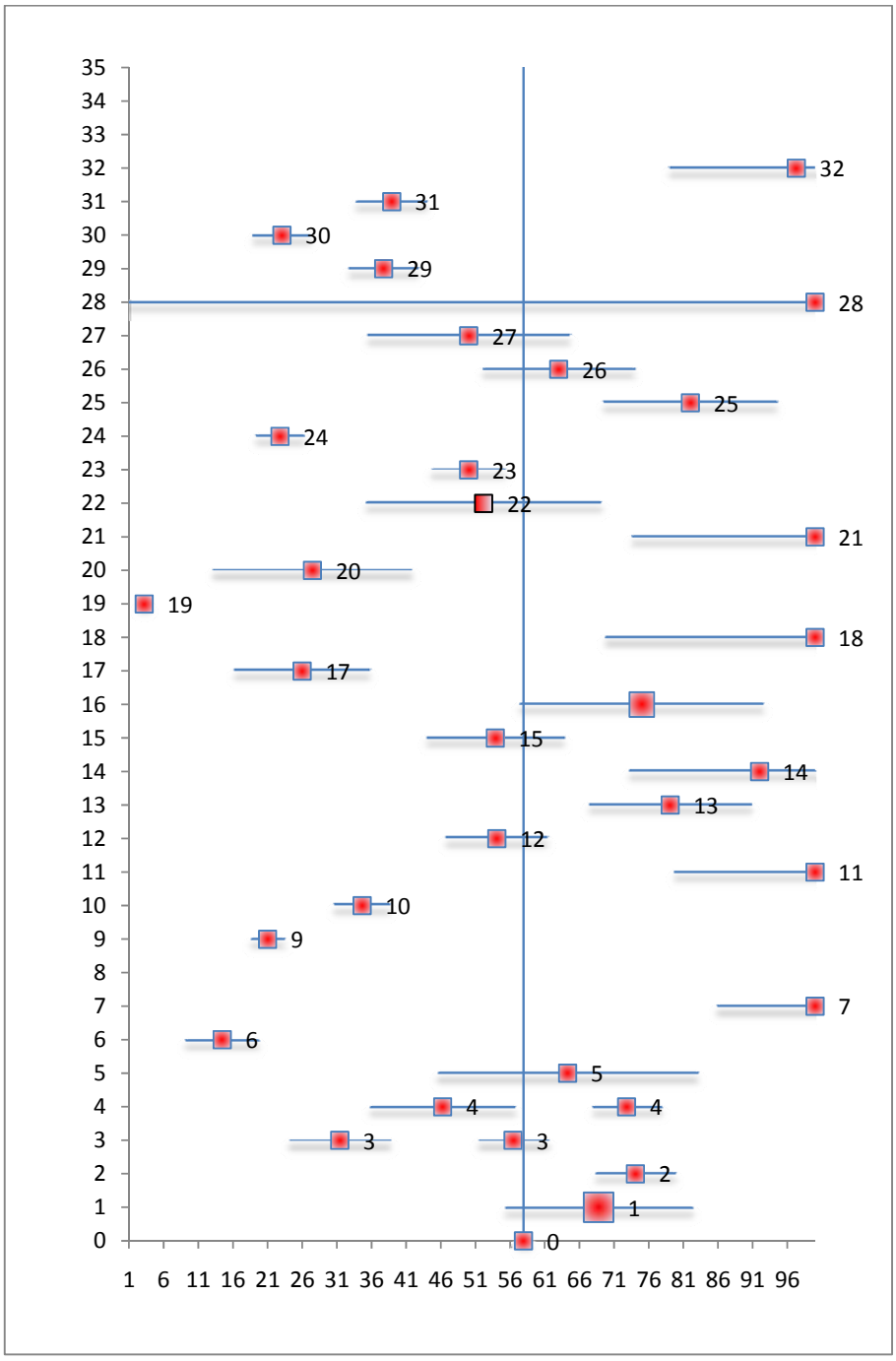


Figure 0.1: Forest plot of *Escherichia coli* prevalence factors in piglets

Table 0.4: *Escherichia coli* virulence factors in piglets based on published information from different countries

Location of study	Events	Sample Size	Outcome (es)	SE	Var	w	w*es	w*(es^2)	w^2	w_v	w_v*es	w_v*(es^2)	w_v^2
US (herd)	108	111	0.973	0.0936	0.0088	114.08333	111	108	13015.007	114.08333	111	108	13015.007
Denmark (Diagnostic samples)	219	563	0.389	0.0263	0.0007	1447.347	563	219	2094813.4	1447.347	563	219	2094813.4
Mexico (Farm)	116	503	0.2306	0.0214	0.0005	2181.1121	503	116	4757249.9	2181.1121	503	116	4757249.9
Japan (Necropsy samples)	214	567	0.3774	0.0258	0.0007	1502.2851	567	214	2256860.4	1502.2851	567	214	2256860.4
India (Farm)	3	3	1	0.5774	0.3333	3	3	3	9	3	3	3	9
Denmark (Research institute)	45	90	0.5	0.0745	0.0056	180	90	45	32400	180	90	45	32400
Vietnam (Farm)	126	200	0.63	0.0561	0.0032	317.46032	200	126	100781.05	317.46032	200	126	100781.05
Canada (Farm)	164	200	0.82	0.064	0.0041	243.90244	200	164	59488.4	243.90244	200	164	59488.4
Korea (Farm)	164	720	0.2278	0.0178	0.0003	3160.9756	720	164	9991766.8	3160.9756	720	164	9991766.8
US (Research institute)	330	660	0.5	0.0275	0.0008	1320	660	330	1742400	1320	660	330	1742400
Spain (Farm)	36	69	0.5217	0.087	0.0076	132.25	69	36	17490.063	132.25	69	36	17490.063
US (Farm)	55	55	1	0.1348	0.0182	55	55	55	3025	55	55	55	3025
England (Farm)	14	51	0.2745	0.0734	0.0054	185.78571	51	14	34516.332	185.78571	51	14	34516.332
Zimbabwe (Farm)	63	1984	0.0318	0.004	0	62480.254	1984	63	3.904E+09	62480.254	1984	63	3.904E+09
Japan (Farm)	42	42	1	0.1543	0.0238	42	42	42	1764	42	42	42	1764
Germany (Farm)	27	104	0.2596	0.05	0.0025	400.59259	104	27	160474.43	400.59259	104	27	160474.43
Vietnam (Farm)	69	92	0.75	0.0903	0.0082	122.66667	92	69	15047.111	122.66667	92	69	15047.111
China (Farm)	112	208	0.5385	0.0509	0.0026	386.28571	208	112	149216.65	386.28571	208	112	149216.65
Brazil (Farm)	92	100	0.92	0.0959	0.0092	108.69565	100	92	11814.745	108.69565	100	92	11814.745
Slovakia (Farm)	174	220	0.7909	0.06	0.0036	278.16092	220	174	77373.497	278.16092	220	174	77373.497
China (Farm)	206	381	0.5407	0.0377	0.0014	704.66505	381	206	496552.83	704.66505	381	206	496552.83
Slovakia (Farm)	92	92	1	0.1043	0.0109	92	92	92	8464	92	92	92	8464
Czech Republic	277	800	0.3463	0.0208	0.0004	2310.4693	800	277	5338268.5	2310.4693	800	277	5338268.5

(Farm)													
Spain (Farm)	280	1334	0.2099	0.0125	0.0002	6355.5571	1334	280	40393107	6355.5571	1334	280	40393107
Denmark (Research Laboratory)	191	191	1	0.0724	0.0052	191	191	191	36481	191	191	191	36481
Denmark (Research Laboratory)	28	194	0.1443	0.0273	0.0007	1344.1429	194	28	1806720	1344.1429	194	28	1806720
Brazil (Farm)	45	70	0.6429	0.0958	0.0092	108.88889	70	45	11856.79	108.88889	70	45	11856.79
Poland (Farm)	819	1125	0.728	0.0254	0.0006	1545.3297	1125	819	2388043.8	1545.3297	1125	819	2388043.8
Argentina (Farm)	70	223	0.3139	0.0375	0.0014	710.41429	223	70	504688.46	710.41429	223	70	504688.46
Sweden (Farm)	634	856	0.7407	0.0294	0.0009	1155.735	856	634	1335723.4	1155.735	856	634	1335723.4
Indonesia (Farm)	484	858	0.5641	0.0256	0.0007	1521	858	484	2313441	1521	858	484	2313441
Slovakia (Farm)	74	160	0.4625	0.0538	0.0029	345.94595	160	74	119678.6	345.94595	160	74	119678.6
Brazil (Farm)	99	144	0.6875	0.0691	0.0048	209.45455	144	99	43871.207	209.45455	144	99	43871.207

Table 0.5: Prevalence of *Escherichia coli* virulence factors in weaners

Serial number	Study	Events	Sample Size	Outcome	SE	CI lower	CI upper	Forest plot ID	Rate
1	south Africa (Farm)	2	2	1	0.707106781	-0.38593	2.385929	1	100
2	China (Lab samples)	63	108	0.583333	0.073493092	0.439287	0.72738	2	58.33333
3	Canada (Farm)	20	50	0.4	0.089442719	0.224692	0.575308	3	40
4	US (Farm)	175	304	0.575658	0.043515647	0.490367	0.660949	4	57.56579
5	China (Field isolates)	140	215	0.651163	0.0550333	0.543298	0.759028	5	65.11628
6	Mexico (Farm)	194	450	0.431111	0.030951974	0.370445	0.491777	6	43.11111
7	Switzerland (Farm)	39	50	0.78	0.12489996	0.535196	1.024804	7	78
8	India	48	720	0.066667	0.009622504	0.047807	0.085527	8	6.666667
9	Cuba (Farm)	36	36	1	0.166666667	0.673333	1.326667	9	100
10	Bulgeria (Farm)	409	619	0.660743	0.032671645	0.596707	0.72478	10	66.07431
11	Canada (Farm)	68	170	0.4	0.048507125	0.304926	0.495074	11	40
12	Hungary (Farm)	126	205	0.614634	0.054755962	0.507312	0.721956	12	61.46341
13	Canada (Animal health Lab)	100	135	0.740741	0.074074074	0.595556	0.885926	13	74.07407
14	Poland (Farm)	46	46	1	0.147441956	0.711014	1.288986	14	100
15	Korea (Farm)	45	604	0.074503	0.011106298	0.052735	0.096272	15	7.450331
16	China (Farm)	21	324	0.064815	0.014143752	0.037093	0.092537	16	6.481481
17	Korea (Pathology Department)	149	476	0.313025	0.025644024	0.262763	0.363287	17	31.30252
18	Korea (Farm)	94	230	0.408696	0.042153738	0.326074	0.491317	18	40.86957
19	Denmark (National Vet Lab)	240	240	1	0.064549722	0.873483	1.126517	19	100
20	Denmark (National Vet Lab)	17	83	0.204819	0.049675971	0.107454	0.302184	20	20.48193
21	Canada (Vet Medicine Faculty)	872	1226	0.711256	0.024086171	0.664047	0.758465	21	71.12561
22	Poland (Farm)	298	372	0.801075	0.046405044	0.710121	0.892029	22	80.10753
23	Poland (Farm)	96	207	0.463768	0.047333135	0.370995	0.556541	23	46.37681
24	Poland (Farm)	608	1146	0.530541	0.021516279	0.488369	0.572713	24	53.0541
25	Poland (Farm)	40	40	1	0.158113883	0.690097	1.309903	25	100
				0.579062	0.0047	0.56985	0.588274	Central Tendency	57.90619

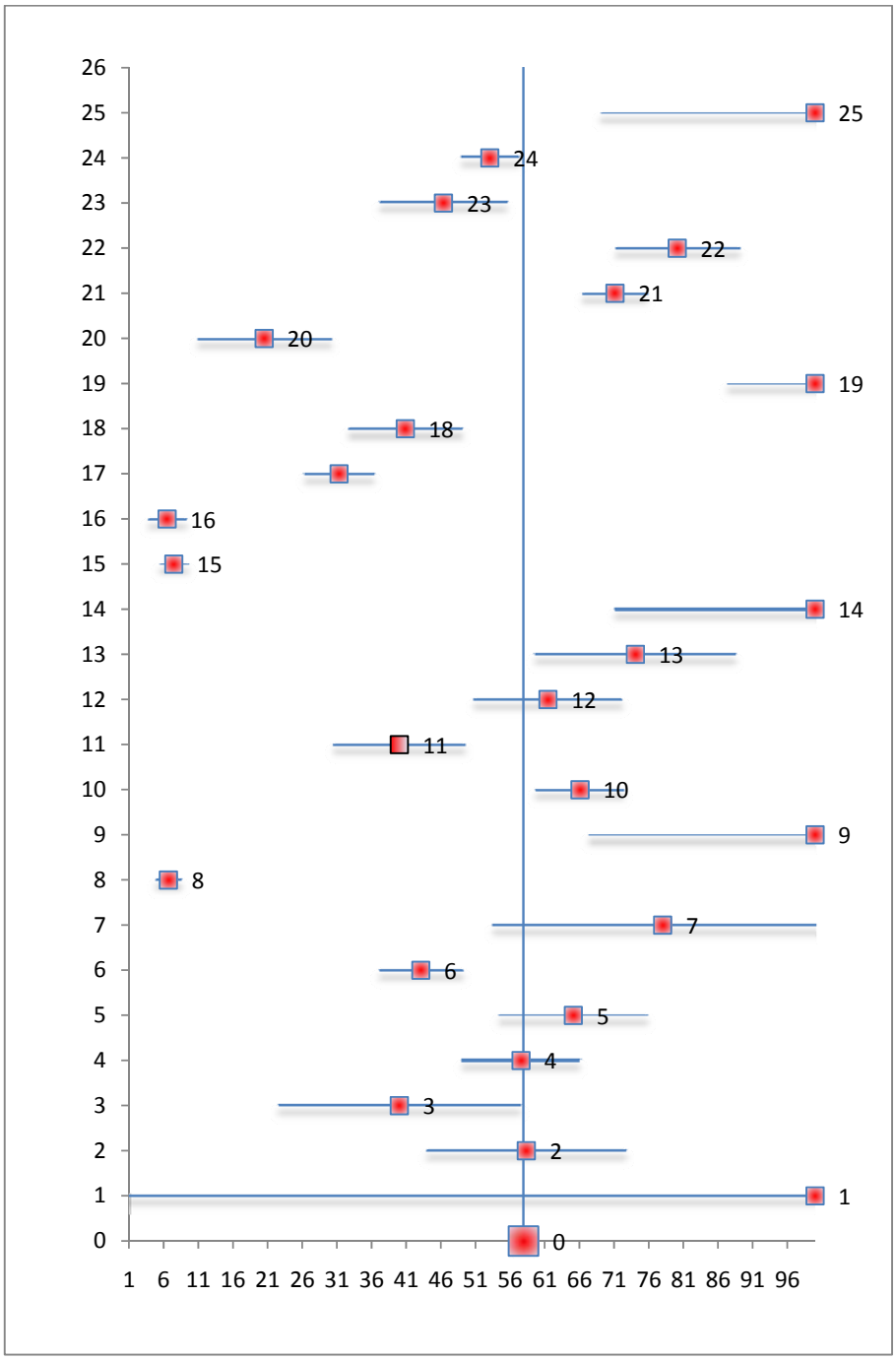


Figure 0.2: Forest plot for prevalence of *Escherichia coli* virulence factors in weaners

Table 0.6: *Escherichia coli* virulence factors in weaners based on published information from different countries

Location of study	Events	Sample Size	Outcome (es)	SE	Var	w	w*es	w*(es^2)	w^2	w_v	w_v*es	w_v*(es^2)	w_v^2
south Africa (Farm)	2	2	1.00	0.71	0.50	2	2	2	4	2.00	2.00	2.00	4
China (Lab samples)	63	108	0.58	0.07	0.01	185.14	108	63	34277.88	185.14	108.00	63.00	34277.88
Canada (Farm)	20	50	0.40	0.09	0.01	125	50	20	15625	125.00	50.00	20.00	15625
US (Farm)	175	304	0.58	0.05	0.00	528.09	304	175	278880.56	528.09	304.00	175.00	278880.56
China (Field isolates)	140	215	0.65	0.06	0.00	330.18	215	140	109017.89	330.18	215.00	140.00	109017.89
Mexico (Farm)	194	450	0.43	0.03	0.00	1043.81	450	194	1089548.6	1043.81	450.00	194.00	1089548.60
Switzerland (Farm)	39	50	0.78	0.12	0.02	64.10	50	39	4109.1387	64.10	50.00	39.00	4109.14
India	48	720	0.07	0.01	0.00	10800	720	48	116640000	10800.00	720.00	48.00	116640000
Cuba (Farm)	36	36	1.00	0.17	0.03	36	36	36	1296	36.00	36.00	36.00	1296
Bulgeria (Farm)	409	619	0.66	0.03	0.00	936.82	619	409	877639.13	936.82	619.00	409.00	877639.13
Canada (Farm)	68	170	0.40	0.05	0.00	425	170	68	180625	425.00	170.00	68.00	180625
Hungary (Farm)	126	205	0.61	0.05	0.00	333.53	205	126	111243.43	333.53	205.00	126.00	111243.43
Canada (Animal health Lab)	100	135	0.74	0.07	0.01	182.25	135	100	33215.06	182.25	135.00	100.00	33215.06
Poland (Farm)	46	46	1.00	0.15	0.02	46	46	46	2116	46.00	46.00	46.00	2116
Korea (Farm)	45	604	0.07	0.01	0.00	8107.02	604	45	65723809	8107.02	604.00	45.00	65723809
China (Farm)	21	324	0.06	0.01	0.00	4998.86	324	21	24988573	4998.86	324.00	21.00	24988573
Korea (Pathology Department)	149	476	0.31	0.03	0.00	1520.64	476	149	2312359.1	1520.64	476.00	149.00	2312359.1
Korea (Farm)	94	230	0.41	0.04	0.00	562.77	230	94	316705.52	562.77	230.00	94.00	316705.52
Denmark (National Vet Lab)	240	240	1.00	0.06	0.00	240	240	240	57600	240.00	240.00	240.00	57600
Denmark (National Vet Lab)	17	83	0.20	0.05	0.00	405.24	83	17	164215.64	405.24	83.00	17.00	164215.64

Canada (Vet Medicine Faculty)	872	1226	0.71	0.02	0.00	1723.71	1226	872	2971179.6	1723.71	1226.00	872.00	2971179.6
Poland (Farm)	298	372	0.80	0.05	0.00	464.38	372	298	215644.92	464.38	372.00	298.00	215644.92
Poland (Farm)	96	207	0.46	0.05	0.00	446.34	207	96	199222.74	446.34	207.00	96.00	199222.74
Poland (Farm)	608	1146	0.53	0.02	0.00	2160.06	1146	608	4665855.8	2160.06	1146.00	608.00	4665855.8
Poland (Farm)	40	40	1.00	0.16	0.03	40	40	40	1600	40.00	40.00	40.00	1600
K	16				Sums:	35706.95	8058	3946	220994363	35706.95	8058	3946	220994363
Df	15												
										v	0.07		
Q	2127.5483			Q_v	2127.548312								
I^2	99.294963			I^2_v	99.29496313								
es (fixed)	0.2256704			es (random)	0.225670351								
SEes (fixed)	0.005292			SEes (random)	0.005292046								
CI (fixed)	0.2152979	0.23604		CI (random)	0.215297941	0.2360428							

Table 0.7: Prevalence of *Escherichia coli* virulence factors in porkers

serial number	Study	Events	Sample Size	Outcome	SE	CI lower	CI upper	Forest plot ID	Rate
1	Norway	3	1976	0.001518219	0.000876544	-0.0002	0.003236	1	0.151822
2	US	6	305	0.019672131	0.008031114	0.003931	0.035413	2	1.967213
3	US	484	687	0.704512373	0.03202329	0.641747	0.767278	3	70.45124
4	Belgium	95	135	0.703703704	0.072198477	0.562195	0.845213	4	70.37037
5	south Africa	106	263	0.403041825	0.039146883	0.326314	0.47977	5	40.30418
				0.36648965	0.0047	0.357278	0.375702	Central Tendency	36.64897

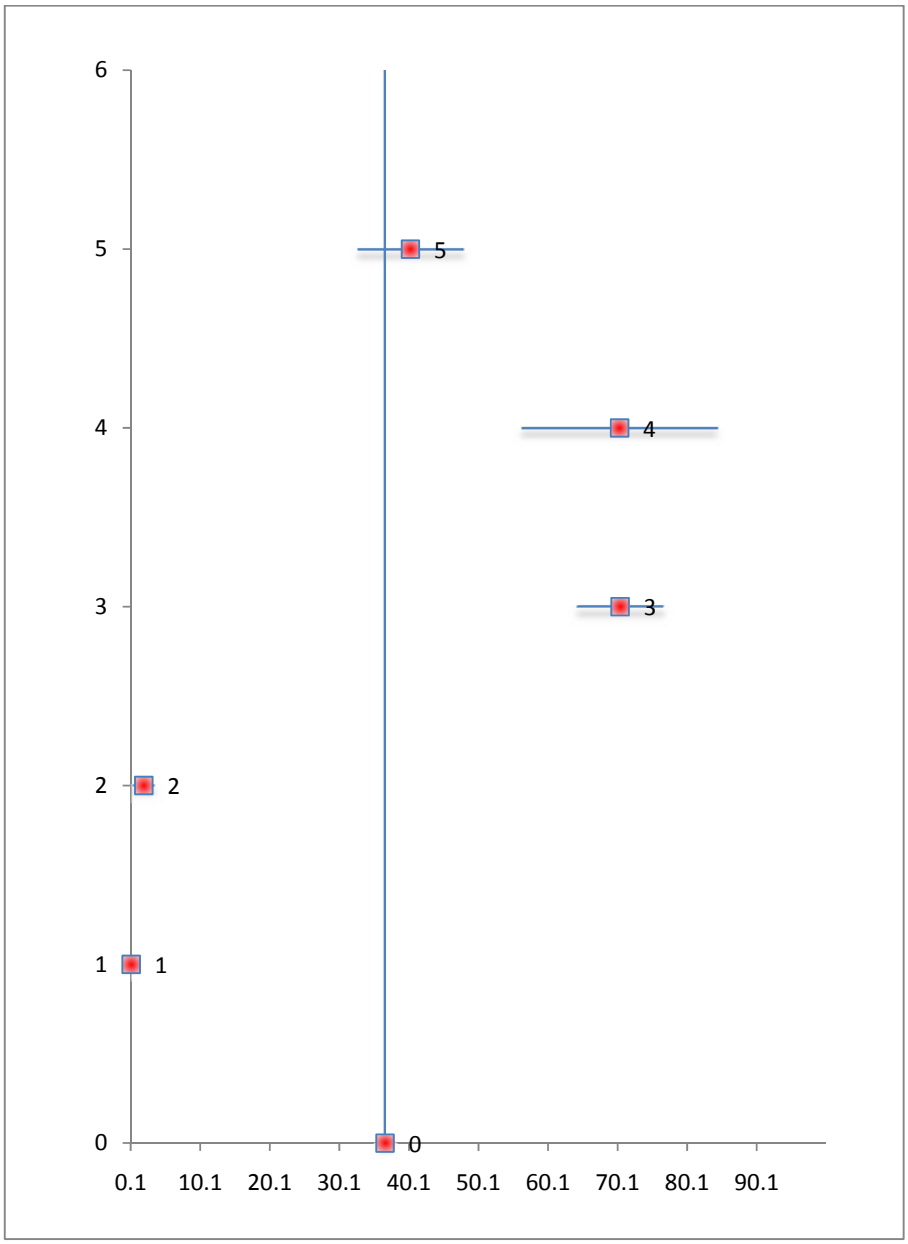


Figure 0.3: Forest plot for prevalence of *Escherichia coli* virulence factors in porkers

Table 0.8: *Escherichia coli* virulence factors in porkers based on published information from different countries

Location of study	Events	Sample Size	Outcome (es)	SE	Var	w	w*es	w*(es^2)	w^2	w_v	w_v*es	w_v*(es^2)	w_v^2
Norway	3	1976	0.00	0.00	0.00	1301525.3	1976	3	1.694E+12	51.07	0.08	0.00	2607.7405
US	6	305	0.02	0.01	0.00	15504.17	305	6	240379184	50.90	1.00	0.02	2590.8494
US	484	687	0.70	0.03	0.00	975.14	687	484	950903.02	48.53	34.19	24.09	2354.8415
Belgium	95	135	0.70	0.07	0.01	191.84	135	95	36803.39	40.33	28.38	19.97	1626.6533
south Africa	106	263	0.40	0.04	0.00	652.54	263	106	425805.5	47.36	19.09	7.69	2243.1118
		3366	0.37										
K	16				Sums:	1318849	3366	694	1.694E+12	238.19	82.74	51.77	11423.197
Df	15												
										v	0.020		
Q	685.40921			Q_v	23.031651								
I^2	97.811526			I^2_v	34.872232								
es (fixed)	0.0025522		es (random)		0.3473616								
SEes (fixed)	0.0008708		SEes (random)		0.064795								
CI (fixed)	0.0008455	0.0042589	CI (random)		0.2203634	0.4743598							

2.9 References

Abramson JH (2011). WINPEPI updated: computer programs for epidemiologists, and their teaching potential. Epidemiol. Perspect. Innov. 8:1.

Agdestein A, Johansen TB, Polaček V, Lium B, Holstad G, Vidanović D, Aleksić-Kovačević S, Jørgensen A, Žultauskas J, Nilsen SF (2011). Investigation of an outbreak of mycobacteriosis in pigs. BMC Vet. Res. 7(1):10.118

Alban L, Baptista F, Møgelmose V, Sørensen L, Christensen H, Aabo S, Dahl J (2012). *Salmonella* surveillance and control for finisher pigs and pork in Denmark: a case study. Food Res. Int. 45(2): 656-665.

Alter T, Gaull F, Kasimir S, Gürtler M, Mielke H, Linnebur M, Fehlhaber K (2005). Prevalences and transmission routes of *Campylobacter* spp. strains within multiple pig farms Vet. Microbiol.108(3):251-261.

Amano H, Shibata M, Kajio N, Morozumi T (1994). Pathologic observations of pigs intranasally inoculated with serovar 1, 4 and 5 of *Haemophilus parasuis* using immunoperoxidase method. J. Vet. Med. Sci.56(4): 639-644.

Amezcua R, Friendship RM, Dewey CE, Gyles, C, Fairbrother JM (2002). Presentation of postweaning *Escherichia coli* diarrhoea in southern Ontario, prevalence of hemolytic *E. coli* serogroups involved and their antimicrobial resistance patterns.Can. J. Vet.Res.66(2): 73-78.

An H, Fairbrother JM, Désautels C, Mabrouk T, Dugourd D, Dezfulian H, Harel J (2000). Presence of the LEE (locus of enterocyte effacement) in pig attaching and effacing *Escherichia coli* and characterization of eae, espA, espB and espD genes of PEPEC (pig EPEC) strain 1390.Microb. Pathog.28(5): 291-300.

Andresen LO (1998). Differentiation and distribution of three types of exfoliative toxin produced by *Staphylococcus hyicus* from pigs with exudative epidermitis.FEMS Immunol. Med. Microbiol.20(4): 301-310.

Andresen LO, Bille-Hansen V, Wegener HC (1997). Staphylococcus hyicusexfoliative toxin: purification and demonstration of antigenic diversity among toxins from virulent strains.Microb. Pathog.22(2):113-122.

Arbuckle J (1970). The location of *Escherichia coli* in the pig intestine.J. Med Microbiol.3(2): 333-340.

Backstrom LR, Brim TA, Collins MT (1988). Development of turbinate lesions and nasal colonization by *Bordetella bronchiseptica* and *Pasteurella multocida* during long-term exposure of healthy pigs to pigs affected by atrophic rhinitis.Can. J. Vet. Res.52(1): 23-29.

Baker AA, Davis E, Rehberger T, Rosener D (2010). Prevalence and diversity of toxigenic *Clostridium perfringens*and *Clostridium difficile* among swine herds in the midwest. Appl. Environ.Microbiol.76(9): 2961-2967.

Barman N, Deb R, Ramamurthy T, Sharma R, Borah P, Wani S, Kalita D (2008). Molecular characterization of shiga like toxin-producing *Escherichia coli* (STEC) isolates from pigs oedema.Indian J. Med. Res.127(6): 602-606.

Barth S, Schwanitz A, Bauerfeind R (2011). Polymerase chain reaction-based method for the typing of F18 fimbriae and distribution of F18 fimbrial subtypes among porcine Shiga toxin-encoding *Escherichia coli* in Germany. J. Vet. Diagn. Invest.23(3): 454-464.

Begum J, Dutta T, Chandra R, Choudhary PR, Varte Z, Bitew M (2014). Molecular and phenotypic characterization of shigatoxigenic *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) from piglets and infants associated with diarrhoea in Mizoram, India.Afr.J. Biotechnol.13(12): 1452-1461

Beiers P, Simmons G (1967). Botulism in pigs. Aust.Vet. J.43(7): 270-271.

Bertschinger H (1999).Postweaning*Escherichia coli* diarrhea and edema disease: Diseases of Swine, Blackwell Science,Oxford.441-454.

Bhaduri S, Wesley IV, Bush EJ (2005). Prevalence of pathogenic *Yersinia enterocolitica* strains in pigs in the United States. Appl. Environ. Microbiol.71(11):7117-7121.

Biet F, Boschiroli ML, Thorel MF, Guilloteau LA (2005). Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC).Vet. Res.36(3): 411-436.

Binder A, Aumüller R, Likitdecharote B, Kirchhoff H (1990). Isolation of *Mycoplasma arthritidis* from the joint fluid of boars. J. Vet. Med. Series B.37(1-10): 611-614.

Blanchard B, Vena MM, Cavalier A, Le Lannic J, Gouranton J, Kobisch M (1992). Electron microscopic observation of the respiratory tract of SPF piglets inoculated with *Mycoplasma hyopneumoniae*.Vet. Microbiol.30(4): 329-341.

Blanco M, Lazo L, Blanco JE, Dahbi G, Mora A, López C, González EA, Blanco J (2006). Serotypes, virulence genes, and PFGE patterns of enteropathogenic *Escherichia coli* isolated from Cuban pigs with diarrhea.Int. Microbiol.9(1): 53-60.

Borch E, Nesbakken T Christensen H (1996). Hazard identification in swine slaughter with respect to foodborne bacteria. Int. J. Food Microbiol.30(1): 9-25.

Boutrup TS, Boesen H, Boye M, Agerholm JS, Jensen TK (2010). Early pathogenesis in porcine proliferative enteropathy caused by *Lawsonia intracellularis*.J. Comp. Pathol.143(2):101-109.

Brockmeier SL (2004). Prior infection with *Bordetella bronchiseptica* increases nasal colonization by*Haemophilus parasuis* in swine.Vet. Microbiol.99(1):75-78.

Brockmeier SL, Palmer MV, Bolin SR, Rimler RB (2001). Effects of intranasal inoculation with *Bordetella bronchiseptica*, porcine reproductive and respiratory syndrome virus, or a combination of both organisms on subsequent infection with *Pasteurella multocida* in pigs. Am. J.Vet. Res.62(4):521-525.

Brugmann M, Peters M, Mumme J (2001). Case report: *Yersinia enterocolitica* septicemia in an American minipig. Dtsch. Tierarztl.Wochenschr108(6): 257-260.

Burrin D, Stoll B (2003). 12 Intestinal nutrient requirements in weanling pigs.Weaning the pig: concepts and consequences. P. 301.

Busch R, Barnes D, Sautter J (1971). Pathogenesis and pathologic changes of experimentally induced listeriosis in newborn pigs. Amer. J. Vet. Res.

Canibe N, Jensen BB (2012). Fermented liquid feed-Microbial and nutritional aspects and impact on enteric diseases in pigs. Anim. Feed Sci.Technol.173(1): 17-40.

Carstensen L, Ersbøll AK, Jensen KH, Nielsen JP (2005). *Escherichia coli* post-weaning diarrhoea occurrence in piglets with monitored exposure to creep feed.Vet.Microbiol.110(1): 113-123.

Chen R, Li B, Zhang X, Liu H, He D (2013). Diversity of *Escherichia coli* Isolated from Southern China. J. Anim. Vet. Adv.12(14):1233-1236.

Chen S, Wang Y, Chen F, Yang H, Gan M, Zheng SJ (2007). A highly pathogenic strain of *Staphylococcus sciuri* caused fatal exudative epidermitis in piglets.PLoS One,2(1): e147.

Chen X, Gao S, Jiao X, Liu XF (2004). Prevalence of serogroups and virulence factors of *Escherichia coli* strains isolated from pigs with post-weaning diarrhoea in eastern China.Vet. Microbiol.103(1):13-20.

Cheng D, Sun H, Xu J Gao S (2005). Prevalence of fimbial colonization factors F18ab and F18ac in *Escherichia coli* isolates from weaned piglets with oedema and/or diarrhoea in China.Vet. Microbiol.110(1):35-39.

Cheon DS, Chae C (1996). Outbreak of diarrhoea associated with *Enterococcus durans* in piglets. J. Vet.Diagn. Invest.8(1):123-124.

Choi C, Cho W, Chung H, Jung T, Kim J, Chae C (2001a). Prevalence of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 (EAST1) gene in isolates in weaned pigs with diarrhea and/or edema disease. Vet. Microbiol81(1): 65-71.

Choi C, Kwon D, Chae C (2001b). Prevalence of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene and its relationship with fimbrial and enterotoxin genes in *E. coli* isolated from diarrheic piglets. J. Vet. diagn. Invest.13(1): 26-29.

Contzen M, Sting R, Blazey B, Rau J (2011). *Corynebacterium ulcerans* from diseased wild boars.Zoonoses Public Health. 58(7): 479-488.

Cox E, Houvenaghel A (1993). Comparison of the in vitro adhesion of K88, K99, F41 and P987 positive *Escherichia coli* to intestinal villi of 4-to 5-week-old pigs.Vet. Microbiol.34(1): 7-18.

Czirók É, Semjen G, Steinrück H, Herpay M, Milch H, Nyomärkay I, Stverteczky Z, Szeness A (1992). Comparison of rapid methods for detection of heat-labile (LT) and heat-stable (ST) enterotoxin in *Escherichia coli*. J. Med. Microbiol.36(6): 398-402.

da Silva AS, Valadares GF, Penatti MPA, Brito BG, da Silva Leite D (2001). *Escherichia coli* strains from oedema disease: O serogroups, and genes for Shiga toxin, enterotoxins, and F18 fimbriae.Vet. Microbiol.80(3): 227-233.

Davies RL, MacCorquodale R, Baillie S, Caffrey B (2003). Characterization and comparison of *Pasteurella multocida* strains associated with porcine pneumonia and atrophic rhinitis.J. Med. Microbiol.52(1): 59-67.

Davies RL, MacCorquodale R, Reilly S (2004). Characterisation of bovine strains of Pasteurella multocida and comparison with isolates of avian, ovine and porcine origin.Vet. Microbiol.99(2):145-158.

De Bruyne E, Flahou B, Chiers K, Meyns T, Kumar S, Vermoote M, Pasmans F, Millet S, Dewulf J, Haesebrouck F (2012). An experimental Helicobacter suis infection causes gastritis and reduced daily weight gain in pigs.Vet. Microbiol.160(3): 449-454.

de Haan L, Hirst TR (2004). Cholera toxin: a paradigm for multi-functional engagement of cellular mechanisms. Molec. Membrane Biol. 21: 77–92.

De Neeling A, Van den Broek M, Spalburg E, Van Santen-Verheuvel M, Dam-Deisz W, Boshuizen H, Van De Giessen A, Van Duijkeren E, Huijsdens X (2007). High prevalence of methicillin resistant*Staphylococcus aureus* in pigs.Vet.Microbiol.122(3): 366-372.

De Smet S, De Zutter L, Houf K (2012). Spatial distribution of the emerging foodborne pathogen *arcobacter* in the gastrointestinal tract of pigs. Foodborne pathog. Dis.9(12): 1097-1103.

DebRoy C, Roberts E, Scheuchenzuber W, Kariyawasam S, Jayarao BM (2009). Comparison of genotypes of *Escherichia coli* strains carrying F18ab and F18ac fimbriae from pigs.J. Vet. Diagn Invest.21(3):359-364.

Deyoe B (1967) Pathogenesis of three strains of Brucella suis in swine.Am J. Vet. Res. 28:951-957.

Diab SS, Uzal FA, Songer JG, Prescott JF, Popoff MR (2016). Diseases produced by *Clostridium difficile*.Clostridial Diseases of Animals, Wiley-Blackwell. pp.177-195.

Do TN, Cu PH, Nguyen HX, Au TX, Vu QN, Driesen SJ, Townsend KM, Chin JJ, Trott DJ (2006). Pathotypes and serogroups of enterotoxigenic *Escherichia coli* isolated from pre-weaning pigs in north Vietnam.J. Med. Microbiol.55(1): 93-99.

Došen R, Prodanov J, Milanov D, Stojanov I, Pušić I (2007). The bacterial infections of respiratory tract of swine.Biotech. Anim. Husbandry.23(5-6-2):237-243.

Dubreuil JD (2012). The whole Shebang: the gastrointestinal tract, *Escherichia coli* enterotoxins and secretion.Curr. Issues Mol. Biol.14(2):71-82.

Duncan JR, Ross RF, Switzer WP, Ramsey FK (1966). Pathology of experimental *Bordetella bronchiseptica* infection in swine: atrophic rhinitis.Am. J. Vet. Res.27(117): 457-466.

Duran C, Walton J (1997). *Clostridium novyi* sudden death in sows: toxaemia or post mortem invader.Pig J. 39: 37- 53

Dziva F, Muhairwa AP, Bisgaard M, Christensen H (2008). Diagnostic and typing options for investigating diseases associated with *Pasteurella multocida*.Vet. Microbiol.128(1):1-22.

Epps SV, Harvey RB, Hume ME, Phillips TD, Anderson, RC, Nisbet DJ (2013). Foodborne *Campylobacter*: infections, metabolism, pathogenesis and reservoirs.Int. J. Environ. Res Public Health, 10(12): 6292-6304.

Evans DJ, Chen LC, Curlin GT, Evans DG (1972). Stimulation of adenyl cyclase by *Escherichia coli* enterotoxin. Nature236(66):137-138.

Faine S (1994).*Leptospira* and leptospirosis.CRC Press Inc. Boca Raton.

Fairbrother, J. (1999) Identification, nomenclature, and diagnosis of pathogenic *Escherichia coli*.In: Proceedings of the Annual Meeting of the Western Canadian Association of Swine Practitioners, Saskatoon, Canada, pp. 21–31.

Fairbrother JM, Nadeau E, Gyles CL (2005). Escherichia coli in post-weaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim. Health Re. Rev. 6(01)): 17-39.

Fairbrother J, Gyles C (2006).Post-weaning *Escherichia coli* diarrhoea and oedema disease:Diseases of Swine. Blackwell Publishing. (9): 649-662.

Fairbrother J, Gyles C (2012). Colibacillosis: Diseases of swine.Blackwell Publishing.10:723-749.

Fairbrother J, Ngeleka M (1994). Extraintestinal *Escherichia coli* infections in pigs: In *Escherichia coli* in domestic animals and humans. CAB international, Wallingford, United Kingdom. pp 221-236

Fasina FO, Bwala DG, Madoroba E (2015). Investigation of multidrug-resistant fatal colisepticaemia in weanling pigs.Onderstepoort J. Vet. Res.82(1): 1-6

Feder I, Wallace FM, Gray JT, Fratamico P, Fedorka-Cray PJ, Pearce RA, Call JE, Perrine R, Luchansky JB (2003). Isolation of *Escherichia coli* O157: H7 from intact colon faecal samples of swine.Emerg Infect Dis.9(3):380-383.

Fedorka-Cray PJ, Gray JT, Wray C (2000). *Salmonella* infections in pigs:*Salmonella* in domestic animals.CABI publishing, Oxon, Great Britain. pp. 191-207.

Fedorka-Cray PJ, Kelley LC, Stabel TJ, Gray JT, Laufer JA (1995). Alternate routes of invasion may affect pathogenesis of *Salmonella typhimurium* in swine.*Infect. Immun.*63(7):2658-2664.

Francis DH (2002). Enterotoxigenic *Escherichia coli* infection in pigs and its diagnosis.J. Swine Health Prod.10(4):171-175.

Frank RK, Chengappa MM, Oberst RD, Hennessy KJ, Henry SC, Fenwick B (1992). Pleuropneumonia caused by *Actinobacillus pleuropneumoniae* biotype 2 in growing and finishing pigs.J. Vet. Diagn Invest.4(3): 270-278.

Fratamico PM, Bagi LK, Bush EJ, Solow BT (2004). Prevalence and characterization of shiga toxin-producing *Escherichia coli* in swine feces recovered in the National Animal Health Monitoring System's Swine 2000 study.Appl. Environ. Microbiol.70(12):7173-7178.

Frydendahl K (2002). Prevalence of serogroups and virulence genes in *Escherichia coli* associated with post-weaning diarrhoea and oedema disease in pigs and a comparison of diagnostic approaches.Vet. Microbiol.85(2):169-182.

Frydendahl K, Jensen TK, Andersen JS, Fredholm M, Evans G (2003).Association between the porcine *Escherichia coli* F18 receptor genotype and phenotype and susceptibility to colonisation and post-weaning diarrhoea caused by *E. coli* O138: F18. Vet Microbiol. 93(1): 39-51.

Garabal J, González E, Vazquez F, Blanco J, Blanco M (1995). Toxigenic *Escherichia coli* in Spanish piggeries from 1986 to 1991. Vet. Microbiol.47(1):17-25.

Garabal J, Vázquez F, Blanco J, Blanco M, González E (1997). Colonization antigens of enterotoxigenic *Escherichia coli* strains isolated from piglets in Spain.Vet. Microbiol.54(3): 321-328.

Giles C (1992). Bordetellosis: Diseases of swine.Iowa State University Press, Ames.pp. 436-445.

Giuffra E, Kijas JM, Amarger V, Carlborg O, Jeon JT, Andersson L (2000). The origin of the domestic pig: independent domestication and subsequent introgression.Genetics154(4): 1785-1791.

Godfroid J (2002). Brucellosis in wildlife. Rev. Sci. Tech.21(1):277-286.

Gosh L (2014). Why pigs are so valuable for medical research*http://a-z-animals.com/animals/pig/*. Accessed:15th january 2016.

Gottschalk M, Segura M, Xu J (2007). *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America.Anim. Health Res. Rev.8(01):29-45.

Goyette-Desjardins G, Auger J, Xu J, Segura M, Gottschalk M (2014). *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing.Emerg.Microbes Infect.3(6): e45.

Grasso G, Ripabelli G, Sammarco M, Ruberto A, Iannitto G (1996). Prevalence of *Helicobacter*-like organisms in porcine gastric mucosa: a study of swine slaughtered in Italy.Comp. Immun. Microbiol. Infect. Dis.19(3): 213-217.

Gray S, Stickler D (1989). Some observations on the faecal carriage of mesophilic *Aeromonas* species in cows and pigs.Epidemiol. Infect.103(03):523-537.

Gray JT, Fedorka-Cray PJ, Stabel TJ, Kramer TT (1996).Natural transmission of *Salmonella choleraesuis* in swine.Appl. Environ. Microbiol.62(1):141-146.

Guedes RM, Gebhart CJ (2003). Comparison of intestinal mucosa homogenate and pure culture of the homologous *Lawsonia intracellularis* isolate in reproducing proliferative enteropathy in swine.Vet Microbiol.93(2):159-166.

Gupta S, Keck J, Ram P, Crump J, Miller M, Mintz E (2008). Part III. Analysis of data gaps pertaining to enterotoxigenic *Escherichia coli* infections in low and medium human development index countries, 1984–2005.Epidemiol. Infect.136(06):721-738.

Gyles CL, Stevens JB, Craven JA (1971). A study of *Escherichia coli* strains isolated from pigs with gastro-intestinal disease.Can, J. Comp. med.35(3):258-266.

Ha SK, Choi C, Chae C (2003). Prevalence of a gene encoding adhesin involved in diffuse adherence among *Escherichia coli* isolates in pigs with post-weaning diarrhoea or oedema disease.J. Vet.Diagn. Invest.15(4):378-381.

Haesebrouck F, Pasmans F, Chiers K, Maes D, Ducatelle R, Decostere A (2004). Efficacy of vaccines against bacterial diseases in swine: what can we expect.Vet. Microbiol.100(3): 255-268.

Hampson D, Hinton M, Kidder D (1985). Coliform numbers in the stomach and small intestine of healthy pigs following weaning at three weeks of age.J. Comp. Pathol.95(3): 353-362.

Harel J, Lapointe H, Fallara A, Lortie LA, Bigras-Poulin M, Lariviere S, Fairbrother JM (1991). Detection of genes for fimbrial antigens and enterotoxins associated with *Escherichia coli* serogroups isolated from pigs with diarrhoea.J. Clin. Microbiol.29(4):745-752.

Heinritzi K (1989). *Eperythrozoon* infection in swine as a disease factor. Berl. Munch. Tierarztl. Wochenschr. 102(10): 337-342.

Helgerson AF, Sharma V, Dow AM, Schroeder R, Post K, Cornick NA (2006). Edema disease caused by a clone of *Escherichia coli* O147. J. Clin. Microbiol.44(9): 3074-3077.

Helie P, Morin M, Jacques M, Fairbrother JM (1991). Experimental infection of newborn pigs with an attaching and effacing *Escherichia coli* O45:K"E65" strain. Infect. Immun.59(3): 814-821.

Hellemans A, ChiersK, Maes D, De Bock M, Decostere A, Haesebrouck F, Ducatelle R (2007). Prevalence of '*CandidatusHelicobacter suis*' in pigs of different ages.Vet. Rec. 161(6): 189-192

Higgins R, Gottschalk M (1999). *Streptococcal* diseases: Diseases of swine.8: 563-570.

Hijazin M, Metzner M, Erhard M, Nagib S, Alber J, Lämmler C, Hassan A, Prenger-Berninghoff E, Zschöck M (2012). First description of *Trueperella* (*Arcanobacterium*) bernardiae of animal origin.Vet. Microbiol.159(3): 515-518.

Hinton M, Hampson D, Hampson E, Linton A (1985). A comparison of the ecology of *Escherichia coli* in the intestine of healthy unweaned pigs and pigs after weaning.J. Appl. Bacteriol.58(5): 471-477.

Hirst R, Patten B (1991). The importance of enterotoxigenic *Escherichia coli* containing the 987P antigen in causing neonatal colibacillosis in piglets in Indonesia.Vet. Microbiol.26(4): 393-400.

Houdijk J, Hartemink R, Verstegen M, Bosch M (2002). Effects of dietary non-digestible oligosaccharides on microbial characteristics of ileal chyme and faeces in weaner pigs.Arch. Anim. Nutr.56(4): 297-307.

Igbinosa IH, Igbinosa EO, Okoh AI (2016). Antibiogram characterization and putative virulence genes in *Aeromonas* species isolated from pig faecal samples.Environ. Sci. Pollut Res.23: 1-7.

Imberechts H, Bertschinger H, Stamm M, Sydler T, Pohl P, De Greve H, Hernalsteens J, Van Montagu M, Lintermans P (1994). Prevalence of F107 fimbriae on *Escherichia coli* isolated from pigs with oedema disease or postweaning diarrhoea.Vet Microbiol.40(3-4): 219-230.

Inderlied CB, Kemper CA, Bermudez LE (1993). The *Mycobacterium avium* complex.Clin Microbiol. Rev.6(3): 266-310

Janczyk P, Pieper R, Smidt H, Souffrant WB (2007). Changes in the diversity of pig ileal *lactobacilli* around weaning determined by means of 16S rRNA gene amplification and denaturing gradient gel electrophoresis.FEMS Microbiol. Ecol.61(1): 132-140.

Janke BH, Francis DH, Collins JE, Libal MC, Zeman DH, Johnson DD (1989). Attaching and effacing *Escherichia coli* infections in calves, pigs, lambs, and dogs.J. VetDiagn Invest1(1): 6-11.

Jensen AN, Dalsgaard A, Baggesen DL, Nielsen E (2006). The occurrence and characterization of *Campylobacter jejuni* and *C. coli* in organic pigs and their outdoor environment.Vet. Microbiol.116(1), pp. 96-105.

Jensen BB. (2001). possible ways of modifying type and amount of products from microbial fermentation in the gut: Manipulation of the gut environment in pigs. Nottingham University Press. pp. 12-14.

Jensen TK, Strijkstra G Gruys E, Baumgärtner W, Schou KK, Boye M (2014). Detection of polytreponemal infection in three cases of porcine ulcerative stomatitis by Fluorescent in situ hybridization.Cutting Edge Pathol.

Jiang H, Huang S, Zhang W, Zhao L, Xu C, Deng S, Zhu X (2013). Seroprevalence of *Chlamydia* infection in pigs in Jiangxi province, south-eastern China. J. Microbiol.62(12): 1864-1867.

Johnsen G, Wasteson Y, Heir E, Berget OI, Herikstad H (2001). *Escherichia coli* O157: H7 in faeces from cattle, sheep and pigs in the southwest part of Norway during 1998 and 1999.Int. J. Food Microbiol.65(3): 193-200.

Karlsson F (2014).*Treponema* spp. in porcine skin ulcers.Phd. Thesis, Swedish University of Agricultural Sciences, Uppsala.

Karlsson F, Svartström O, Belák K, Fellström C, Pringle M (2013). Occurrence of Treponema spp. in porcine skin ulcers and gingiva.Vet.Microbiol.165(3): 402-409.

Kennedy M, Rosnick D, Ulrich R, Yancey J R (1988). Association of *Treponema hyodysenteriae* with porcine intestinal mucosa.Microbiol.134(6):1565-1576.

Kenworthy R, Crabb W (1963). The intestinal flora of young pigs, with reference to early weaning, *Escherichia coli* and scours. J. Comp. Pathol Ther.73: 215-228.

Komijn RE, de Haas PE, Schneider MM, Eger T, Nieuwenhuijs JH, van den Hoek RJ, Bakker D, van Zijd Erveld FG, van Soolingen D (1999). Prevalence of *Mycobacterium avium* in slaughter pigs in The Netherlands and comparison of IS1245 restriction fragment length polymorphism patterns of porcine and human isolates.J.Clin.Microbiol.37(5): 1254-1259.

Konstantinov SR, Awati AA, Williams BA, Miller BG, Jones P, Stokes CR, Akkermans AD, Smidt H, De Vos WM (2006). Post-natal development of the porcine microbiota composition and activities.Environ. Microbiol.8(7): 1191-1199.

Konstantinov SR, Favier CF, Zhu WY, Williams BA, Klüß J, Souffrant W, de Vos WM, Akkermans AD, Smidt H (2004). Microbial diversity studies of the porcine gastrointestinal ecosystem during weaning transition. Anim. Res.53(4): 317-324.

Kudo Y, Yanagawa R (1987). Isolation and numerical taxonomic study of urease-positive aerobic *corynebacteria* from lower urinary tract of healthy swine. Jpn. J. Vet. Res.35(3): 181-193.

Kwon D, Choi C, Jung T, Chung HK, Kim JP, Bae SS, Cho WS, Kim J, Chae C (2002). Genotypic prevalence of the fimbrial adhesins (F4, F5, F6, F41 and F18) and toxins (LT, STa, STb and STx2e) in *Escherichia coli* isolated from post-weaning pigs with diarrhoea or oedema disease in Korea.Vet. Rec.150(2): 35-37.

Ladd P, Dennis S, Njoku C (1974). Pathology of listeric infection in domestic animals.Vet. Bulletin.44(2): 67-74.

Laukkanen R (2010).Enteropathogenic *Yersinia* in pork production. Academic dessertation, Department of Food Hygiene and Environmental Health Faculty of Veterinary Medicine University of Helsinki Helsinki, Finland

Lawson PA, Falsen E, Åkervall E, Vandamme P, Collins MD (1997). Characterization of some *Actinomyces*-like isolates from human clinical specimens: reclassification of *Actinomyces suis* (Soltys and Spratling) as *Actinobaculum suis* comb. nov. and description of *Actinobaculum schaalii* sp. nov.Int. J. Syst Evol Microbiol.47(3):899-903.

Lecce JG, Clare DA, Balsbaugh RK, Collier DN (1983). Effect of dietary regimen on rotavirus-*Escherichia coli* weanling diarrhoea of piglets. J. Clin Microbiol.17(4): 689-695.

Liu W, Yuan C, Meng X, Du Y, Gao R, Tang J, Shi D (2014). Frequency of virulence factors in *Escherichia coli* isolated from suckling pigs with diarrhoea in China.Vet. J.199(2): 286-289.

Long JR, Dukes TW (1972). Listeriosis in newborn swine.Can. Vet. J.13(2): 49-52.

Lopez A, Bildfell R (1989). Neonatal porcine listeriosis.Can Vet J. 30(10): 828-829.

Lungu B, Ricke SC, Johnson MG (2010). Occurrence of Listeria monocytogenes in Raw and Ready-to-Eat Foods and Food-Processing Environments and Intervention Strategies for Control. Perspectives on Food-Safety Issues of Animal-Derived Foods.P.129.

Lyutskanov M (2011). Epidemiological characteristics of post-weaning diarrhoea associated with toxin-producing *Escherichia coli* in large intensive pig farms.Trakia J. Sci.9(3): 68-73.

Madec F, Bridoux N, Bounaix S Jestin A (1998). Measurement of digestive disorders in the piglet at weaning and related risk factors. Prev. Vet.Med.35(1): 53-72.

Madoroba E, Van Driessche E, De Greve H, Mast J, Ncube I, Read J, Beeckmans S (2009). Prevalence of enterotoxigenic *Escherichia coli* virulence genes from scouring piglets in Zimbabwe.Trop. Anim. Health Prod.41(7): 1539-1547.

Madzimure J, Chimonyo M, Zander KK Dzama K (2012). Potential for using indigenous pigs in subsistence-oriented and market-oriented small-scale farming systems of Southern Africa.Trop. Anim. Health Prod.45(1):135-142.

Mainil JG, Jacquemin E, Pohl P, Kaeckenbeeck A, Benz I (2002). DNA sequences coding for the F18 fimbriae and AIDA adhesin are localised on the same plasmid in *Escherichia coli*isolates from piglets. Vet. Microbiol.86(4): 303-311.

Martins MF, Martinez-Rossi NM, Ferreira A, Brocchi M, Yano T, Castro AFP, Silveira, WD (2000). Pathogenic characteristics of *Escherichia coli* strains isolated from newborn piglets with diarrhea in Brazil.Vet Microbiol.76(1): 51-59.

Maynard C, Fairbrother JM, Bekal S, Sanschagrin F, Levesque RC, Brousseau R, Masson L, Lariviere S, Harel (2003). Antimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149:K91 isolates obtained over a 23-year period from pigs.Antimicrob. Agents Chemother.47(10): 3214-3221.

Mazumder Y, Das A, Kar D, Shome B, Dutta B, Rahman H (2012). Isolation of *Bordetella bronchiseptica* from pigs in North East India. J. Anim. Sci. Adv.2(4): 396-406.

Mead, P.S. and Griffin, P.M. (1998). *Escherichia coli* O157: H7.Lancet.352(9135): 1207-1212.

Meseko C, Oluwayelu D (2012). Clinical Tetanus in Pigs in a Pig Farming Complex, Lagos, Nigeria.Nig Vet. J.33(4): 666-669

Messick JB (2004). *Hemotrophic mycoplasmas* (hemoplasmas): a review and new insights into pathogenic potential.Vet. Clin. Pathol.33(1): 2-13.

Mijs W, de Haas P, Rossau R, Van Der Laan T, Rigouts L, Portaels F, van Soolingen D (2002). Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and'*M. avium* subsp*. hominissuis*' for the human/porcine type of *M. avium*. Int J. Syst. Evol. Microbiol.52(5):1505-1518.

Mohlatlole RP, Madoroba E, Muchadeyi FC, Chimonyo M, Kanengoni AT, Dzomba EF (2013). Virulence profiles of enterotoxigenic, shiga toxin and enteroaggregative *Escherichia coli* in South African pigs.Trop. Anim Health Prod.45(6):1399-1405.

Moon HW, Kohler EM, Schneider RA, Whipp SC (1980). Prevalence of pilus antigens, enterotoxin types, and enteropathogenicity among K88-negative enterotoxigenic *Escherichia coli* from neonatal pigs. Infect. Immun.27(1): 222-230.

Moxley RA, Duhamel GE (1999). Comparative pathology of bacterial enteric diseases of swine: Mechanisms in the Pathogenesis of Enteric Diseases 2. Springer. 83-101.

Myers LL, Shoop DS (1987). Association of enterotoxigenic *Bacteroides fragilis* with diarrheal disease in young pigs.Am. J. Vet. Res.48(5):774-775.

Nagy B, Casey TA. Moon HW (1990). Phenotype and genotype of *Escherichia coli* isolated from pigs with post-weaning diarrhoea in Hungary.J. Clin. Microbiol.28(4): 651-653.

Nagy B, Fekete PZ (1999). Enterotoxigenic *Escherichia coli* (ETEC) in farm animals.Vet. Res.30(2-3): 259-284.

Nagy B, Fekete PZ (2005). Enterotoxigenic *Escherichia coli* in veterinary medicine.Int. J. Med. Microbiol.295(6):443-454.

Nakamine M, Kono Y, Abe S, Hoshino C, Shirai J, Ezaki T (1998). Dual infection with enterotoxigenic *Escherichia coli* and porcine reproductive and respiratory syndrome virus observed in weaning pigs that died suddenly.J. Vet. Med Sci.60(5):555-561.

Nakazawa M, Sugimoto C, Isayama Y, Kashiwazaki M (1987). Virulence factors in *Escherichia coli* isolated from piglets with neonatal and post-weaning diarrhea in Japan.Vet. Microbiol.13(4): 291-300.

Neyeloff JL, Fuchs SC, Moreira LB (2012). Meta-analyses and forest plots using a Microsoftexcel spreadsheet: step-by-step guide focusing on descriptive data analysis. BMC Res. Notes. 5 (52) 0500-5-52.

Ngeleka M, Pritchard J, Appleyard G, Middleton DM, Fairbrother JM (2003). Isolation and association of *Escherichia coli* AIDA-I/STb, rather than EAST1 pathotype, with diarrhea in piglets and antibiotic sensitivity of isolates.J. Vet. Diagn Invest.15(3): 242-252.

Nicoletti P (2010). Brucellosis: past, present and future. Contributions of Macedonian Academy of Sciences & Arts (1) 21-32

Nielsen EM, Engberg J, Madsen M (1997). Distribution of serotypes of Campylobacter jejuni and C. coli from Danish patients, poultry, cattle and swine.FEMS Immun Med Microbiol.19(1): 47-56.

Nietfeld JC, Franklin CL, Riley LK, Zeman DH. Groff BT (1995) .Colonization of the tracheal epithelium of pigs by filamentous bacteria resembling cilia-associated respiratory bacillus.J. Vet. Diagn Invest.7(3): 338-342.

Niewerth U, Frey A, Voss T, Le Bouguenec C, Baljer G, Franke S, Schmidt MA (2001). The AIDA autotransporter system is associated with F18 and stx2e in Escherichia coli isolates from pigs diagnosed with edema disease and postweaning diarrhea.Clin. Diagn. Lab. Immun.8(1): 143-149.

Noamani BN, Fairbrother JM, Gyles CL (2003). Virulence genes of O149 enterotoxigenic *Escherichia coli* from outbreaks of postweaning diarrhea in pigs.Vet. Microbiol.97(1): 87-101.

Oanh TKN, Nguyen VK, Do TN, Goddeeris BM, De Greve H (2010). *Escherichia coli* strains causing oedema disease in northern Vietnam share an identical verotoxin 2e.Trop. Anim. Health Prod.42(8): 797-1804.

Ojeniyi B, Ahrens P, Meyling A (1994). Detection of fimbrial and toxin genes in *Escherichia coli* and their prevalence in piglets with diarrhoea. The application of colony hybridization assay, polymerase chain reaction and phenotypic assays.J.Vet. Med.41(1-10): 49-59.

Okello E, Moonens K, Erume J, De Greve H (2015). Enterotoxigenic *Escherichia coli* strains are highly prevalent in Ugandan piggeries but disease outbreaks are masked by antibiotic prophylaxis.Trop. Anim. Health Prod.47(1): 117-122.

Oliveira M, Barroco C, Mottola C, Santos R, Lemsaddek A, Tavares L, Semedo-Lemsaddek T (2014). First report of *Corynebacterium pseudotuberculosis* from caseous lymphadenitis lesions in Black Alentejano pig (*Sus scrofa domesticus*).BMC Vet. Res.10: 218

Oliveira S, Blackall P, Pijoan C (2003). Characterization of the diversity of *Haemophilus parasuis* field isolates by use of serotyping and genotyping.Am. J. Vet. Res.64(4): 435-442.

Oliveira S, Pijoan C (2002). Diagnosis of *Haemophilus parasuis* in affected herds and use of epidemiological data to control disease. J. Swine Health Prod.10(5): 221-225.

Omar A, Kheong CK, Mahendranathen T (1962). Observations on porcine melioidosis in Malaya. Br.Vet. J.118: 421-429.

Opriessnig T, Bender JS, Halbur PG (2010). Development and validation of an immunohistochemical method for rapid diagnosis of swine erysipelas in formalin-fixed, paraffin-embedded tissue samples. J. Vet Diagn Invest. 22(1): 86-90.

Opriessnig T, Hoffman LJ, Harris DL, Gaul SB, Halbur PG (2004). *Erysipelothrix rhusiopathiae*: genetic characterization of midwest US isolates and live commercial vaccines using pulsed-field gel electrophoresis. J. Vet. Diagn. Invest.16(2): 101-107.

Osek J (1999). Prevalence of virulence factors of *Escherichia coli* strains isolated from diarrhoeic and healthy piglets after weaning. Vet. Microbiol.68(3): 209-217.

Osek J (2000). Virulence factors and genetic relatedness of *Escherichia coli* strains isolated from pigs with post-weaning diarrhea.Vet. Microbiol.71(3): 211-222.

Osek J (2003). Detection of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 (EAST1) gene and its relationship with fimbrial and enterotoxin markers in *E. coli* isolates from pigs with diarrhoea.Vet. Microbiol.91(1): 65-72.

Osek J, Truszczyński M (1992). Occurrence of fimbriae and enterotoxins in *Escherichia coli* strains isolated from piglets in Poland.Comp. Immun. Microbiol. Infect. Dis.15(4): 285-292.

Osek J, Gallien P, Truszczyñski M, Protz D (1999). The use of polymerase chain reaction for determination of virulence factors of *Escherichia coli* strains isolated from pigs in Poland.Comp. Immun. Microbiol. Infect. Dis.22(3): 163-174.

Park J, Seok S, Cho S, Baek M, Lee H, Kim D, Park J (2004) .The high prevalence of *Helicobacter* sp. in porcine pyloric mucosa and its histopathological and molecular characteristics. Vet. Microbiol.104(3): 219-225.

Parma A, Sanz M, Vinas M, Cicuta M, Blanco J, Boehringer S, Vena M, Roibon W, Benitez M, Blanco J (2000). Toxigenic *Escherichia coli* isolated from pigs in Argentina.Vet. Microbiol.72(3): 269-276.

Pedersen K, Sørensen G, Löfström C, Leekitcharoenphon P, Nielsen B, Wingstrand A, Aarestrup FM, Hendriksen RS, Baggesen DL (2015).Reappearance of *Salmonella serovar Choleraesuis var. Kunzendorf*in Danish pig herds.Vet. Microbiol.176(3): 282-291.

Peet R, Fry J, Lloyd J, Henderson J, Curran J, Moir D (1983). *Haemophilus parasuis* septicaemia in pigs.Aust. Vet. J.60(6): 187-187.

Penner JL (1988). The genus *Campylobacter:* a decade of progress.Clin. Microbiol. Rev.1(2): 157-172.

Pieper R, Janczyk P, Zeyner A, Smidt H, Guiard V Souffrant WB (2008). Ecophysiology of the developing total bacterial and *Lactobacillus* communities in the terminal small intestine of weaning piglets. Microb. Ecol.56(3): 474-483.

PluskeJR, Pethick DW, Hopwood DE, Hampson DJ (2002). Nutritional influences on some major enteric bacterial diseases of pig.Nutr.Res. Rev 15(02): 333-371.

Pringle M, Backhans A, Otman F, Sjölund M, Fellström C (2009). Isolation of spirochetes of genus *Treponema* from pigs with ear necrosis.Vet. Microbiol.139(3): 279-283.

Qi X, Huang N, Zhao B, Wang C. Zhao X (2012). Prevalence of serogroups and genotypes for fimbriae and enterotoxins in *Escherichia coli* isolated from diarrheic piglets in western China. J. Swine Health Prod20(6): 290-293.

Radostits O, Gay C, Blood D, Hinchcliff K (2000). Actinobacillosis in pigs. Veterinary Medicine. A Text book of the disease of cattle, horses, sheep, pigs and goats. Bailler, Tindal, London. pp. 942.

Ramos-Vara JA, Wu CC, Mitsui I, Lin TL, Miller MA (2008). Metritis, valvular endocarditis, and septicemia by *Actinobacillus equuli*in a gilt in the United States.Vet. Pathol. 45(4): 495-499.

Rampling A (1964). Porcine melioidosis in the territory of Papua and New Guinea. Aust.Vet. J. 40(6):241-241.

Riley MG, Russell EG, Callinan RB (1977). *Haemophilus parasuis* infection in swine.J. Am.Vet. Med. Assoc.171(7): 649-651.

Ross RF, Zimmerman BJ, Wagner WC, Cox DF (1975). A field study of coliform mastitis in sows. J. Am. Vet. Med. Assoc.167(3): 231-235.

Ryley J, Simmons G (1954). Leptospirosis of pigs. Aust. Vet. J.30(7): 203-208.

Rzewuska M, Witkowski L, Cisek AA, Stefańska I, Chrobak D, Stefaniuk E, Kizerwetter-Świda M ,Takai S (2014). Characterization of *Rhodococcus equi* isolates from submaxillary lymph nodes of wild boars (*Sus scrofa*), red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*). Vet. Microbiol.172(1): 272-278.

Salmon D, Smith T (1886). The bacterium of swine plague.Am.Month.Microbiol. J.7: 204.

Schrauwen E, Devriese L, Hoorens J, Takahashi T (1993). *Erysipelothrix-tonsillarum* endocarditis in a dog-a case-report.Vlaams Diergen.Tijds62(5): 160-161.

Skjerve E, Lium B, Nielsen B, Nesbakken T (1998). Control of *Yersinia enterocolitica* in pigs at herd level. Int. J. Food Microbiol.45(3): 195-203.

Smith HW, Jones J (1963). Observations on the alimentary tract and its bacterial flora in healthy and diseased pigs.J. Pathol. Bacteriol.86(2): 387-412.

Smith S, McOrist S (1997). Development of persistent intestinal infection and excretion of *Lawsonia intracellularis* by piglets.Res. Vet. Sci.62(1): 6-10.

Soderlind O, Thafvelin B, Mollby R (1988). Virulence factors in *Escherichia coli* strains isolated from Swedish piglets with diarrhea. J. Clin. Microbiol.26(5): 879-884.

Songer JG, Meer RR (1996). Genotyping of*Clostridium perfringens*by Polymerase Chain Reaction is a Useful Adjunct to Diagnosis of Clostridial Enteric Disease in Animals.Anaerobe.2(4): 197-203.

Songer JG, Post KW, Larson DJ, Jost BH, Glock RD (2000). Diagnostic Notes: Infection of neonatal swine with *Clostridium difficile*.Swine Health Prod.8: 185-190.

SongerJG, Uzal FA (2005). Clostridial enteric infections in pigs.J. Vet. Diagn Invest.17(6): 528-536.

Sørum H, Sunde M (2001). Resistance to antibiotics in the normal flora of animals.Vet. Res.32(3-4): 227-241.

Staats J, Feder I, Okwumabua O, Chengappa M (1997). *Streptococcus suis*: past and present.Vet. Res. Commun.21(6): 381-407.

Statista (2016). Number of pigs world wide from 1990 to 2013.*http://www.statista.com/statistics/263963/number-of-pigs-worldwide-since-1990/*.Accessed: 9th February, 2016.

Stevens MP, Gray JT (2013). 13 *Salmonella* Infections in Pigs: *Salmonella* in Domestic Animals.P.263.

Stewart CS (1997). Microorganisms in hindgut fermentors: Gastrointestinal microbiology. Chapman and Hall, New York.2: 142-186.

Szeredi L, Schiller I, Sydler T, Guscetti F, Heinen E, Corboz L, Eggenberger E, Jones GE, Pospischil A (1996). Intestinal *Chlamydia* in finishing pigs.Vet. Pathol.33(4): 369-374.

Takeshi K, Makino S, Ikeda T, Takada N, Nakashiro A, Nakanishi K, Oguma K, Katoh Y, Sunagawa H, Ohyama T (1999). Direct and rapid detection by PCR of *Erysipelothrix* sp. DNAs prepared from bacterial strains and animal tissues. J. Clin. Microbiol.37(12): 4093-4098.

Tancrede C (1992). Role of human microflora in health and disease. Eur. J. Clin. Microbiol. Infect. Dis.11(11): 1012-1015.

Tang X, Zhao Z, Hu J, Wu B, Cai X, He Q,Chen H (2009). Isolation, antimicrobial resistance, and virulence genes of *Pasteurella multocida* strains from swine in China. J. Clin Microbiol. 47(4): 951-958.

Taylor, D.J. (2013) Pig diseases .9th ed, North Birbiston Road, Lennoxtown

Thacker EL (2006). Mycoplasmal diseases. Diseases of swine 9: 701-717.

Thiagarajah JR,Verkman AS (2003). CFTR pharmacology and its role in intestinal fluid secretion. Curr. Opin. Pharmacol. 3: 594–599.

Thorel MF, Huchzermeyer HF, Michel AL (2001). *Mycobacterium avium* and *Mycobacterium intracellulare* infection in mammals.Rev. Sci. Tech.20(1): 204-218.

Toledo A, Gomez D, Cruz C, Carreon R, Lopez J, Giono S.Castro AM (2012). Prevalence of virulence genes in *Escherichia coli* strains isolated from piglets in the suckling and weaning period in Mexico. J. Med. Microbiol.61(1): 148-156.

Townsend KM, O'Boyle D, Phan TT, Hanh TX, Wijewardana TG, Wilkie I, Trung NT, Frost AJ (1998). Acute septicaemic pasteurellosis in Vietnamese pigs.Vet. Microbiol.63(2): 205-215.

Tzipori S, Hayes J, Sims L,Withers M (1984). *Streptococcus durans*: an unexpected enteropathogen of foals.J. Infect. Dis.150(4): 589-593.

Van den Broeck W, Cox E, Goddeeris BM. (1999). Seroprevalence of F4 enterotoxigenic *Escherichia coli* in regions with different pig farm densities.Vet. Microbiol.69(3): 207-216.

Vannier P (1999). Infectious causes of abortion in swine.Reprod. Domest. Anim.34(3-4): 367-376.

Verbrugghe E, Van Parys A, Leyman B, Boyen F, Arnouts S, Lundberg U, Ducatelle R, Van den Broeck W, Yekta MA, Cox E (2015). Heat-labile enterotoxin of *Escherichia coli* promotes intestinal colonization of *Salmonella enterica*.Comp. Immun. Microbiol. Infect. Dis.43: 1-7.

Vidotto MC, Lima N, Fritzen JT, Freitas JCD, Venâncio EJ, Ono MA (2009). Frequency of virulence genes in *Escherichia coli* strains isolated from piglets with diarrhoea in the North Parana State, Brazil. Braz. J.Microbiol.40(1): 199-204.

Vu-Khac H, Holoda E, Majerčiak M, Gašpar G, Pilipčinec E (2004). Genotyping of fimbrial adhesins in*Escherichia coli* strains isolated from Slovak piglets suffering from diarrhoea. Folia microbiol.49(1): 59-63.

Vu-Khac H, Holoda E, Pilipcinec E, Blanco M, Blanco J, Dahbi G, Mora A, López C, González E, Blanco J (2007). Serotypes, virulence genes, intimin types and PFGE profiles of *Escherichia coli* isolated from piglets with diarrhoea in Slovakia.Vet.J.174(1): 176-187.

Wathes C, Miller B, Bourne F (1989). Cold stress and post-weaning diarrhoea in piglets inoculated orally or by aerosol.Anim Prod.49(03): 483-496.

Wegener HC, Andresen LO, Bille-Hansen V (1993). *Staphylococcus hyicus* virulence in relation to exudative epidermitis in pigs.Can. J. Vet. Res57(2): 119-125.

Whyte P, Dobson K, Ratcliff R, Cargill C (1982). Protection of pregnant swine by vaccination against Leptospira infection. Aust. Vet. J.59(2): 41-45.

Wilcock BP (1979). Experimental *Klebsiella* and *Salmonella* infection in neonatal swine.Can. J .Comp. Med.43(2): 200-206.

Wills RW (2000). Diarrhea in growing-finishing swine.Vet. Clin. North Am Food Anim. Pract.16(1): 135-161.

Witkowski L, Rzewuska M, Takai S, Kizerwetter-Świda M, Kita J (2016). Molecular epidemiology of *Rhodococcus equi* in slaughtered swine, cattle and horses in Poland. BMC Microbiol16(1): 10.1186

Woldemeskel M, Drommer W, Wendt M (2002). Microscopic and ultrastructural lesions of the ureter and renal pelvis in sows with regard to *Actinobaculum suis* infection. J. Vet. Med. Series A. 49(7): 348-352.

Wood. RL, Steele JH (1994). *Erysipelothrix* infections: Handbook of Zoonoses.pp. 83-91.

Xavier NM, Paixao AT, den Hartigh BA, TsolisMR, SantosLR (2010). Pathogenesis of *Brucella spp*. Open Vet. Sci. J.4(1): 109-118

Zajacova ZS, Konstantinova L, Alexa P (2012). Detection of virulence factors of *Escherichia coli* focused on prevalence of EAST1 toxin in stool of diarrhoeic and non-diarrhoeic piglets and presence of adhesion involving virulence factors in astA positive strains.Vet.Microbiol.154(3): 369-375.

Zhang W, Zhao M, Ruesch L, Omot A, Francis D (2007). Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US.Vet. Microbiol.123(1): 145-152.

Zhao L, Chen X, Xu X, Song G, Liu X (2009). Analysis of the AIDA-I gene sequence and prevalence in *Escherichia coli* isolates from pigs with post-weaning diarrhoea and oedema disease.Vet. J.180(1): 124-129.

Zhu C, Harel J, Jacques M, Desautels C, Donnenberg MS, Beaudry M, Fairbrother JM (1994). Virulence properties and attaching-effacing activity of *Escherichia coli* O45 from swine postweaning diarrhea. Infect.Immun62(10): 4153-4159.

Zhu W, Lomsadze A, Borodovsky M (2010). Ab initio gene identification in metagenomic sequences. Nucleic acids Res.38(12): e132.

Chapter Three

Antimicrobials usage in pig production: effects on *Escherichia coli* resistance and virulence profiles.
R.H. Abubakar, E. Madoroba, F.O. Fasina

Preface

An overview of gut bacteria pathogens in pigs was established in the previous chapter. In this chapter the first and second objectives of this study which were to characterize the virulence profile of *Escherichia coli* (indicator organism for this study) and determine distribution of antibiotic resistance pattern of *Escherichia coli* in a commercial farm setting in growing pigs was achieved. The text has been submitted to the Journal of infection in developing countries and currently under review.

3.1 Abstract

Antibiotics are used as growth promoters and for therapeutic purposes in pig production. Misuse, abuse and overuse of antibiotics lead to development of resistant bacterial strains. This study investigated the frequency in which growing pigs harbour ETEC and VTEC virulence genes and compared phenotypic and genotypic antibiotic resistance patterns of fecal *E. coli* in samples collected from (i) pigs that received routine farm treatment without antibiotics usage, and (ii) pigs treated with antibiotics on the farm, with both groups monitored over a 70-day period. A total of 241 *E. coli* were isolated from both groups and used for antibiotics resistance testing through disk diffusion and extracted genomic DNA was amplified by PCR. Virulence genes were detected in 24.8% of the antibiotic group isolates and 43.5% of the non-antibiotics group with a significant difference (P=0.002). Proportion of individual virulence genes; STa, STb, EAST1 and Stx2e were 18.1%, 0%, 78.7% and 3%; and 14.8%, 8.5%, 85.1% and 12.7% in the antibiotics and non-antibiotics groups respectively. Resistance to oxytetracycline was most common and were significant (P = 0.03) in samples of days 10 (P = 0.02) and 21 (P = *0.01*). Significant resistance to amoxicillin on days 56 (P = 0.04) and 70 (P = 0.01) and trimethoprim on days 5, 10, 21, 56 and 70 ($P<0.05$) were observed. Seventeen phenotypic antibiotics resistance combinations were observed and eight were multidrug resistant. Furthermore, 63.9% possessed at least one of the four tested tetracycline resistance genes. *TetA* (23.3%) was the most common in the antibiotics group whereas tetB (43.5%) was common in the non-antibiotics group. The study showed that usage/non usage of antibiotics in growing pigs does not prevent occurrence of disease causing virulence genes and other factors may be involved. Oxytetracycline, Amoxicillin and Trimethoprim have the highest level of resistance. Tetracycline

resistance genes in pigs can be found at any point during the growth period with or without antibiotic usage.

Key words

Virulence gene; Antibiotic resistance; growing pigs; *Escherichia coli*.

3.2 Introduction

Escherichia coli is a major cause of diarrhea in pigs (piglets and weaners) at different levels of intensity worldwide (Henton and Engelbrecht, 1997; Nagy and Fekete, 1999; Khac *et al.,* 2006). In piglets, *E. coli* diarrhea may be followed by terminal septicemia, which is an important cause of economic loss for pig producers globally (Toledo *et al.,* 2012). The estimated pig population in South Africa as at 2010 was about 1.5 million (Meissner *et al.*, 2013), while population worldwide is about 1 billion (Statista, 2016). Pork serves as an important source of protein for human beings in developing countries (Madzimure *et al.,* 2012).

Diarrheagenic *E. coli* pathovars involved in pig enteric infections include mainly enterotoxigenic *E. coli* (ETEC) encoding heat stable (STa, STb, EAST1) and/or heat labile (LT) enterotoxins, causing secretory diarrhea in newborn and weaned piglets (Nagy *et al.,* 1997; Gyles, 2010). In addition Shiga toxin *E. coli* (STEC) strains encode the Shiga toxin type 2e (Stx2e) which causes edema disease but not diarrhea (MacLeod *et al.*, 1991). Interestingly, some strains harbor both the Stx2e genes and enterotoxin genes capable of causing symptoms of both edema disease and diarrhea in the same animal (STEC/ ETEC) (Barth *et al.,* 2007; Barth *et al.*, 2011). Many porcine ETEC and STEC strains have fimbrial structures on their surface, that like LT, STa, and STb enterotoxins, are usually plasmid mediated (Dubreuil et al., 2016). These fimbriae are termed colonization antigens and they enable the bacteria to colonize the epithelial surface of the pig small intestine namely F4 (K88), F5 (K99), F6 (P987), F18 and F41 usually found in pig ETEC (Chen *et al.,* 2004; Frydendahl, 2002; Nagy *et al.*, 1999; Dean *et al.*, 1989; Garabal *et al.,* 1997; Blanco *et al.,* 2006; Madoroba *et al.,* 2009). Antibiotics are frequently used in the treatment and control of these enteric infections in pigs.

Some studies have shown that administration of antibiotics increases the risk of antibiotic resistance (Taylor *et al.,* 2009; Varga *et al.,* 2009; Lutz *et al.,* 2011; Burow *et al.,* 2014). Other factors like stress from temperature, crowding, and management also seem to contribute to the

occurrence of antibiotic resistance in animals (Sørum and Sunde, 2001). The commensal bacteria in animals may become a reservoir of resistance genes for pathogenic bacteria. This may contaminate meat and meat products meant for human consumption (van den Bogaard *et al.*, 2000). Recent reports have indicated that prevalence and isolation of antimicrobial-resistant *E. coli* are on the increase (Toledo *et al.,* 2012; Enne *et al.,* 2008; Luppi *et al.,* 2015) and the infections caused by the resistant bacteria usually fail to respond to treatment by specific antibiotics (Rice, 2009). This may be associated with, increased proliferation of bacterial pathogens, re-infection rates, chronicity, opportunistic infections with resistant organisms and reduced life span (Capita and Alonso-Calleja, 2013).

Tetracycline resistance phenotypically have been reported more frequently among bacteria isolated from pigs than previously known (Scott *et al.,* 2005; Funk *et al.,* 2006; Alali *et al.,* 2008; Tadesse, 2012). The resistance is known to be inducible and occurs basically due to acquisition of *tet* or *otr* genes (Roberts, 2011) and many isolates from pigs have shown multidrug resistance genes located on plasmids (Lutz *et al.,* 2011).

E. coli infections have been identified to be a challenge in South African pig production industry (Fasina *et al.,* 2015; Kanengoni *et al.,* 2017). A recent study showed that the prevalence of ETEC, VTEC and EAST1 and associated fimbrial genes in indigenous South African breeds to be high (Mohlatlole *et al.,* 2013), an outbreak of multidrug resistance coliceptisaemia in weanling pigs was reported (Fasina *et al.*, 2015) and an investigation on piglet mortality in a farm was characterized to be associated with shigatoxin *E.coli* (Kanengoni *et al.,* 2017). Treatment and control of disease outbreaks in South African pig industry involves the use of antibiotics (Henton *et al.,* 2011). The purpose of this investigation was to determine the effect of antibiotic treatment on the prevalence of virulence genes and Antibiotic resistance in intestinal *E. coli* in growing pigs.

3.3 Materials and Methods

3.3.1 Approvals, animal care and welfare

Prior to the commencement of study, a completed study protocol was submitted to the National Department of Agriculture, Forestry and Fisheries, South Africa for approval to carry out responsible infectious disease research with approval reference number: 12/11/1/1/8 of the Section 20 of the Animal Disease Act 35 of 1984, South Africa. This approval ensures the strict

regulation and control of infectious pathogens, and minimizes the risk of contamination of the environment and other pig farms. In addition other necessary permits associated with the control of infectious materials were strictly adhered to including the "permission to move animal products from the farm" and approval of the farm management. Secondly, protocol on the adherence to animal welfare was submitted to the Animal Ethics Committee of the University of Pretoria and an approval number V029-16 was granted (Appendix 1, page 130).

All pigs involved in the study were placed under a 24-hour monitoring program conducted by the pig farm team (attendant and manager) for the duration of the study using the assessment and control of the severity of scientific procedures on laboratory animals scoring system and the guide to defining and implementing protocols for the welfare assessment of laboratory animals (Wallace *et al.,* 1990; Hawkins *et al.*, 2011). All piglets were housed in the farrowing unit with crates, creep area, heating lamps and unlimited access to the dam's teats, creep feed and water *ad-libitum* (Figure 1). A total of 4 out of 10 piglets were removed in the last two weeks of the study due to laboratory-confirmed colisepticaemia (edema disease). For each animal to be removed by euthanasia or sudden death, the humane endpoint was set with a Severity Index (SI) score of > 20 on the Laboratory Animal Science Association (LASA) Working Party Scale (Wallace *et al.,* 1990) and or a Score of ≥ 6 on the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group Scale (Hawkins P, Morton DB, Burman O, Dennison N, Honess P, Jennings M, Lane S, Middleton V, Roughan JV, Wells S, Westwood K, 2011). No other unexpected occurrence was recorded in the course of the experiment. Situation that triggered the scores for removal include pain incompatible with animal welfare like prostration, nervous manifestation that affects normal movement and loss of ability to ingest food for 24-48 hours.

3.3.2 Study design

A small scale commercial pig farm was identified in the Gauteng province of South Africa, and two pregnant sows were monitored clinically and physiologically until the day of farrowing. Piglets (n = 10) were randomly selected (5 from each sow together with their unselected litter mates) and placed into 2 groups. All 10 selected piglets were tagged into groups A (Non-antibiotics group: with 5 tagged piglets and other non-tagged litter mates which were kept in one farrowing pen under routine farm management practices but without any form of antibiotics usage and B (Antibiotics group: with 5 piglets and other non-tagged litter mates which were kept

under the routine management practices of the farm, which included administration of multi vitamins, deworming, tail docking, vaccination, provision of warmth and antibiotic administration to the sick animals). Effort was made to ensure prevention of cross contamination from the environment and between the groups by leaving vacant at least three farrowing pens (5.4 m width) between the 2 groups and piglets were randomly selected from the litters for uniformity.

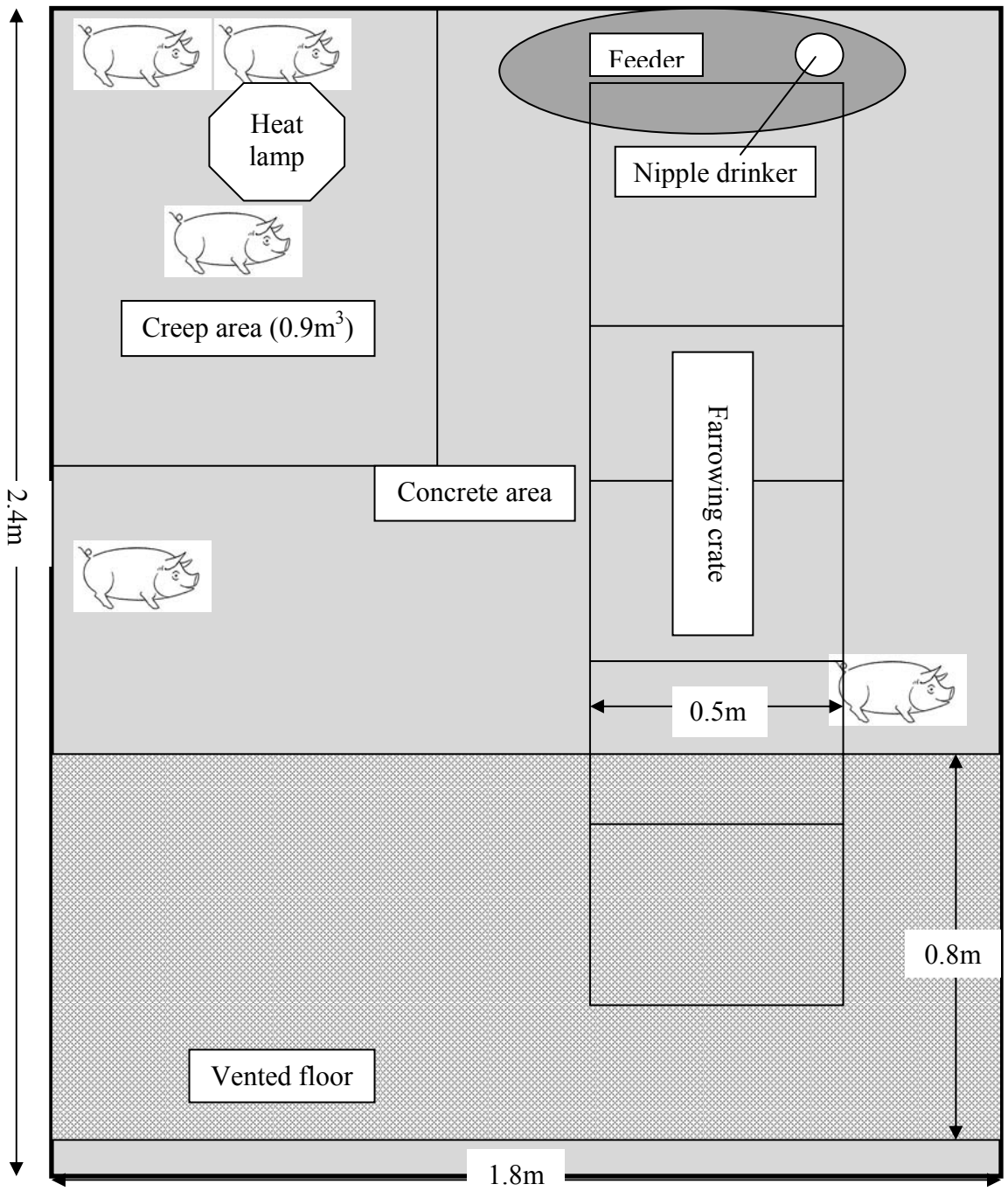


Figure 0.1: Standard farrowing pen with creep area, farrowing crate, concrete and vented floor.

The sow (dam) is restricted to the farrowing crate but the piglets move freely within the pen. The creep area is warm with temperature range from 32°C (week 1) to 27°C (week 4). All pigs within a pen have access to feed and water liberally; however, the piglets depended primarily on sow's milk for the first 10-14 days. Only five piglets per litter were tagged for the experiment. After 4 weeks, the piglets are weaned into the weaners pen.

3.3.3 Sample collection

Rectal swabs were taken from all 10 piglets (4 swabs per animal at each collection) with a sterile swab stick and each swab labeled with the specific pig identification number and age (days), and transported to the Agricultural Research Council-Onderstepoort Veterinary Research Feed and Food Analysis laboratory (Bacteriology section) on ice. All samples were processed in the laboratory within 2 hours of sample collection. The samples were collected periodically on days 1, 5, 10, 21, 28, 35, 56 and 70 from all pigs.

3.3.4 Classical Microbiological analysis

3.3.4.1 *E. coli* Isolation

The swabs were streaked directly on MacConkey agar plates and incubated aerobically overnight at 37°C. Lactose fermenting colonies (n = 4-6) were selected and sub-cultured on MacConkey agar. The pure colonies were then transferred to nutrient agar plates. The isolates on nutrient agar plates were subjected to indole test together with with other biochemical reactions for *E. coli* identification. For this purpose, 10ml of tryptone water was inoculated with pure culture and incubated over night at 37°C. Kovacs reagent (1 to 2 drops) was added and formation of a red ring was indicative of *E. coli*. In addition, Methyl red, Voges-Proskauer and citrate (IMViC) tests were also conducted. Subcultures were also cultured on 5% sheep blood agar to check the hemolytic characteristics of the *E. coli. E. coli* ATCC 25922 and *E.coli* O157 were used as controls.

3.3.4.2 Antibiotics resistance testing.

Antimicrobial susceptibility testing was done using the Kirby-Bauer disk diffusion method. The following antibiotics discs were selected according to standard regulations (Clinical Laboratory Standards Institute, 2015; World Health Organization, 2012; Food and Drug Administration, 2012): Amoxicillin (AML) 10µg; Cefotaxime (CTX) 30µg; Oxytetracycline (OT) 30µg; Kanamycin (K) 30µg; Florfenicol (FFC) 30µg; Enrofloxacin (ENR) 5µg and Trimethoprim (W) 5µg (Table 3.1). For this purpose, 1-3 colonies from a nutrient agar plate were transferred into a tube containing 5 ml Ringers solution, vortexed and adjusted to 0.5 McFarland turbidity standard. A sterile cotton swab was dipped into the adjusted suspension and excess fluid on the swab was removed by pressing on the inside of the bottle neck. Mueller Hinton agar plate surface was then swabbed making sure that the entire surface was covered in order to obtain confluent growth. The Mueller Hinton agar plates were left for 3 to 5 minutes, to allow for any excess moisture on the

surface to dry off. The antibiotics disk sticks (Oxoid), were placed into the disk dispenser (Oxoid) and gently dispensed on the inoculated Mueller Hinton agar plate. The plates were incubated at 37^{o}C for 24 hours. The zones were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines (Clinical Laboratory Standards Institute, 2015).

3.3.5 Molecular characterization of *E. coli* isolates

3.3.5.1 DNA Extraction.

The DNA from *E. coli* isolates was obtained using the cell-lysis method. Briefly, 1ml of sterile distilled water was dispensed in labeled eppendorf tubes. Loopfuls of bacterial cultures from nutrient agar plates were suspended in sterilized distilled water and vortexed, then washed twice by centrifugation at 13,000 rpm for 5 minutes (Eppendorf mini spin; Eppendorf, Germany). The washed *E. coli* cells were boiled at 95^{o}C for 20 minutes to lyse the bacteria. The lysate was cooled at 4^{o}C and centrifuged at 13,000rmp for 5 minutes. The supernatant that contained the crude genomic DNA was used as templates for PCR.

3.3.5.2 DNA amplification using polymerase chain reaction (PCR).

E. coli isolates were tested for virulence genes and tetracycline resistance genes, *tet* (A, B, C and E) using sets of forward and reverse primers (Table 2). For detection of enterotoxins, STa, STb and LT a multiplex PCR assay, Cheng 2006 (Cheng *et al.,* 2006) was adapted using a total 25µl of reaction volume including the PCR master mix (DreamTaq^{TM} Green PCR Master Mix), 0.3µl of each primer, nuclease free PCR water (Fermentas) and 3µl DNA. DNA amplification was carried out using Eppendorf thermocycler (Eppendorf, Germany) and the cycling conditions were initial denaturation at 94^{o}C for 3 min, followed by 10 cycles of denaturation at 94^{o}C for 30 sec, annealing at 60-56^{o}C (1^{o}C decrease for every 2 cycles) for 30 sec and extension at 72^{o}C for 1 minute, followed by another 22 cycles of similar thermocycling conditions but annealing at 56^{o}C, and a final extension at 72^{o}C for 10 minutes.

Enteroaggregative heat stable enterotoxin (EAST-1) was detected using a monoplex PCR assay as described by Ngeleka, 2003 with slight modifications. The reaction mixture consisted of 0.3µl of each primer, 12.5µl of 2× PCR master mix (DreamTaq^{TM} Green PCR Master Mix, Fermentas), 3µl DNA nuclease free PCR water (Fermentas) to make a 25µl reaction. Thermocycling conditions were initial denaturation at 94^{o}C for 3minutes, followed by 35 cycles of 94^{o}C for 30 seconds, annealing at 60^{o}C for 30 seconds and elongation at 72^{o}C for 30 seconds,

then a final elongation step at 72°C for 5 minutes. A multiplex assay for stx2e (including stx1, stx2) was carried out using similar reaction mixtures and thermocyling conditions for the protocol above except for the annealing temperature which was 58°C. The adhesins AIDA 1, *eae* and *paa* were amplified as a multiplex PCR using similar protocols as that of EAST 1. The fimbriae F4, F5, F6 and F41 (Fimbriae set 1) and F18ab and F18ac (Fimbriae set 2) (Cheng *et al.,* 2006; Cheng *et al.,* 2005) were amplified using multiplex PCR. The reaction mixtures were similar to those of the enterotoxins, however the thermocyling conditions were initial denaturation at 94°C for 3 minutes, followed by first 10 cycles of denaturation at 94°C for 30 seconds annealing at 66-62°C (1°C decrease for every 2 cylcles) for 30 seconds, and elongation at 72°C for 60 seconds, then 22 cycles of similar conditions but except for annealing at 62°C for 30 seconds and a final elongation at 72°C for 10 minutes.

Tetracycline resistance genes *tet* (A), B, C and E were detected using a multiplex PCR assay as described by Agga et al., 2014 (Agga *et al.,* 2014) with slight modifications. The 25µl reaction mixture consisted of 12.5µl of Dreamtaq mastermix, 0.3µl of each primer, 3µl crude DNA and 7.1µl of nuclease free PCR grade water (Table 2). The thermal cycling conditions consisted of an initial denaturation at 95°C for 10 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1.5 minutes, and elongation at 72°C for 1.5 minutes, followed by a final elongation step at 72°C for 10 minutes. Positive controls were obtained from ARC-OVR Bacteriology and Feed and Food laboratories culture collections. They include: B41 (F5, F41 and STa), RCM39a (Stx1, Stx2 and eae), WL 187/16(Stx2e), TPNB 137/16 (STa, STb and LT), and *E.coli* ATCC25922 (negative control).

3.3.5.3 Agarose gel electrophoresis, gel documentation and interpretation of the results

Two percent agarose (Thermo scientific) gel was prepared using 1x TAE buffer (Tris, acetic acid and EDTA mixture) (Bioland Scientific) and stained in ethidium bromide. Approximately 8µl of PCR amplicons were pipetted into the gel wells and electophorosed at 3V/cm for about 1 hour, this was followed by visualization under ultraviolet light using a gel documentation system (Bio-Rad, Japan). The expected band sizes for each gene were estimated using a 100-bp ladder (BioLabs)

3.3.5.4 Statistical analysis

All output data including the management and field parameters were entered into Microsoft Excel® (Microsoft Inc., Redmond, WA). Data were filtered, harmonized and aligned with bacteriological results based on days of sample collections (positive and negative results) from antibiotics and non- antibiotics groups. Descriptive and analytical statistics were conducted using Minitab® 16. (Minitab Inc., State College, PA). Specifically, two by two tables were generated for results and classical test of hypothesis were conducted using Chi square (χ^2) for all categorical variables. *P*-value was set at an alpha of 0.05 as the cut-off for significance and 95% confidence interval. Proportions were calculated with 95% confidence interval in Openepi® version 3.01online calculator (Dean, Sullivan and Soe, 2015).

3.4 Results

Based on the evidence gathered from the farm, routine antibiotic administrations include the intra-uterine suppository of Oxytetracycline within 6 hours post-partum in sows, and the parenteral or intramuscular injection of antibiotics (amoxicillin and penicillin-streptomycin combinations to diarrhoeic piglet during the growth phase). One ml of iron dextran and two ml of multivitamins were also injected on day 3 of birth to all piglets. The piglets were allowed unlimited access to milk from the dam's teats for the first 14 days after which creep starter feed is introduced to reduce suckling stress on the sows. Grower feed is introduced from about the fifth week of life of the weaned piglets and these feeds are changed to pig fatteners feed after the seventh weeks.

Table 0.1: Disk contents of antibiotics and resistance break points used for disk diffusion testing of the E. coli isolates.

Antimicrobial class (FDA, 2012)	Antibacterial agent	Abbreviations	Disk content (μg)	Resistance break point (mm)	WHO Classification (WHO, 2012)
Penicillins	Amoxicillin	AML	10	≤ 13	Critically important
Cephems	Cefotaxime	CTX	30	≤ 22	Critically important
Tetracyclines	Oxytetracycline	OT	30	≤ 11	Highly important
Aminoglycosides	Kanamycin	K	30	≤ 13	Critically important
Phenicols	Florfenicol	FFC	30	≤ 14	Highly important
Flouroquinolones	Enrofloxacin	ENR	5	≤ 16	Critically important
Folate pathway inhibitors	Trimethoprim	W	5	≤ 10	Highly important

Breakpoints were based on Clinical Laboratory standards guideline (CLSI, 2015) and Performance Standards for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated from Animals.

Table 0.2: Primer sequences and amplicon sizes used for PCR detection of virulence genes, virulence factors and tetracycline resistance genes

Primer sequences for virulence genes and factors

Target gene	Primer	Primer sequence (5'- 3')	Amplicon size (bp)	References
Toxins				
EAST-1 (*astA*)	EAST-1-F	TCG GAT GCC ATC AAC ACA GT	125	Ngeleka et al, 2003
	EAST-1-R	GTC GCG AGT GAC GGC TTT GTA G		
STa (*estI*)	STa-F	GGG TTG GCA ATT TTT ATT TCT GTA	183	Cheng et al, 2006
	STa-R	ATT ACA ACA AAG TTC ACA GCA GTA		
STb (*estII*)	STb-F	ATG TAA ATA CCT ACA ACG GGT GAT	360	Cheng et al, 2006
	STb-R	TAT TTG GGC GCC AAA GCA TGC TCC		
LT (*elt*)	LT-F	TAG AGA CCG GTATTA CAG AAATCT GA	282	Cheng et al, 2006
	LT-R	TCA TCC CGA ATT CTG TTA TAT ATGTC		
Stx1(*stxI*)	Stx1-F	ATT CGC TGA ATG TCATTC GCT	664	Cheng et al, 2006
	Stx1-R	ACG CTT CCC AGA ATT GCA TTA		
Stx2 (*stxII*)	Stx2-F	GAA TGA AGA AGA TGT TTA TAG CGG	281	Cheng et al, 2006
	Stx2-R	GGT TAT GCC TCA GTC ATT ATT AA		
Stx2e (*stx2e*)	Stx2e-F	GAA TGA AGA AGA TGT TTA TAG CGG	454	Cheng et al, 2006
	Stx2e-R	TTT TAT GGA ACG TAG GTA TTA CC		
Fimbriae				
F4 (K88) (*faeG*)	F4 (K88)-F	GAT GAA AAA GAC TCT GAT TGC A	841	Cheng et al, 2006
	F4 (K88)-R	GAT TGC TAC GTT CAG CGG AGC G		
F5 (K99) (*fanA*)	F5 (K99)-F	CTG AAA AAA ACA CTG CTA GCT ATT	543	Cheng et al, 2006
	F5 (K99)-R	CAT ATA AGT GAC TAA GAA GGA TGC		
F6 (987P) (*fasA*)	F6 (987P)-F	GTT ACT GCC AGT CTA TGC CAA GTG	463	Cheng et al, 2006
	F6 (987P)-R	TCG GTG TAC CTG CTG AAC GAA TAG		
F41 (*fim41a*)	F41-F	GAT GAA AAA GAC TCT GAT TGC A	682	Cheng et al, 2006
	F41-R	TCT GAG GTC ATC CCA ATT GTG G		
F18 (*fedA*)	F18-F1 (b)	ATG AAA AGA CTA GTG TTT ATT TCT T	513 or 516	Cheng et al, 2005
	F18-F2 (c)	CGT GAA CGG TAA AAC ACA GGG	170	
	F18-R	TTA CTT GTA AGT AAC CGC GTA AGC C		
Adhesins				
AIDA-1 (*aidA*)	AIDA-1-F	ACA GTA TCA TAT GGA GCC A	585	Ngeleka et al, 2003
	AIDA-1-R	TGT GCG CCA GAA CTA TTA		
EAE (*eae*)	EAE-F	CAT TAT GGA ACG GCA GAG GT	790	Ngeleka et al, 2003
	EAE-R	ATC TTC TGC GTA CTG CGT TCA		
PAA (*paa*)	PAA-F	ATG AGG AAC ATA ATG GCA GG	360	Ngeleka et al, 2003

	PAA-R	TCT GGT CAG GTC GTC AAT AC		
Primer sequences for tetracycline resistance genes				
Target gene	Primer	Primer sequence (5'- 3')	Amplicon size (bp)	References
tetA (*tetA*)	tetA-F	GCT ACA TCC TGC TTG CCT TC	210	Agga et al, 2014
	tetA-R	CAT AGA TCG CCG TGA AGA GG		
tetB (*tetB*)	tetB-F	TTG GTT AGG GGC AAG TTT TG	659	Agga et al, 2014
	tetB-R	GTA ATG GGC CAA TAA CAC CG		
tetC (*tetC*)	tetC-F	CTT GAG AGC CTT CAA CCC AG	418	Agga et al, 2014
	tetC-R	ATG GTC GTC ATC TAC CTG CC		
tetE (*tetE*)	tetE-F	AAA CCA CAT CCT CCA TAC GC	278	Agga et al, 2014
	tetE-R	AAA TAG GCC ACA ACC GTC AG		

Primer sequences were also partially adapted from Mohlatlole et al., 2013; Agga et al., 2014; Fasina et al., 2015; Kanengoni et al., 2017

3.4.1 Virulence genes and adhesion factors

A total of 241 *E. coli* isolates were obtained from both groups, of which 55.2% (n = 133) were from the antibiotics group and 44.8% (n = 108) were from the non-antibiotics group (P = 0.02). From the 241 isolates, 33% (n = 80) harbored virulence genes, 24.8% ($Cl_{95\%}$: 18.2 - 32.7) and 43.5% ($Cl_{95\%}$: 34.5 - 52.9) coming from the antibiotics (n = 33) and non-antibiotics (n = 47) group isolates respectively ($P = 0.002$).

EAST-1 was the most prevalent virulence gene in both groups with 78.7% ($Cl_{95\%}$: 62.25 - 89.32) and 85.1% ($Cl_{95\%}$: 72.32-92.59) of the virulence genes observed in the antibiotics and non-antibiotics groups respectively ($P = 0.46$) (Table 3). The STa observed were 18.1% ($Cl_{95\%}$: 8.61-34.39) and 14.8% ($Cl_{95\%}$: 7.40-27.68) in the antibiotics and non-antibiotics groups respectively ($P = 0.70$). No STb was identified in the antibiotics group and 8.5% ($Cl_{95\%}$: 3.36-19.93) of the STbs were seen in the non-antibiotics group ($P = 0.09$). The stx2e gene was identified in both groups; 3% ($Cl_{95\%}$: 0.53-15.32) in the antibiotics group and 12.7% ($Cl_{95\%}$: 5.98-25.17) in the non- antibiotics group ($P = 0.13$; table 3.3).

Of the adhesion factors for virulence genes, F6 [4.25% ($Cl_{95\%}$: 1.17-14.25)] and EAE [2.10% ($CI_{95\%}$ 0.37-11.11)] were observed in the non-antibiotics group with none in the antibiotics group. However, AIDA1 incidence were 3.0% ($Cl_{95\%}$: 0.53-15.32) and 23.4% ($CI_{95\%}$: 13.6-37.22) in the antibiotic and non-antibiotic group respectively ($P = 0.01$); and for PAA, the incidence were 18.1% ($CI_{95\%}$: 8.61-34.39) and 0% ($CI_{95\%}$. 0-7.55) for antibiotic and non-antibiotic group respectively ($P < 0.005$); (Table 3.3).

We observed 12 pathotypes with EAST1 being the most common in both groups; 60.6% ($Cl_{95\%}$: 43.64-75.32) and 61.7% ($Cl_{95\%}$: 47.43-74.21) of the antibiotics group and non-antibiotics group respectively (P = 0.92) (Table 3). The trend for recovery of virulence genes is available in Figure 2 with significance differences in recovery rates between antibiotic and non-antibiotic groups on days 5, 28 and 35.

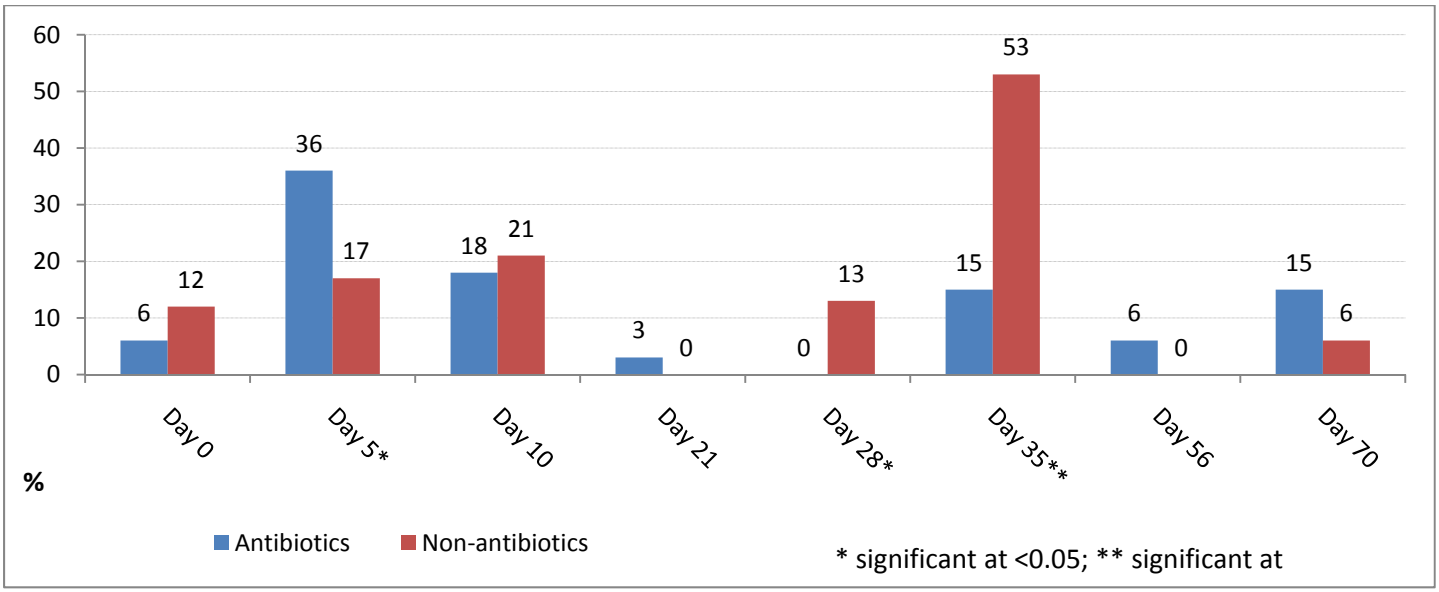


Figure 0.2: . Frequency of isolation of virulence genes in percentages based on days of sampling

Table 0.3: Frequency of *E. coli* isolates, virulence genes, adhesion factors and pathotypes from *E. coli* isolates with virulence genes

Frequency of virulence genes at sampling points			
Day	**Antibiotic group (n=33)**	**Non-antibiotic group (n=47)**	**P-value**
0	6.0 (1.67-19.61)	11.6 (4.63-22.59)	0.48
5	36.3 (22.19-53.38)	17.0 (8.88-30.14)	**0.05**
10	18.1(8.61-34.39)	21.2 (11.99-34.9)	0.73
21	3.0 (0.53-15.32)	0 (0-7.55)	0.23
28	0 (0.0-10.43)	12.7 (5.98-25.17)	**0.03**
35	15.1 (6.65-30.92)	53.1 (39.23-66.67)	**0.001**
56	6 (1.67-19.61)	0 (0-7.55)	0.09
70	15.1 (6.65-30.92)	6.3 (2.19-17.16)	0.20
Virulence genes			
Genes			
STa	18.1(8.61-34.39)	14.8 (7.40-27.68)	0.70
STb	0 (0.0-10.43)	8.5 (3.36-19.93)	0.09
EAST 1	78.7 (62.25-89.32)	85.1 (72.32-92.59)	0.46
StX2e	3 (0.53-15.32)	12.7 (5.98-25.17)	0.13
Adhesion factors from isolates that carried virulence genes			
AIDA	3.0 (0.53-15.32)	23.4 (13.6-37.22)	**0.01**
PAA	18.1(8.61-34.39)	0 (0-7.55)	**<0.005**
EAE	0 (0.0-10.43)	2.1 (0.37-11.11)	0.40
F6	0 (0.0-10.43)	4.25(1.17-14.25)	0.23
Pathotype combinations of isolates that carried virulence genes			
Pathotypes			
EAST1	60.6 (43.64-75.32)	61.7 (47.43-74.21)	0.92
STa	18.1(8.61-34.39)	0 (0-7.55)	**<0.005**
STa/F6	0 (0.0-10.43)	2.1 (0.37-11.11)	0.4
STb/EAST1/AIDA1	0 (0.0-10.43)	8.5 (3.36-19.93)	0.09
Stx2e	3.0 (0.53-15.32)	0 (0-7.55)	0.23
EAST1/EAE	0 (0.0-10.43)	2.1 (0.37-11.11)	0.4
EAST1/PAA	15.1 (6.65-30.92)	0 (0-7.55)	**<0.01**
EAST1/AIDA1	3.0 (0.53-15.32)	8.5 (3.36-19.93)	0.32
EAST1/STa	0 (0.0-10.43)	2.1 (0.37-11.11)	0.4
EAST1/STa/F6	0 (0.0-10.43)	2.1 (0.37-11.11)	0.4
STa/Stx2e/AIDA	0 (0.0-10.43)	6.3 (2.19-17.16)	0.14
STa/Stx2e	0 (0.0-10.43)	6.3 (2.19-17.16)	0.14

Significant values are presented in bold.

3.4.2 Antibiotic resistance

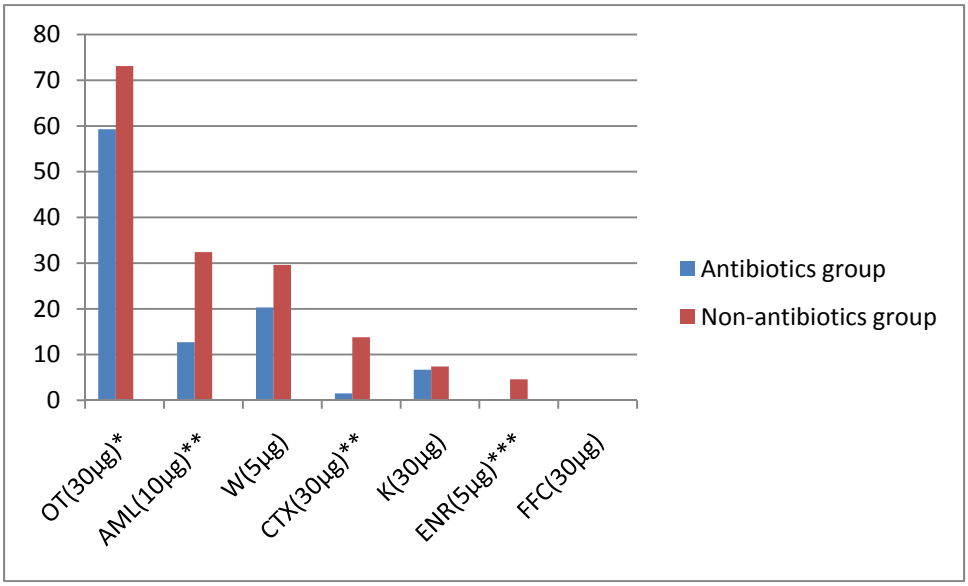
In total 164 (68%) of the isolates showed phenotypic resistance to the seven antibiotics tested, there was a significant difference (P = 0.02) between the antibiotic group; 61.6% ($Cl_{95\%}$: 53.17-69.48) and the non-antibiotic group; 75.9% ($Cl_{95\%}$: 67.06-83.01). Resistance to oxytetracycline was most common in the antibiotics group, it amounted to 59.3% ($Cl_{95\%}$: 50.9-67.3). In the non-antibiotics group 73.1% ($Cl_{95\%}$: 64.1-80.6) isolates also showed resistance to oxytetracycline (Figure 3). The difference between the two groups was statistically significant ($p < 0.05$). Trimethoprim was the second antibiotic that showed predominant resistance 20.3% ($Cl_{95\%}$: 14.3-27.9), followed by, amoxicillin 12.7% ($Cl_{95\%}$: 8.1-19.5), kanamycin 6.7% (3.6-12.3), cefotaxime 1.5% ($Cl_{95\%}$: 0.4-5.3) and no resistance for enrofloxacin and florfenicol was observed in the antibiotics group. Amoxicillin was the second most resistant antibiotic 32.4% (24.3-41.7), followed by trimethoprim 29.6% ($Cl_{95\%}$: 21.8-38.8), cefotaxime 13.8% ($Cl_{95\%}$: 8.6-21.6), kanamycin 7.4% ($Cl_{95\%}$: 3.8-13.9), enrofloxacin 4.6% ($Cl_{95\%}$: 1.9-10.3) and no resistance to florfenicol was observed within the non-antibiotics group isolates (Figure 3.3).

During the experimental period (day 1-70), resistance to oxytetracycline was more common on day 21 (P = 0.01), which constituted about 25.3% ($Cl_{95\%}$: 17.0-35.8) in the antibiotics group. However in the non-antibiotics group oxytetracycline resistance was more frequent on day 10, constituting 22.7% ($Cl_{95\%}$: 14.9-33.1). Amoxicillin resistance was more common on day 10, and constituted 23.5% ($Cl_{95\%}$: 9.5-47.2) in the antibiotic group and on days 10 and 35; resulting in 28.5% ($Cl_{95\%}$: 16.3.45.0) in the non-antibiotics group. Trimethoprim resistance was more frequent on the 21st day, and constituted 37.0% ($Cl_{95\%}$: 21.5-55.7) in the isolates of the antibiotics group and on the 10th day, 40.6% ($Cl_{95\%}$: 25.5-57.7) in the non-antibiotics group. Cefotaxime resistance was observed only on days 0 and 28 in the isolates of the antibiotics group. *E. coli* resistance to cefotaxime was observed only on days 5, 10 and 21 in the non-antibiotics group. Kanamycin resistance was observed only on days 21 and 56 among the antibiotics group isolates and on days 5, 10 and 21 among the non-antibiotics group isolates (Table 3.4).

A total of 17 phenotypic antibiotic resistance combinations were observed. Oxytetracycline (ot) phenotype was most common in the two groups, with 54.8% ($Cl_{95\%}$: 44.1-65.1) in the antibiotics group and 40.2% ($Cl_{95\%}$: 30.3-51.0) among the non-antibiotics group isolates (P = 0.06).

However, ot-w-k and ot-aml-w-ctx-k were statistically significant (P>0.05) among all the phenotypes observed in the two groups (Figure 4a)

Figure 0.3: Frequency of occurrence of antibiotic resistance for each of the antibiotics tested in this study



Significant values (<0.05, **<0.005, ***<0.01).*

Table 0.4: Frequency of occurrence of phenotypic antibiotic resistance during the growing period

Antibiotics agent	Age(days)	Antibiotic group*	Non-antibiotic group#	*P*-value
Oxytetracycline	0	15.1 (8.9-24.7)	13.9 (7.9-23.2)	0.82
	5	7.5 (3.5-15.5)	15.1 (8.9-24.7)	0.13
	10	8.8 (4.3-17.1)	22.7 (14.9-33.1)	**<0.05**
	21	25.3 (17.0-35.8)	10.1 (5.2-18.7)	**0.01**
	28	12.6 (7.0-21.7)	15.1 (8.9-24.7)	0.65
	35	16.4 (9.8-26.1)	13.9 (7.9-23.2)	0.66
	56	6.3 (2.7-13.9)	3.7 (1.3-10.5)	0.47
	70	7.5 (3.5-15.5)	5.0 (1.9-12.3)	0.51
Amoxicillin	0	17.6 (6.1-41.0)	2.8 (0.5-14.5)	0.06
	5	5.8 (1.0-26.9)	11.4 (4.5-25.9)	0.53
	10	23.5 (9.5-47.2)	28.5 (16.3-45.0)	0.7
	21	11.7 (3.2-34.3)	20 (10.0-35.8)	0.46
	28	5.8 (1.0-26.9)	8.5 (2.9-22.3)	0.73
	35	5.8 (1.0-26.9)	28.5 (16.3-45.0)	0.06
	56	11.7 (3.2-34.3)	0 (0.0-9.8)	**<0.05**
	70	17.6 (6.1-41.0)	0 (0.0-9.8)	**0.01**
Trimethoprim	0	11.1 (3.8-28.0)	6.2 (1.7-20.1)	0.5
	5	0 (0.0-12.4)	15.6 (6.8-31.7)	**<0.05**
	10	0 (0.0-12.4)	40.6 (25.5-57.7)	**<0.0005**
	21	37.0 (21.5-55.7)	3.1 (0.5-15.7)	**0.001**
	28	7.4 (2.0-23.3)	12.5 (4.9-28.0)	0.52
	35	11.1 (3.8-28.0)	21.8 (11.0-38.7)	0.27
	56	18.5 (8.1-36.7)	0 (0.0-10.7)	**0.01**
	70	14.8 (5.9-32.4)	0 (0.0-10.7)	**<0.05**
Cefotaxim	0	50(9.4-90.5)	0 (0.0-20.3)	**0.005**
	5	0 (0.0-65.7)	20 (7.0-45.1)	0.49
	10	0 (0.0-65.7)	73.3 (48.0-89.1)	**<0.05**
	21	0 (0.0-65.7)	6.6 (1.1-29.8)	0.7
	28	50 (9.4-90.5)	0 (0.0-20.3)	**0.005**
	35	-	-	-
	56	-	-	-
	70	-	-	-
Kanamycin	0	-	-	-
	5	0 (0.0-29.9)	12.5 (0.1-49.2)	0.27
	10	0 (0.0-29.9)	75.0 (40.1-93.7)	**0.001**
	21	88.8 (56.5-98.0)	12.5 (0.1-49.2)	**<0.005**
	28	-	-	-
	35	-	-	-

	56	11 (1.9-43.5)	0 (0.0-32.4)	0.33
	70	-	-	-
Enrofloxacin	0	0	0	-
	5	0	0	-
	10	0	0	-
	21	0	80.0 (36.0-98.0)	-
	28	0	20.0 (2.0-64.0)	-
	35	0	0	-
	56	0	0	-
	70	0	0	-
Florfenicol	0	0	0	-
	5	0	0	-
	10	0	0	-
	21	0	0	-
	28	0	0	-
	35	0	0	-
	56	0	0	-
	70	0	0	-

**For the antibiotic groups, the total numbers of samples that showed resistance were 79, 17, 27, 2 and 9 for Oxytetracycline, Amoxicillin, Trimethoprim, Cefotaxim and Kanamycin respectively. #For the non-antibiotic groups, the total numbers of samples that showed resistance were 79, 35, 32, 15,8,5 and 0 for Oxytetracycline, Amoxicillin, Trimethoprim, Cefotaxim , Kanamycin, Enrofloxacin and florfenicol respectively. Significant values are presented in bold. All isolated bacteria were sensitive to florfenicol and enrofloxacin except for four and one isolates against enrofloxacin on days 21 and 28 in the non-antibiotic group.*

3.4.3 Tetracycline resistance genes

Only 154 (64%) of the isolates possessed one or more of the four tetracycline resistance genes tested, it constituted 64.6% ($Cl_{95\%}$: 56.2-72.2) of the antibiotics group isolate population and 62.9% ($Cl_{95\%}$: 53.5-71.4) of the non-antibiotics group isolates population.

The most frequently observed *tet* gene in the antibiotics group is the tet (A), which constituted 23.3% ($Cl_{95\%}$: 16.9-31.1) of the isolates population, followed by *tet (B)* that made up 21.0% ($Cl_{95\%}$: 14.9-28.7), *tet (C)* [20.3% ($Cl_{95\%}$: 14.3-27.9)] and *tet (E)* [12.7% ($Cl_{95\%}$: 8.1-19.5)]. In the non-antibiotics group isolates *tet (B)* was more frequent [43.5% ($Cl_{95\%}$: 34.5-52.9)], followed by *tet (A)* [18.5% ($Cl_{95\%}$: 12.3-26.8)], *tet (C)* [9.2% ($Cl_{95\%}$: 5.1-16.2)] and *tet* (E) [1.8% ($Cl_{95\%}$: 0.5-6.5)], *tet* (B), C and E had significant statistical difference between the 2 groups (Table 3.5)

During the growing period *tet* A was more common; on day 0, with 32.2% ($Cl_{95\%}$: 18.5 -49.8) in the antibiotics group and 25% ($Cl_{95\%}$: 11.1-46.8) on days 5 and 35 in the non-antibiotics group isolates. *Tet* B was more frequent on the 35th day, 32.1% ($Cl_{95\%}$: 17.9-50.6) and 21.2% ($Cl_{95\%}$: 11.9-34.9) on day 0 in the antibiotics and non-antibiotics groups respectively. *Tet* C was more common on day 5, with 29.6% ($Cl_{95\%}$: 15.8-48.4) in the antibiotics group and 50% ($Cl_{95\%}$: 23.6-76.3) in the non antibiotics group on day 70. Tet E was more common, 29.4% ($Cl_{95\%}$: 13.2-53.1) in the antibiotics group on day 28 but observed only on days 0 and 5 in the non antibiotics group (Table 3.5).

Eleven *tet* gene combinations among the four *tet* genes were observed with *tet (A)* genotype being the most common in the antibiotics group 33.7% ($Cl_{95\%}$: 24.6-44.2) and *tet (B)* genotype 55.8% ($Cl_{95\%}$: 44.0-67.0) among the non-antibiotics group (Figure 3.4b).

Table 0.5: Frequency of each of tet genes observed and occurrence of tetracycline resistance genes during the growing period

Frequency of each of the *tet* genes observed			
tet genes	Antibiotic group(n=133)	Non-antibiotic group (n=108)	P-value
tet A	23.3(16.9-31.1)	18.5 (12.3-26.8)	0.37
tet B	21.0 (14.9-28.7)	43.5 (34.5-52.9)	**<0.0005**
tet C	20.3 (14.3-27.9)	9.2 (5.1-16.2)	**0.02**
tet E	12.7 (8.1-19.5)	1.8(0.5-6.5)	**0.002**
occurence of tetracycline resistance genes during the growing period			
Gene	Day	*Antibiotic group	#Non-antibiotic group	P-value
tetA	0	32.2 (18.5 -49.8)	0 (0.0-16.1)	**0.005**
	5	16.1 (7.0-32.6)	25 (11.1-46.8)	0.44
	10	0 (0.0 -11.0)	20 (8.0-41.6)	**0.009**
	21	19.3 (9.1-36.2)	0 (0.0-16.1)	**0.04**
	28	3.2 (0.5-16.1)	30 (14.5-51.9)	**0.007**
	35	6.4 (1.7-20.7)	25 (11.1-46.8)	0.06
	56	9.6 (3.3-24.9)	0 (0.0-16.1)	0.15
	70	12.9 (5.1-28.8)	0 (0.0-16.1)	0.09
tetB	0	7.1 (1.9-22.6)	21.2 (11.9-34.9)	0.11
	5	3.5 (0.6-17.7)	17 (8.8-30.1)	0.08
	10	25 (12.6-43.3)	19.1 (10.4-32.5)	0.55
	21	14.2 (5.6-31.4)	2.1 (0.3-11.1)	**0.04**
	28	0 (0.0-12.0)	10.6 (4.6-22.5)	0.07
	35	32.1 (17.9-50.6)	12.7 (5.9-25.1)	**0.04**

	56	3.5 (0.6-17.7)	6.3 (2.1-17.1)	0.6
	70	14.0 (5.6-31.4)	10.6 (4.6-22.5)	0.64
tetC	0	14.8 (5.9-32.4)	20 (5.6-50.9)	0.7
	5	29.6 (15.8-48.4)	0 (0.0-27.7)	**0.05**
	10	25.9 (13.1-44.6)	0 (0.0-27.7)	0.07
	21	-	-	-
	28	0 (0.0-12.4)	10 (1.7-40.4)	0.1
	35	25.9 (13.1-44.6)	20 (5.6-50.9)	0.71
	56	-	-	-
	70	3.7 (0.6-18.2)	50 (23.6-76.3)	**0.001**
tetE	0	17.6 (6.1-41.0)	50 (9.4-90.5)	0.23
	5	23.5 (9.5-47.2)	50 (9.4-90.5)	0.43
	10	23.5 (9.5-47.2)	0 (0.0-65.7)	0.44
	21	0 (0.0-18.4)	0 (0.0-65.7)	-
	28	29.4 (13.2-53.1)	0 (0.0-65.7)	0.37
	35	5.8 (1.0-26.9)	0 (0.0-65.7)	-
	56	-	-	-
	70	-	-	-

**For the antibiotic groups, the total numbers of samples positive were 31, 28, 27 and 17 for tetA, tetB, tetC and tetE respectively. #For the non-antibiotic groups, the total numbers of samples positive were 20, 47, 10 and 2 for tetA, tetB, tetC and tetE respectively. Significant values are presented in bold.*

Figure 4.2a

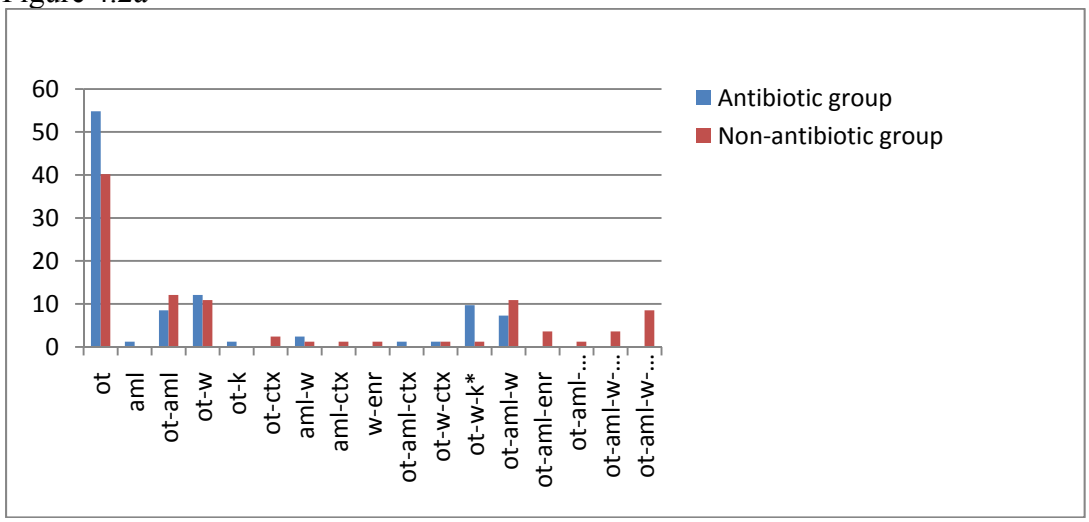


Figure 4.2b

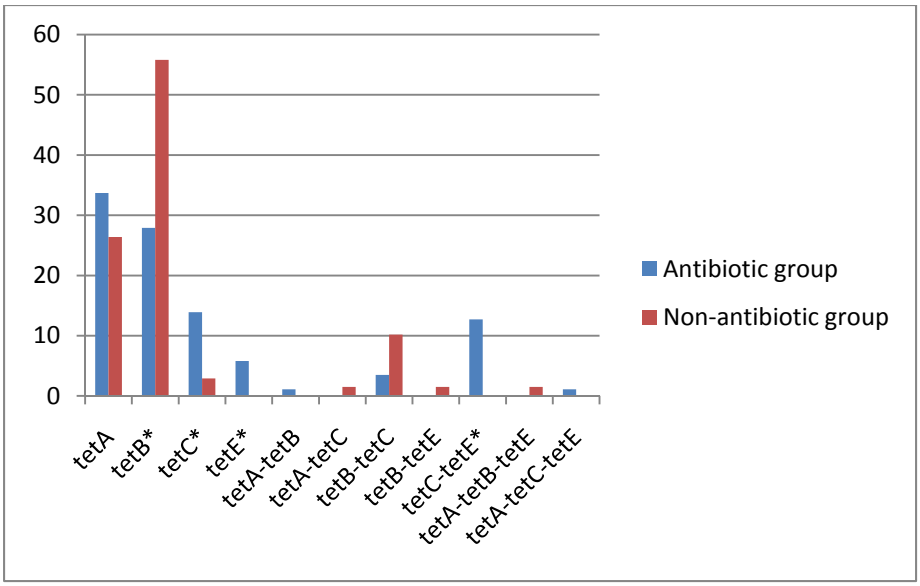
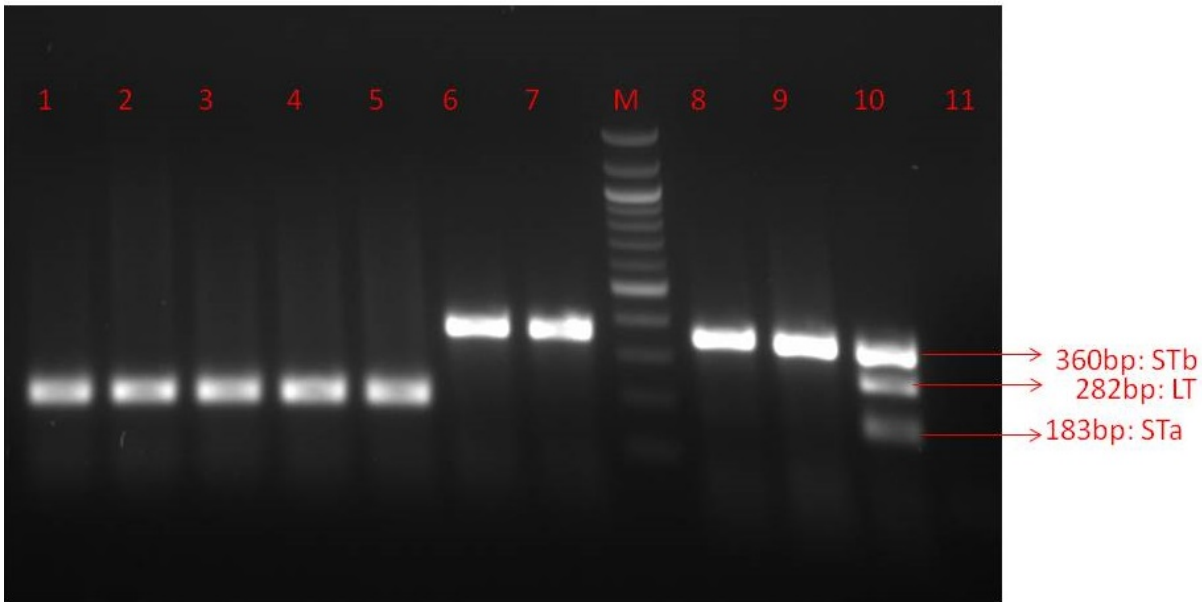
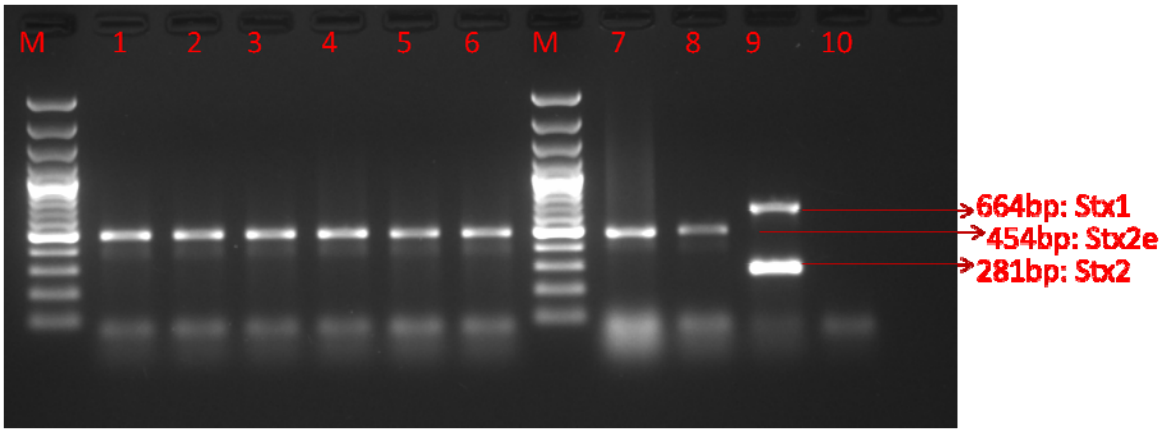


Figure 0.4a and b: Frequency of phenotypic combination of resistance among the seven antibiotics tested and genotypic combination of tet genes observed within each of the resistant E. coli isolates for both groups.



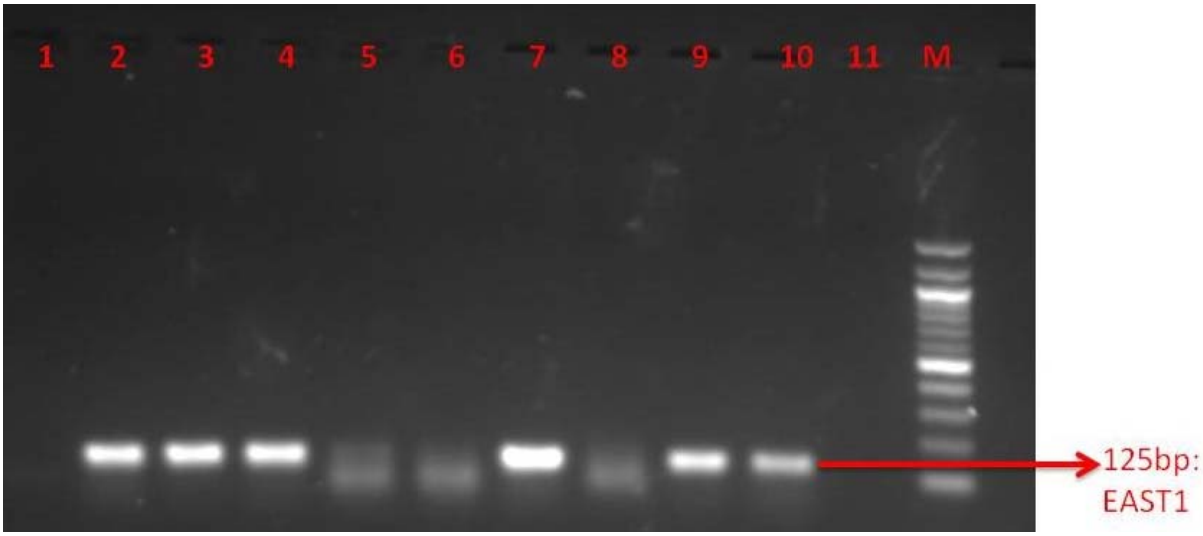
0.5: Gel picture showing STa, STb and LT bands for *E. coli* isolates that carried virulence genes.

Lane M, 100-bp ladder; Lane 1-5, Field isolates positive for STa; lane 6-9, Field isolates positive for STb; lane 10, STa, STb and LT positive control; Lane 1



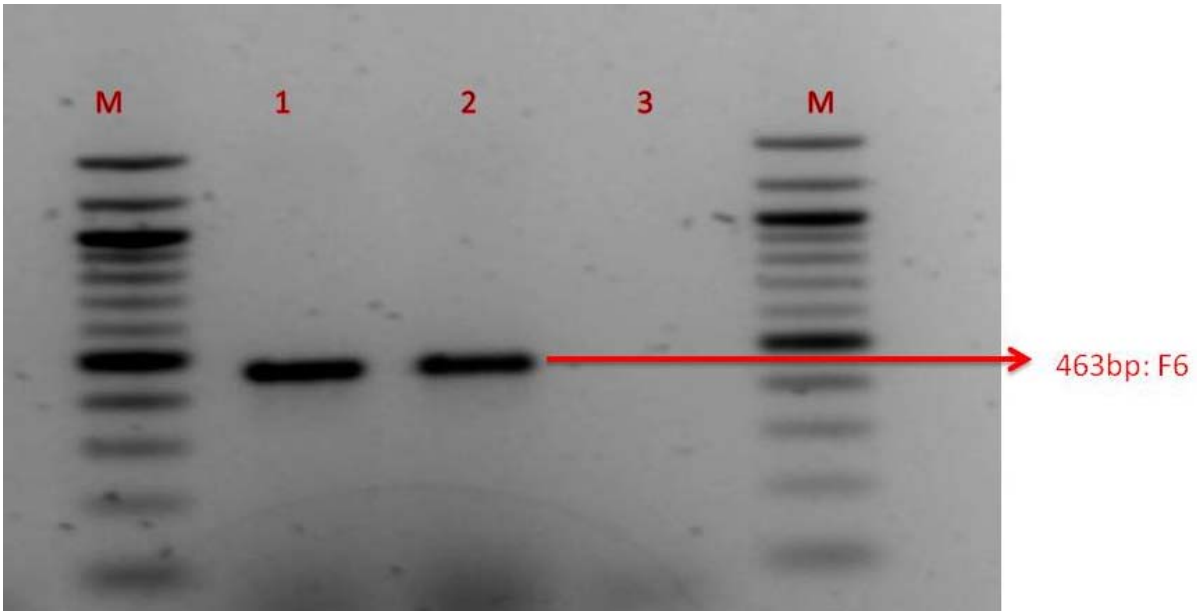
0.6: Gel picture showing Stx1, Stx2 and Stx2e bands for *E. coli* that harbored virulence genes.

Lane M, 100-bp ladder; Lane 1-7, Field isolates positive for Stx2e; Lane 8, Stx2e positive control; Lane 9 Stx1 and Stx2 positive control; Lane 10, negative control



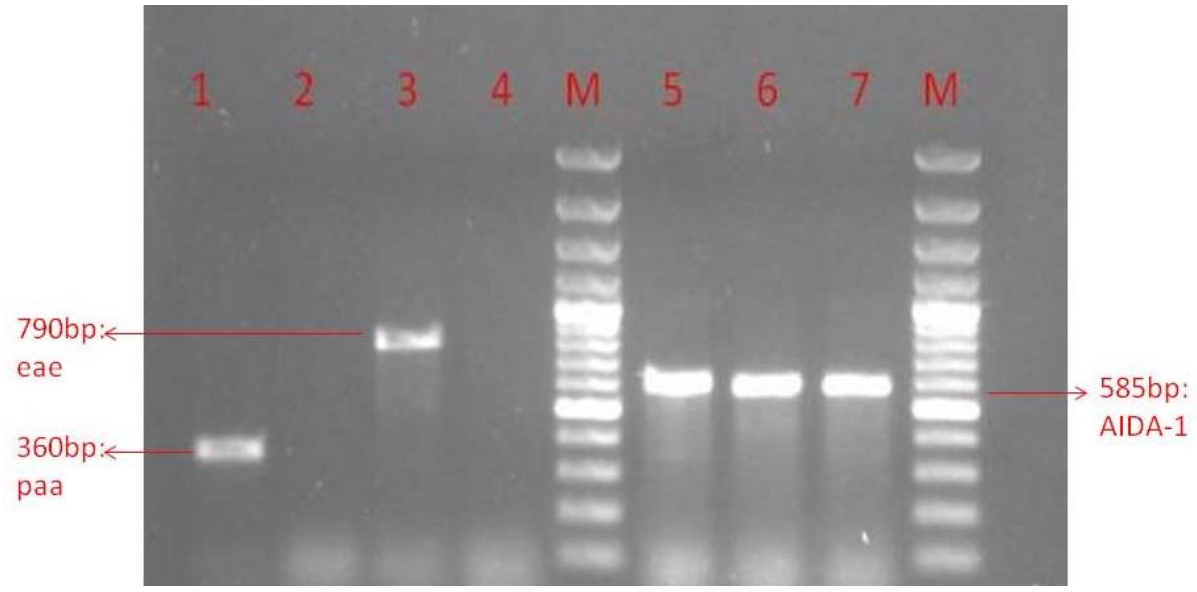
0.7:Gel picture showing bands for *E. coli* isolates that harbored EAST1 gene.

Lane M, 100-bp ladder; Lanes 1-4, 7 and 9, Field isolates positive for EAST1; lane 5,6 and 8, Field isolates negative for EAST; Lane 11, negative control.



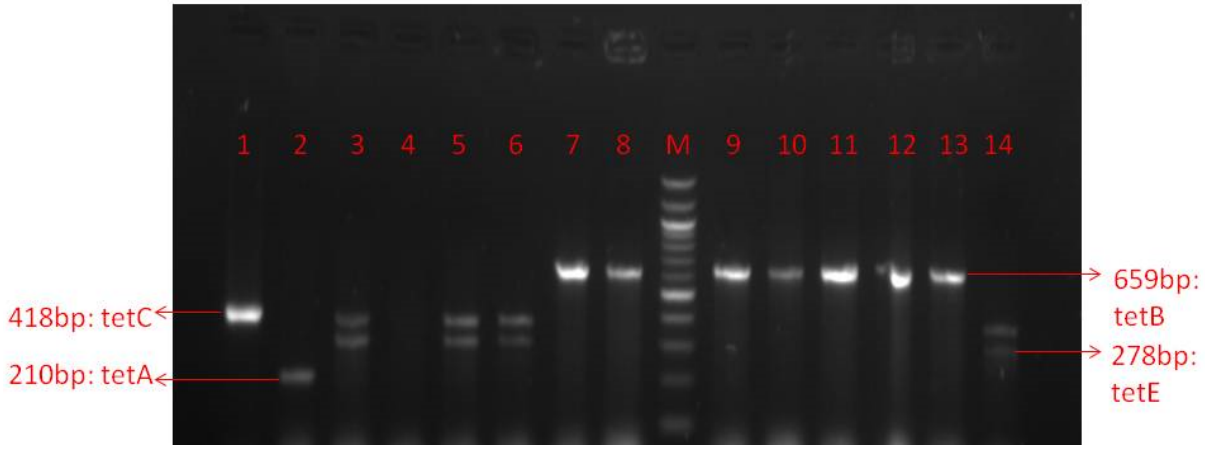
0.8:Gel picture showing bands for *E.coli* isolates that carried the F6 gene

Lane M, 100-bp ladder; Lane 1and 2 F6 positive isolates, lane 3, negative isolate.



0.9: Gel picture showing bands for *E.coli* isolates that harbored AIDA, eae and paa virulence factors.

Lane M, 100-bp ladder; Lane 1, isolate positive for paa; lane 3, isolates paa; lanes 5-7, AIDA-1 positive isolates; Lanes 2 and 4, negative isolates.



0.10: Gel picture showing PCR bands of *E. coli* isolates that carried the tet A, B, C and E resistance genes

Lane M, 100-bp ladder; Lanes 1, 3, 5, 6, and 14, isolates positive for tet C; lanes 3,5,6 and 14, isolates positive for tet E; lanes7,8-13, isolates positive for tet B and Lane 2, isolate positive for tet A.

3.5 Discussion

In this work, we have compared the effect of usage and non-usage of antibiotics in pig production from piglet to porker stage (1 to 70-110 days) using phenotypic and genotypic characteristics. Although location-specific differences exist in production systems globally, the use of antibiotics as growth promoters and for therapeutic purposes is widespread. *E. coli* isolates were obtained in both groups of pigs (with or without antibiotics) and differential resistance levels were observed in both groups. A total of 241 resident *E. coli* isolates were obtained from the samples but no distinction was made between the commensal and pathogenic organisms in this study. Previous study had confirmed that animals with intense antibiotic administration are more likely to present with antibiotic resistance clinical isolates compared with the non-antibiotics treated group which may have more commensal *E. coli* (Enne *et al.,* 2008). Because pigs are slaughtered as from day 70, the possibility of transferring resistance genes in the human food chain remains. Whereas a distance of over 5m was created between the two experimental pens during this experiment, resistance gene pattern was randomly observed in the two groups. Other workers have confirmed that mobile genetic elements (MGE) allows horizontal gene transfer (HGT) of resistance genes to other pathogens, commensal and environmental strains (Muniesa, et al., 2013; von Wintersdorff *et al.,* 2016; Tripathi and Tripathi,2017).Virulence genes have been identified in both groups irrespective of whether antibiotics were applied or not, with higher prevalence in the non-antibiotics group. It is likely that such genes are inclusive of

the environmentally acquired HGT of commensal *E. coli.* This observation is in agreement with the findings of other workers (Chapman *et al.,* 2006), which stated that although commensal *E. coli* isolates are non-pathogenic, they may potentially contain virulence genes that are capable of causing disease (Chapman *et al.,* 2006).

EAST1 was the predominant virulence gene with no significant difference between the antibiotic and non-antibiotic groups. Previous studies have concluded that EAST1 was a major determinant in *E. coli* associated diarrhea of pigs (Choi *et al.,* 2001; Osek, 2003; Vu-Khac, *et al.*, 2004). Whereas F4 which is associated with more severe diarrhea is said to be more predominant fimbria found in pigs (Madoroba *et al.,* 2009; Ikwap *et al.,* 2016; Luppi *et al.,* 2016), our study identified only F6, which cause milder form of diarrhea in pigs. In addition, AIDA1, and PAA were the predominant adhesion factors in this study but other factors were similarly recovered. AIDA1 has been associated with ETEC toxin genes (Sta, STb and EAST1) and Stx2e. AIDA1 association with toxin genes have been identified in previous studies and indicated as an important marker gene for the causation of diarrhea and edema disease in pigs (Ngeleka *et al.,* 2003; Ha, Choi and Chae, 2003; Zhang *et al.,* 2007; Zhao *et al.,* 2009).

While a number of combination of pathotypes were recovered in this study, no Stx1 and stx2 was identified. These toxins have been more commonly isolated in bovine, ovine and in humans in cases of haemolytic ureamic syndrome (Paton and Paton, 1998). Furthermore, pigs have not been known as reservoirs for human pathogenic STEC (Khac *et al.,* 2006). Stx2e isolation was higher in the non-antibiotics group and was commonly associated with AIDA, perhaps due lack of maternal immunity and development of edema disease since no antibiotic was used to clear the pathogenic organisms. Edema disease affects pigs during the post weaning period with high mortality and no recorded commercial vaccine is available but reports have shown that high sero-prevalence for stx2e in sows may provide mild protective immunity to pre-weaned pigs (Bertschinger, 1999; Oanh *et al.,* 2012).

Overall, virulence genes isolation was significantly high in the first week of life and from after the fourth weeks (post-weaning). These periods of increase isolation of virulence genes roughly coincide with period of initiation of immunity (colostral) and waned maternal immunity (Toledo *et al.,* 2012; Oanh *et al.,* 2012). This study has indicated that virulence gene distribution in pigs

from birth to porker stage is diversely random and that EAST1 remains the most common during the growing period.

Antibiotic usage in animals affects resistance patterns (Mathew *et al.,* 1999; McEwen and Fedorka-Cray, 2002; Lanz, *et al.*, 2003). Phenotypic antibiotic resistance in *E. coli* was associated with pigs in both groups, an evidence that even without antibiotic usage in pigs, resistance levels are high. Since antimicrobials are used in commercial pig farms during farrowing as uterine suppositories or parenterally, vertical transmission of antibiotic resistance from the dam to piglets is highly likely with implications for genetic transfer of resistance genes among bacteria and consequent increased morbidity and mortality (Callens *et al.,* 2015). Among the seven tested antibiotics, *E. coli* were most resistant to oxytetracycline, this is consistent with findings of previous studies (Mathew *et al.,* 1999; van Den Bogaard *et al.*, 2000). Tetracyclines are widely used in the treatment of commonly observed pig diseases (Li *et al.,* 2014) and presence of high concentrations of tetracycline in pig manure has been observed following prophylactic use in sows (Pan *et al.,* 2011). In addition to oxytetracycline, the *E. coli* were also resistant in high levels to amoxicillin and trimethoprim similar to the finding in the Netherlands (van Den Bogaard *et al.*, 2000). In South Africa, the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act 36 of 1947) subsists with laws that regulate antibiotics administration in animals. Under this Act, tetracyclines, sulphonamides and penicillins are freely accessible over the counter and records of use may not always be available (Henton *et al.,* 2011).

Although resistance to oxytetracycline and Amoxicillin was significantly higher in the non-antibiotics group, Callens et al have earlier reached the same conclusion (Callens *et al.,* 2015), however, it contrasted the findings of another study (Österberg *et al.,* 2016). High levels of *tet (A),*B,C and E resistance genes were similarly observed in the study (Figure 4a&b) and these confirmed the phenotypic patterns of oxytetracycline resistance observed. The feeding of low levels of tetracycline such as in growth promotion may increase the chances of *E. coli* resistance genes development (Agga *et al.,* 2014). Hence, the abundance of *tet* A and *tet* B may be due to the spread of *E. coli* clones carrying these genes as a result of selective pressures for the 2 genes. Whether this observation also has environmental component to it is unknown. However more piglets succumb to late-stage infection in this group and have to be humanely sacrificed. Because

the piglets live in an environment where in the dam can pass antibiotic resistance gene in their faeces and milk, these factors may serve as predisposing conditions for environmentally acquired resistance organisms, with possibility of multi-drug resistance isolates. Enrofloxacin, and cefotaxime presented with the lowest phenotypically resistant strains. It should be understood that these substances are restricted for use in animals, and are only permitted under the stricter Act 101 which requires mandatory prescription by a competent medical or veterinary personnel in South Africa (Henton *et al.,* 2011).

Age-specific resistance patterns of isolates was observed but was more pronounced within the second to fifth weeks of sampling. An association with increase usage of antimicrobials at this stage is feasible, as increase risk of diarrhea is observed during this period due to increased colonization of the gut by pathogenic microorganisms (Vidotto *et al.,* 2009). Piglets may also inadvertently ingest resistance strains on the dams' teat during the process of suckling. Abundance of *tet* genes were observed in the period between birth and day 35 of age, possibly due to frequent use of antibiotics during this period to control and treat common diseases associated with piglets such as neonatal diarrhea, post-weaning diarrhea and edema disease (Mathew *et al.,* 1999; Wellington *et al.,* 2013).

In conclusion, this study has shown that virulence genes in pigs can develop and be observed at any point during the growth phase with or without direct antibiotic administration for prophylaxis or metaphylaxis. Perhaps a restriction of antibiotics in growing meat-type pigs should be accompanied by similar restriction in the associated production-type pigs. Phenotypic resistances to antibiotics are abundant and random throughout the growing period. *Tet* genes are common in pigs due to high levels of use of tetracyclines.

3.6 References

Agga, G., Scott, H., Amachawadi, R., Nagaraja, T., Vinasco, J., Bai, J., Norby, B., Renter, D., Dritz, S. and Nelssen, J. (2014) 'Effects of chlortetracycline and copper supplementation on antimicrobial resistance of fecal *Escherichia coli* from weaned pigs', *Preventive veterinary medicine,* 114(3), pp. 231-246.

Alali, W.Q., Scott, H.M., Harvey, R.B., Norby, B., Lawhorn, D.B. and Pillai, S.D. (2008) 'Longitudinal study of antimicrobial resistance among *Escherichia coli* isolates from integrated multisite cohorts of humans and swine', *Applied and Environmental Microbiology,* 74(12), pp. 3672-3681.

Barth, S., Schwanitz, A. and Bauerfeind, R. (2011) 'Polymerase chain reaction-based method for the typing of F18 fimbriae and distribution of F18 fimbrial subtypes among porcine Shiga toxin-encoding *Escherichia coli* in Germany', *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc,* 23(3), pp. 454-464.

Barth, S., Tscholshiew, A., Menge, C., Weiss, R., Baljer, G. and Bauerfeind, R. (2007) 'Virulence and fitness gene patterns of Shiga toxin-encoding *Escherichia coli* isolated from pigs with edema disease or diarrhea in Germany', *Berliner und Munchener tierarztliche Wochenschrift,* 120(7-8), pp. 307-316.

Bertschinger, H. (1999) 'Postweaning *Escherichia coli* diarrhea and edema disease', *BE Straw, S. D'Allaire, WL Mengeling, DJ Taylor (Eds.), Diseases of Swine, Blackwell Science, Oxford,* , pp. 441-454.

Blanco, M., Lazo, L., Blanco, J.E., Dahbi, G., Mora, A., López, C., González, E.A. and Blanco, J. (2006) 'Serotypes, virulence genes, and PFGE patterns of enteropathogenic *Escherichia coli* isolated from Cuban pigs with diarrhea', *International Microbiology,* 9(1), pp. 53-60.

Burow, E., Simoneit, C., Tenhagen, B. and Käsbohrer, A. (2014) 'Oral antimicrobials increase antimicrobial resistance in porcine *E. coli*–A systematic review', *Preventive veterinary medicine,* 113(4), pp. 364-375.

Callens, B., Faes, C., Maes, D., Catry, B., Boyen, F., Francoys, D., de Jong, E., Haesebrouck, F. and Dewulf, J. (2015) 'Presence of antimicrobial resistance and antimicrobial use in sows are risk factors for antimicrobial resistance in their offspring', *Microbial Drug Resistance,* 21(1), pp. 50-58.

Capita, R. and Alonso-Calleja, C. (2013) 'Antibiotic-resistant bacteria: a challenge for the food industry', *Critical reviews in food science and nutrition,* 53(1), pp. 11-48.

Chapman, T.A., Wu, X.Y., Barchia, I., Bettelheim, K.A., Driesen, S., Trott, D., Wilson, M. and Chin, J.J. (2006) 'Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine', *Applied and Environmental Microbiology,* 72(7), pp. 4782-4795.

Chen, X., Gao, S., Jiao, X. and Liu, X.F. (2004) 'Prevalence of serogroups and virulence factors of *Escherichia coli* strains isolated from pigs with postweaning diarrhoea in eastern China', *Veterinary microbiology,* 103(1), pp. 13-20.

Cheng, D., Sun, H., Xu, J. and Gao, S. (2006) 'PCR detection of virulence factor genes in *Escherichia coli* isolates from weaned piglets with edema disease and/or diarrhea in China', *Veterinary microbiology,* 115(4), pp. 320-328.

Cheng, D., Sun, H., Xu, J. and Gao, S. (2005) 'Prevalence of fimbial colonization factors F18ab and F18ac in *Escherichia coli* isolates from weaned piglets with edema and/or diarrhea in China', *Veterinary microbiology,* 110(1), pp. 35-39.

Choi, C., Cho, W., Chung, H., Jung, T., Kim, J. and Chae, C. (2001) 'Prevalence of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 (EAST1) gene in isolates in weaned pigs with diarrhea and/or edema disease', *Veterinary microbiology,* 81(1), pp. 65-71.

Clinical Laboratory Standards Institute (2015) 'Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals', *3rd Edition (VET01S), Available at: https://clsi.org/media/1530/vet01s,* , pp. 128.

Dean, A., Sullivan, K. and Soe, M. (2015) *OpenEpi: Open source epidemiologic statistics for public health, version,* .

Dean, E.A., Whipp, S.C. and Moon, H.W. (1989) 'Age-specific colonization of porcine intestinal epithelium by 987P-piliated enterotoxigenic *Escherichia coli*', *Infection and immunity,* 57(1), pp. 82-87.

Dubreuil, J.D., Isaacson, R.E. and Schifferli, D.M. (2016) 'Animal Enterotoxigenic *Escherichia coli*', *EcoSal Plus,* 7(1), pp. 10.

Enne, V.I., Cassar, C., Sprigings, K., Woodward, M.J. and Bennett, P.M. (2008) 'A high prevalence of antimicrobial resistant *Escherichia coli* isolated from pigs and a low prevalence of antimicrobial resistant *E. coli* from cattle and sheep in Great Britain at slaughter', *FEMS microbiology letters,* 278(2), pp. 193-199.

Fasina, F.O., Bwala, D.G. and Madoroba, E. (2015) 'Investigation of multidrug-resistant fatal colisepticaemia in weanling pigs', *Onderstepoort J Vet Res,* 82(1), pp.1- 6.

Food and Drug Administration (2012) '2012 Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals.', *Department of Health and Human Services. Available at: https://www.fda.gov/downloads/forindustry/userfees/animaldruguserfeeact,* .

Frydendahl, K. (2002) 'Prevalence of serogroups and virulence genes in *Escherichia coli* associated with postweaning diarrhoea and edema disease in pigs and a comparison of diagnostic approaches', *Veterinary microbiology,* 85(2), pp. 169-182.

Funk, J.A., Lejeune, J.T., Wittum, T.E. and Rajala-Schultz, P.J. (2006) 'The effect of subtherapeutic chlortetracycline on antimicrobial resistance in the fecal flora of swine', *Microbial drug resistance,* 12(3), pp. 210-218.

Garabal, J., Vázquez, F., Blanco, J., Blanco, M. and González, E. (1997) 'Colonization antigens of enterotoxigenic *Escherichia coli* strains isolated from piglets in Spain', *Veterinary microbiology,* 54(3), pp. 321-328.

Gyles CL, F.J. (2010) *'Escherichia coli*', *Pathogenesis of bacterial infections in animals,* Blackwell Publishing. pp. 267-308.

Ha, S.K., Choi, C. and Chae, C. (2003) 'Prevalence of a gene encoding adhesin involved in diffuse adherence among *Escherichia coli* isolates in pigs with postweaning diarrhea or edema disease', *Journal of veterinary diagnostic investigation,*15(4), pp. 378-381.

Hawkins P, Morton DB, Burman O, Dennison N, Honess P, Jennings M, Lane S, Middleton V, Roughan JV, Wells S, Westwood K (2011) 'A guide to defining and implementing protocols for the welfare assessment of laboratory animals', *eleventh report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. Laboratory Animals,* 45, pp. 1-13.

Henton, M.M., Eagar, H.A., Swan, G.E. and Van Vuuren, M. (2011) 'Part VI. Antibiotic management and resistance in livestock production', *SAMJ: South African Medical Journal,* 101(8), pp. 583-586.

Henton, M.M. and Engelbrecht, M. (1997) *'Escherichia coli* serotypes in pigs in South Africa'.

Ikwap, K., Larsson, J., Jacobson, M., Owiny, D.O., Nasinyama, G.W., Nabukenya, I., Mattsson, S., Aspan, A. and Erume, J. (2016) 'Prevalence of adhesin and toxin genes in *E. coli* strains isolated from diarrheic and non-diarrheic pigs from smallholder herds in northern and eastern Uganda', *BMC microbiology,* 16(1), pp. 178.

Kanengoni, A.T., Thomas, R., Gelaw, A.K. and Madoroba, E. (2017) 'Epidemiology and characterization of *Escherichia coli* outbreak on a pig farm in South Africa', *FEMS microbiology letters,* 364(3), pp. 10.

Khac, H.V., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J.E., Mora, A., Dahbi, G., López, C., González, E.A. and Blanco, J. (2006) 'Serotypes, virulence genes, and PFGE profiles of *Escherichia coli* isolated from pigs with postweaning diarrhoea in Slovakia', *BMC Veterinary Research,* 2(1), pp. 10.

Lanz, R., Kuhnert, P. and Boerlin, P. (2003) 'Antimicrobial resistance and resistance gene determinants in clinical *Escherichia coli* from different animal species in Switzerland', *Veterinary microbiology,* 91(1), pp. 73-84.

Li, P., Wu, D., Liu, K., Suolang, S., He, T., Liu, X., Wu, C., Wang, Y. and Lin, D. (2014) 'Investigation of antimicrobial resistance in *Escherichia coli* and enterococci isolated from Tibetan pigs', *PloS one,* 9(4), pp. e95623.

Luppi, A., Bonilauri, P., Dottori, M., Gherpelli, Y., Biasi, G., Merialdi, G., Maioli, G. and Martelli, P. (2015) 'Antimicrobial resistance of F4 *Escherichia coli* isolated from swine in Italy', *Transboundary and emerging diseases,* 62(1), pp. 67-71.

Luppi, A., Gibellini, M., Gin, T., Vangroenweghe, F., Vandenbroucke, V., Bauerfeind, R., Bonilauri, P., Labarque, G. and Hidalgo, Á. (2016) 'Prevalence of virulence factors in enterotoxigenic *Escherichia coli* isolated from pigs with post-weaning diarrhoea in Europe', *Porcine Health Management,* 2(1), pp. 20.

Lutz, E.A., McCarty, M.J., Mollenkopf, D.F., Funk, J.A., Gebreyes, W.A. and Wittum, T.E. (2011) 'Ceftiofur use in finishing swine barns and the recovery of fecal *Escherichia coli* or *Salmonella* spp. resistant to ceftriaxone', *Foodborne pathogens and disease,* 8(11), pp. 1229-1234.

MacLeod, D., Gyles, C. and Wilcock, B. (1991) 'Reproduction of edema disease of swine with purified Shiga-like toxin-II variant', *Veterinary pathology,* 28(1), pp. 66-73.

Madoroba, E., Van Driessche, E., De Greve, H., Mast, J., Ncube, I., Read, J. and Beeckmans, S. (2009) 'Prevalence of enterotoxigenic *Escherichia coli* virulence genes from scouring piglets in Zimbabwe', *Tropical animal health and production,* 41(7), pp. 1539-1547.

Madzimure, J., Chimonyo, M., Zander, K.K. and Dzama, K. (2012) 'Potential for using indigenous pigs in subsistence-oriented and market-oriented small-scale farming systems of Southern Africa', *Tropical animal health and production,* 45(1), pp. 135-142.

Mathew, A.G., Saxton, A.M., Upchurch, W.G. and Chattin, S.E. (1999) 'Multiple antibiotic resistance patterns of *Escherichia coli* isolates from swine farms', *Applied and Environmental Microbiology,* 65(6), pp. 2770-2772.

McEwen, S.A. and Fedorka-Cray, P.J. (2002) 'Antimicrobial use and resistance in animals', *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* 34 Suppl 3, pp. S93-S106.

Meissner, H., Scholtz, M. and Palmer, A. (2013) 'Sustainability of the South African Livestock Sector towards 2050 Part 1: Worth and impact of the sector', *South African Journal of Animal Science,* 43(3), pp. 282-297.

Mohlatlole, R.P., Madoroba, E., Muchadeyi, F.C., Chimonyo, M., Kanengoni, A.T. and Dzomba, E.F. (2013) 'Virulence profiles of enterotoxigenic, shiga toxin and enteroaggregative *Escherichia coli* in South African pigs', *Tropical animal health and production,* 45(6), pp. 1399-1405.

Muniesa, M., Colomer-Lluch, M. and Jofre, J. (2013) 'Could bacteriophages transfer antibiotic resistance genes from environmental bacteria to human-body associated bacterial populations?', *Mobile genetic elements,* 3(4), pp. 739-751.

Nagy, B., Whipp, S., Imberechts, H., Bertschinger, H., Dean-Nystrom, E., Casey, T. and Salajka, E. (1997) 'Biological relationship between F18ab and F18ac fimbriae of enterotoxigenic and verotoxigenic *Escherichia coli* from weaned pigs with oedema disease or diarrhoea', *Microbial pathogenesis,* 22(1), pp. 1-11.

Nagy, B. and Fekete, P.Z. (1999) 'Enterotoxigenic *Escherichia coli* (ETEC) in farm animals', *Veterinary research,* 30(2-3), pp. 259-284.

Nagy, B., Wilson, R.A. and Whittam, T.S. (1999) 'Genetic diversity among *Escherichia coli* isolates carrying f18 genes from pigs with porcine postweaning diarrhea and edema disease', *Journal of clinical microbiology,* 37(5), pp. 1642-1645.

Ngeleka, M., Pritchard, J., Appleyard, G., Middleton, D.M. and Fairbrother, J.M. (2003) 'Isolation and association of *Escherichia coli* AIDA-I/STb, rather than EAST1 pathotype, with diarrhea in piglets and antibiotic sensitivity of isolates', *Journal of veterinary diagnostic,* 15(3), pp. 242-252.

Oanh, T.K., Nguyen, V.K., De Greve, H. and Goddeeris, B.M. (2012) 'Protection of piglets against Edema disease by maternal immunization with Stx2e toxoid', *Infection and immunity,* 80(1), pp. 469-473.

Osek, J. (2003) 'Detection of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 (EAST1) gene and its relationship with fimbrial and enterotoxin markers in *E. coli* isolates from pigs with diarrhoea', *Veterinary microbiology,* 91(1), pp. 65-72.

Österberg, J., Wingstrand, A., Jensen, A.N., Kerouanton, A., Cibin, V., Barco, L., Denis, M., Aabo, S. and Bengtsson, B. (2016) 'Antibiotic Resistance in *Escherichia coli* from Pigs in Organic and Conventional Farming in Four European Countries', *PloS one,* 11(6), pp. e0157049.

Pan, X., Qiang, Z., Ben, W. and Chen, M. (2011) 'Residual veterinary antibiotics in swine manure from concentrated animal feeding operations in Shandong Province, China', *Chemosphere,* 84(5), pp. 695-700.

Paton, J.C. and Paton, A.W. (1998) 'Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli infections*', *Clinical microbiology reviews,* 11(3), pp. 450-479.

Rice, L.B. (2009) 'The clinical consequences of antimicrobial resistance', *Current opinion in microbiology,* 12(5), pp. 476-481.

Roberts, M.C. (2011) 'Environmental macrolide–lincosamide–streptogramin and tetracycline resistant bacteria', *Frontiers in microbiology,* 2, pp. 40.

Scott, H., Campbell, L., Harvey, R., Bischoff, K., Alali, W., Barling, K. and Anderson, R. (2005) 'Patterns of antimicrobial resistance among commensal *Escherichia coli* isolated from integrated multi-site housing and worker cohorts of humans and swine', *Foodbourne Pathogens & Disease,* 2(1), pp. 24-37.

Sørum, H. and Sunde, M. (2001) 'Resistance to antibiotics in the normal flora of animals', *Veterinary research,* 32(3-4), pp. 227-241.

Statista (2016) 'Number of pigs world wide from 1990 to 2013', *http://www.statista.com/statistics/263963/number-of-pigs-worldwide-since-1990/,*. Accessed: 9th February, 2016.

Tadesse, D.A. (2012) 'Antimicrobial Drug Resistance in *Escherichia coli* from Humans and Food Animals, United States, 1950–2002-Volume 18, Number 5—May 2012-Emerging Infectious Disease journal-CDC', .

Taylor, N., Clifton-Hadley, F., Wales, A., Ridley, A. and Davies, R. (2009) 'Farm-level risk factors for fluoroquinolone resistance in *E. coli* and thermophilic *Campylobacter* spp. on finisher pig farms', *Epidemiology and infection,* 137(08), pp. 1121-1134.

Toledo, A., Gomez, D., Cruz, C., Carreon, R., Lopez, J., Giono, S. and Castro, A.M. (2012) 'Prevalence of virulence genes in *Escherichia coli* strains isolated from piglets in the suckling and weaning period in Mexico', *Journal of medical microbiology,* 61(1), pp. 148-156.

Tripathi, V. and Tripathi, P. (2017) 'Antibiotic Resistance Genes: An Emerging Environmental Pollutant'*Perspectives in Environmental Toxicology* Springer, pp. 183-201.

van den Bogaard, Anthony E and Stobberingh, E.E. (2000) 'Epidemiology of resistance to antibiotics: links between animals and humans', *International journal of antimicrobial agents,* 14(4), pp. 327-335.

van Den Bogaard, A.E., London, N. and Stobberingh, E.E. (2000) 'Antimicrobial resistance in pig faecal samples from the Netherlands (five abattoirs) and Sweden', *The Journal of antimicrobial chemotherapy,* 45(5), pp. 663-671.

Varga, C., Rajić, A., McFall, M.E., Reid-Smith, R.J. and McEwen, S.A. (2009) 'Associations among antimicrobial use and antimicrobial resistance of *Salmonella* spp. isolates from 60 Alberta finishing swine farms', *Foodborne pathogens and disease,* 6(1), pp. 23-31.

Vidotto, M.C., Lima, N., Fritzen, J.T., Freitas, J.C.d., Venâncio, E.J. and Ono, M.A. (2009) 'Frequency of virulence genes in *Escherichia coli* strains isolated from piglets with diarrhea in the North Parana State, Brazil', *Brazilian Journal of Microbiology,* 40(1), pp. 199-204.

von Wintersdorff, C.J., Penders, J., van Niekerk, J.M., Mills, N.D., Majumder, S., van Alphen, L.B., Savelkoul, P.H. and Wolffs, P.F. (2016) 'Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer', *Frontiers in microbiology,* 7, pp. 173.

Vu-Khac, H., Holoda, E. and Pilipčinec, E. (2004) 'Distribution of virulence genes in *Escherichia coli* strains isolated from diarrhoeic piglets in the Slovak Republic', *Journal of Veterinary Medicine, Series B,* 51(7), pp. 343-347.

Wallace, J., Sanford, J., Smith, M. and Spencer, K. (1990) 'The assessment and control of the severity of scientific procedures on laboratory animals.', *Laboratory animals,* 24(2), pp. 97-130.

Wellington, E.M., Boxall, A.B., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D.L., Lee, N.M. and Otten, W. (2013) 'The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria', *The Lancet infectious diseases,* 13(2), pp. 155-165.

World Health Organization (2012) *Critically important antimicrobials for human medicine.* http://apps.who.int/iris/bitstream/10665/77376/1/9789241504485_eng.pdf (accessed 9.2.2017).

Zhang, W., Zhao, M., Ruesch, L., Omot, A. and Francis, D. (2007) 'Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US', *Veterinary microbiology,* 123(1), pp. 145-152.

Zhao, L., Chen, X., Xu, X., Song, G. and Liu, X. (2009) 'Analysis of the AIDA-I gene sequence and prevalence in *Escherichia coli* isolates from pigs with post-weaning diarrhoea and oedema disease', *The Veterinary Journal,* 180(1), pp. 124-129.

Chapter Four

Effect of farm antibiotics usage on fecal microbiome of growing pigs

R.H. Abubakar, C.N Kamutando, A. Valverde, E. Madoroba, D. A. Cowan, F.O. Fasina

Preface

This chapter addresses the third objective of this study which was to monitor the development of enteric bacteria population in growing pigs through metagenomics analysis. The text in this chapter is being prepared for submission to a high impact peer reviewed journal.

4.1 Abstract

Microbial community in the gut of pigs provides a vast and complex microbial network of community diversity important for its health and development. Antibiotics are normally used to maintain or improve health and feed efficiency in pigs; but this practice may cause development of disease resistance in the gut microbiome, which may impact negatively on future disease treatment. Here, we report on the metagenomics analysis of fecal samples collected from; (i) pigs receiving normal farm treatment without antibiotics usage, over a 70 day period, and (ii) pigs treated on the farm with antibiotics and monitored over a 70 day period. Our hypothesis is that the use of antibiotics in commercial farms affect guts microbial development in growing pigs. Results revealed differences between community composition and structure in the two groups irrespective of age. Pairwise comparisons at each growth stage between the antibiotics group and non-antibiotics were significant for days 0- 35 but not significant for days 56 and 70. Both groups were dominated by the phylum; *Firmicutes* (41.99% and 54.04%), *Bacteriotedes* (32.88% and 26.75%) and *Proteobacteria* (14.13% and 9.94%) in the antibiotics and non-antibiotic groups respectively. only *Firmicutes* was enriched in the untreated metagenomes. The most abundant classes were *Bacteroidia* (32.9% and 26.6%), *Clostridia* (26.4% and 26.2%), *Bacilli* (11.2% and 18.8%), *Gammaproteobacteria* (11.6% and 8.1%) for the antibiotics and non-antibiotics groups respectively. The most abundant genera were *Prevotella* (21.3% and 12.7%), *Lactobacillus* (11.6% and 21.1%) and *Bacteroides* (9.1% and 5.8) in the antibiotics and non antibiotics groups respectively and were enriched in the antibiotics treated bacterial community but not significant. This shows that antibiotics usage increases gut bacterial population in growing pigs when used for a period of time and non treated pigs could acquire resistance strains as they age which could be due to environmental factors.

4.2 Introduction

Neonate animals usually lack microbial contamination particularly in their gut at the point of birth; however the process of delivery and their subsequent exposure to the environment introduces microorganisms into their body system (Turnbaugh and Gordon, 2008). The microbial population in the gut establishes through succession and it is affected by changes in diet, environment, disease and stress (Isaacson and Kim, 2012). Following the evolutional changes, a climax of a microbial community may be attained which remain in stable association with the host. However, changes in microbial composition may continue to occur as a result of new microbes in the individual's environment (Dethlefsen *et al.,* 2008). This constant co-evolution of the microbial community in the gut provides a vast and complex microbial network of community diversity important for the health and development of animals (Isaacson and Kim, 2012).

Gut microorganisms may play essential roles that may be lacking in the animal , for example; carbohydrate metabolism and immune development (Greenblum, Turnbaugh and Borenstein, 2012; Backhed *et al.,* 2005; Ley, Peterson and Gordon, 2006), stimulation of water transport in the colon (Yolton and Savage, 1976), re-cycling of bile salts (Shimada, Bricknell and Finegold, 1969; Gilliland and Speck, 1977), production of vitamin K (Ramotar *et al.,* 1984), provision of exogenous alkaline phosphatases (Yolton and Savage, 1976), cellulose degradation (Ilmberger *et al.,* 2014), epithelium development, and action as a natural defense against pathogens (Zoetendal *et al.,* 2004).

Exploring the gut microbial community composition and functional capacity in animals is important in understanding the role they play in the host animal health and physiology as well as how this may impact on the safety of water safety meant for human consumption as it may be contaminated by animal fecal microbial pathogens (Ley *et al.,* 2008). Previous studies showed that the bulk of the culturable bacteria are gram-positive, strict anaerobic *Streptococci*, *Lactobacilli*, *Eubacteria*, *Clostridia*, and *Peptostreptococci*, and that the gram-negative microbes are dominated by *Bacteroides*, however information on population dynamics and community responses to environmental disturbances is still limited (Isaacson and Kim, 2012).

Recent studies have used next generation sequencing (NGS) technology in elucidating the intestinal or fecal microbiome of different animals including, cow, reindeer, wallaby, yak, giant panda, buffalo, elephant, pig, Iberian lynx and termite (Ilmberger *et al.,* 2014; Hess *et al.,* 2011;

Brulc *et al.,* 2009; Pope *et al.,* 2012; Pope *et al.,* 2010; Dai *et al.,* 2012; Zhu *et al.,* 2011; Yang *et al.,* 2010; Singh *et al.,* 2012; Lamendella *et al.,* 2011; Alcaide *et al.,* 2012; Warnecke *et al.,* 2007). All this gave significant information on the fecal and gut microbial communities, the metabolism and the genetic potential of the intestinal microbiota and their importance in host survival, fitness, physiology and nutrient utilization (Jacobs and Braun, 2014; Buffie and Pamer, 2013; Varel, 1987). A study in humans also found that adult and infant type gut microbiomes have enriched gene families sharing little overlap, suggesting different core functions within the adult and infant gut microbiota (Kurokawa *et al.,* 2007), hence understanding the gene content of multiple gut microflora can help explain the ecological differences of gut systems. Microbial composition variations have been reported in pigs at different segments of their small intestines, for fecal samples that were collected during the stages of growth and development, as well as from different sex and age groups, thereby stipulating that host genetics may influence the pig gut microbiome (Isaacson and Kim, 2012; Xiao *et al.,* 2016).

Pigs are a major source of food and means of livelihood to farmers globally (Kumar et al 2015). These animals are also widely used as an ideal model species for analyses of several human physiological functions, diseases and perfection of surgical procedures (Xiao *et al.,* 2016; Lunney, 2007). However, large-scale pig farmers experience environmental challenges such as the spread of antibiotic-resistant bacteria and their associated genes (Xiao *et al.,* 2016). Antibiotics have been used in agricultural animals for over 50 years and considered the most cost-effective way to maintain or improve health and feed efficiency of animals raised with conventional agricultural techniques (Cromwell, 2002; Dibner and Richards, 2005; Looft *et al.,* 2012). The impact of such antibiotic use on the treatment of human diseases is of great concern. Furthermore, previous studies showed that antibiotics administered to germ-free animals did not have growth-promoting effects (Coates *et al.,* 1963) which indicated that growth promotion may be due to changes in the gut microflora (Kim *et al.,* 2012). This may be as a result of selective pressure exerted by antibiotics on commensal microorganisms. Furthermore, even in the absence of antibiotics usage, antibiotic resistance genes reservoirs have been shown to be stable in bacterial communities (Gotz *et al.,* 1996; Salyers and Amabile-Cuevas, 1997; Stanton and Humphrey, 2011; Stanton, Humphrey and Stoffregen, 2011). Similarly, the gut microbiome may

also antagonize future disease treatment by facilitating the dissemination of resistance genes to distantly related organisms (Karami *et al.,* 2007; Shoemaker *et al.,* 2001).

Recent studies of environmental and gut microbiomes reveal enormous diversity of antibiotic resistance genes (Allen *et al.,* 2010; Sommer, Dantas and Church, 2009; Martinez *et al.,* 2009) and microbial shifts in response to the use of antimicrobial growth promoters in pigs (Looft *et al.,* 2012; Rettedal *et al.,* 2009; Allen *et al.,* 2011). Furthermore, virulence factors associated with antibiotic resistance genes with highest sequence similarity to genes in *Bacteroidetes*, *Clostridia*, and *Methanosarcina* were abundant within the gene families unique to the pig fecal metagenomes, suggesting that the pig gut microbiome are shaped by husbandry practices (Looft *et al.,* 2012).Therefore exposure of the phylogenetic composition of microbial community and the potential functional capacity of microbiome in different gut locations is of great importance to pig production (Yang *et al.,* 2016). This particular study aim to explore the effects of routine antibiotics usage for disease treatment and prevention, using a longitudinal study design, in order to detect changes in the fecal microbiome of pigs being raised over time (ie., 0-70 days period) in a typical commercial farm setting. The main objective of the study was to test if antibiotics use in commercial farms affect gut microbial development in growing pigs. We hypothesize that age differences and antibiotics usage may influence the pig gut microbiome.

4.3 Materials and methods

4.3.1 Sample collection

A small scale commercial pig farm was identified in the Gauteng province of South Africa. Two estrous synchronized pregnant sows were monitored till the day of farrowing. Five piglets were randomly selected from each sow and these selected 10 piglets were tagged. The first group (ie., the sow with five tagged piglets and other non-tagged litter mates) were kept in one pen under normal farm management practices but without any form of antibiotics usage, and this group was identified as the non-antibiotics group (NAG). The second group (ie. sow with five piglets and other non-tagged litter mates) were kept under the normal management practices of the farm, which included administration of multi vitamins, deworming, tail docking, vaccination, provision of warmth and antibiotic administration for treatment and prevention of diseases, and this group was identified as antibiotics-treated group (ATG). Rectal swabs were taken from the 10 tagged piglets in NAG and ATG and were labeled with the specific pig identification number and age

(in days). The samples were collected periodically on days 1, 5, 10, 21, 28, 35, 56 and 70 and were stored at -20^{o}C until the last samples were collected.

4.3.2 DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from swabs using the QIAamp Stool DNA Mini kit (Qiagen, Hilden, Germany), following manufacturer's instructions after a bead beating step. Extracted DNA was quantified using Nanodrop 1000 spectrophotometer. The primer sequence used to amplify the bacterial 16S rRNA V4 hypervariable region were 515F (5′-GTGYCAGCMGCCGCGGRA-3′) and 909R (5′-CCCCGYCAATTCMTTTRAG-3′). PCR was done in a single step using a barcoded forward primer and a HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) as described by (Oloo *et al.,* 2016). The thermocycling conditions were initial denaturation at 94°C for 3 minutes (5 cycles), followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, then a final elongation at 72°C for 5 minutes. PCR products were electrophoresed on 2% agarose gel to ensure expected band sizes. All samples were pooled in equal proportions and purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, MA, USA). Sequencing was performed on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) at the Molecular Research LP next generation sequencing service (http://www.mrdnalab.com, Shallowater, TX, USA) according to the manufactures guidelines.

4.3.3 16S rRNA sequence analysis

Generated sequence data was analyzed using QIIME software version 1.9.1 (Caporaso *et al.,* 2010). Briefly, the sequence files were joined, sequences with < 200 bp, more than 2 ambiguous bases, had quality scores < 25, or had more than one mismatch to the sample-specific barcode or to the primer sequences were removed. Chimeric sequences were detected using usearch61 version 6.1 (Edgar, 2010) and the operational taxonomic units (OTUs) were clustered at 97% similarity level based on the greengenes reference sequences and taxonomic databases. Final OTUs were taxonomically classified using BLAST. Singletons and Archaea species were filtered out from the OTU table. Samples were rarefied to 36,490, which was the lowest number of sequences obtained for a single sample and used for further downstream analysis.

4.3.4 Statistical Analysis

Shared phylotypes between the two treatment groups were visualized using a venn diagram. The alpha diversity indices (ie., shannon,, simpson, inverse simpson, richness and pielou's evenness) were calculated using the *diversity* function in the vegan package for R(RDevelopmentCoreTeam, 2013, Oksanen et al., 2007). Differences in diversity as well as in taxa abundances between the NAG and the ATG treatment and age groups were tested using Kruskal–Wallis tests. Differences in bacterial structure and composition between the treatment groups were visualized using the non-metric multidimensional scaling (nMDS) and PCoA ordination plots, and permutation tests were done using the Bray–Curtis dissimilarity matrices (Bray and Curtis, 1957) after Hellinger transformation (Legendre and Gallagher, 2001), in order to detect statistical differences between the two bacterial communities. The dissimilarity distance matrices were obtained using the *vegdist* fuction in vegan and the permutation tests were done using the *adonis* function in the vegan package and MANOVA functiont in the RVAideMemoire package. The differences in bacterial structure and composition within the treatment groups were detected using the *betadisper* function again in vegan. The Analysis Of Similarity (ANOSIM) test was also used to explore differences in structure and composition between the age groups, and this was achieved using the *anosim* function in the vegan package.

4.4 Results and discussion

A total of 5,898 OTUs were obtained and these constituted about 6,645,227 sequences. Samples were rarefied using a sampling depth of 22,081 which was the smallest sample, and this resulted in a total of 4,324 OTUs that constituted, approximately 1,435,265 sequences after chloroplast, mitochondria and blanks were removed to ensure that only the Kingdom bacteria were left. A total of 2,560 (59.2 %) OTUs were shared between the two treatment groups, while 966 (22.3%) and 798 (18.5%) OTUs were unique to the antibiotics and non-antibiotics groups respectively. However, during the growing period the shared OTUs were 36.1%, 45.2%, 50.4%, 42.1%, 35.9%, 39.4%, 41.3% and 45.4% on days 0, 5, 10, 21, 28, 35, 56 and 70 respectively. The antibiotics group had 24.6%, 31.4%, 22.6%, 32.9%, 38.7%, 33.7%, 43.4% and 39.8% while the non antibiotics group had 39.4%, 23.5%, 26.9%, 25%, 25.4%, 26.9%, 15.3% and 14.7% of the OTUs unique on days 0, 5, 10, 21, 28, 35, 56 and 70 respectively (figure 4.1). The ATG had higher unique OTUs on all the days except days 0 and 10 (may be due to antibiotics effect) which is expected due to development of resistant strains however, with the exception of day 0,

NAG had most of its OTUs shared with the ATG. This may suggest that the pigs acquired some resistant bacterial strains irrespective of antibiotics application.

A rarefaction curve generated approached a plateau for all the samples, suggesting that most of the bacterial sequences were captured (supplementary figure S1). In general, the means of the alpha diversity measures (ie., richness, Shannon, inverse Simpson or peilou) for the different age groups tended to be higher in the ATG compared to the NAG (Table2). Bacterial richness between the different age groups for both groups was significant (Kruskal-Wallis χ^2 = 41.7, p = 0.0002499), but bacterial richness for the two groups irrespective of age was not significant (Kruskal-Wallis χ^2 = 1.3795, p = 0.2402). Bacterial richness between the different age groups, within the antibiotics-treated group was significant (Kruskal-Wallis χ^2 = 24.831, p = 0.0008129). This may be as a result of increase in bacterial population due to resistance from antibiotic treatments as the pigs get older. This agrees with the findings of looft et al 2012, which showed that antibiotic resistance genes increases in abundance and diversity in antibiotics treated pigs. Bacterial diversity showed to increase during the pre-weaning (day 0-21) and post weaning periods (day 35-56), and declined during the weaning period (day 28-35) (Table 4.1). No statistical differences were observed in bacterial richness between the age groups, within the non-antibiotics treated group (Kruskal-Wallis χ^2 = 12.966, p = 0.07295). Similar trends in bacterial diversity within the ATG was observed except on day five in which bacterial alpha diversity reduced, this may be associated with the stress of neonatal diarrhoea found in pigs after birth (Table 1). Gut bacterial community increases to a stable point as an animal develops, but that stability can be disturbed in response to certain environmental changes such as diet and stress which returns to normal when the agent of disturbance is removed (Dethlefsen *et al.,* 2008). This is in agreement with the findings of (Looft *et al.,* 2012; Kim *et al.,* 2011), who found that microbial community of pigs changes with age.

A total of 17 bacterial phyla were observed across all the 65 samples (Supplimentary table S1a). During the growing period, the bacterial phylum, *Firmicutes* (41.99%), *Bacteriotedes* (32.88 %) and *Proteobacteria* (14.13 %) in the ATG and *Firmicutes* (54.04%), *Bacteriotedes* (26.75 %) and *Proteobacteria* (9.94 %) in the NAG and accounted for the majority of the reads for all the treatment groups and across the age groups (Fig 4.2a; Appendix). The most dominant phyla *Firmicutes* was enriched in the NAG. The average relative abundance for the remaining 15 phyla

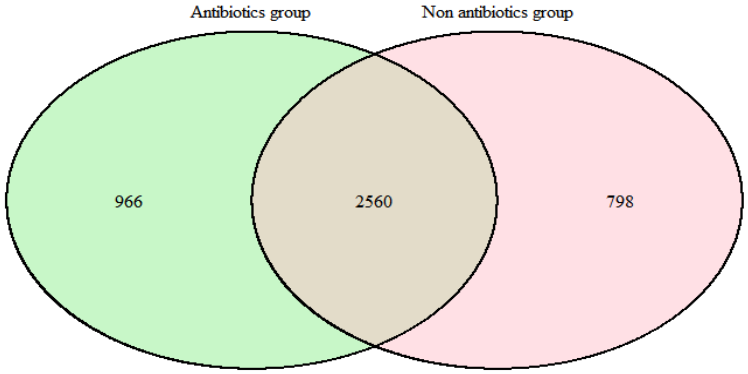
is listed in the appendix 2 (page 131-145). The abundance between the different age groups was significant for *Firmicutes* (Kruskal-Wallis = 33.638, P = 0.003827) *Bacteroidetes* (Kruskal-Wallis χ^2 = 34.502, P = 0.002894) *Proteobacteria* (Kruskal-Wallis χ^2 = 36.301, P = 0.001599) *Spirochaetes* (Kruskal-Wallis χ^2 = 37.557, P = 0.001049) *Fusobacteria* (Kruskal-Wallis χ^2 = 38.91, P = 0.0006609) *Actinobacteria* (Kruskal-Wallis χ^2 = 27.851, P = 0.02252) *Chlamydiae* (Kruskal-Wallis χ^2 =34.715, P = 0.002699) *Verrucomicrobia* (Kruskal-Wallis χ^2 =32.079, P = 0.006281) *Planctomycetes* (Kruskal-Wallis χ^2 = 39.056, P = 0.0006286) *Lentisphaerae* (Kruskal-Wallis χ^2 = 32.255, P = 0.005943) *Fibrobacteres*(Kruskal-Wallis χ^2 =.613, P= 0.02412) *TM7* (Kruskal-Wallis χ^2 = 30.977, P = 0.008849) *Tenericutes* (Kruskal-Wallis χ^2 = 27.514, P = 0.02482) except for *Synergistetes, Deferribacteres, Thermi, Elusimicrobia* that were not significant (P > 0.05). Only the phylum *Firmicutes* and *Fibrobacteres* showed significant difference in abundance between the treatment groups (Kruskal-Wallis χ^2 = 5.8323, P = 0.01573 and Kruskal-Wallis χ^2 = 4.9893, P = 0.02551) respectively. Previous studies indicated that > 90% of bacteria in the pig gut are dominated by the *Firmicutes* and *Bacteroidetes* phyla (Isaacson and Kim, 2012; Kim *et al.,* 2012; Yang *et al.,* 2016). This suggests that bacteria belonging to these phyla may develop resistance to antimicrobial agents since use of antibiotics did not decrease their relative abundance in the ATG and may be a reservoir of antibacterial resistance genes.

At the class level, a total of 33 distinct classes were identified, with the class *Bacteroidia* (32.9% and 26.6%), *Clostridia* (26.4% and 26.2%), *Bacilli* (11.2% and 18.8%), *Gammaproteobacteria* (11.6% and 8.1%), *Erysipelotrichi* (4.4% and 9.0%), *Spirochaetes* (3.7% and 1.8%), *Fusobacteriia* (3.4% and 2.7%) and *Actinobacteria* (0.3% and 1.2%) representing majority of the reads in the ATG and NAG respectively (Fig 4.2b, Appendix 2). Classes *Bacilli, Erysipelotrichi and Actinobacteria* were enriched in the NAG. The difference in class abundance between the two treatment groups across all the ages was not significant (P < 0.05) for all the classes mentioned above. Bacterial abundance between the age groups were significant, *Bacteroidia* (Kruskal-Wallis χ^2 = 35.031, P = 0.002434), *Clostridia* (Kruskal-Wallis χ^2 = 27.022, P = 0.02855), *Bacilli* (Kruskal-Wallis χ^2 = 36.057, P = 0.001735), *Gammaproteobacteria* (Kruskal-Wallis χ^2 = 39.172, P= 0.000604), *Erysipelotrichi* (Kruskal-Wallis χ^2 = 35.762, P = 0.001913), *Spirochaetes* (Kruskal-Wallis χ^2 = 37.334, P = 0.001131), *Fusobacteriia* (Kruskal-Wallis χ^2 =

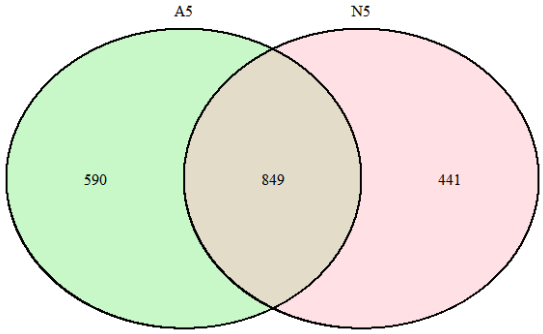
38.91, P = 0.0006609) and *Actinobacteria* (Kruskal-Wallis χ^2 = 35.777, P = 0.001904). At the genus level, a of total 217 distinct genera were identified, with the genus, *Prevotella* (21.3% and 12.7%), *Lactobacillus* (11.6% and 21.1%) and *Bacteroides* (9.1% and 5.8) showing to be the most dominant in the ATG and NAG respectively (Fig 4.2c, Appendix 2) and they were statistically significant between the age groups (Kruskal-Wallis χ^2 =43.944, P = 0.0001122; Kruskal-Wallis χ^2 =42.44 P = 0.0001923; Kruskal-Wallis χ^2 = 46.895, P = 3.828e-05, respectively). This agrees with (Yang *et al.,* 2016) who stated that finer phylogenetic resolution shows *Prevotella* as the most dominant genus in the swine fecal metagenome but contradicts with findings of (Zhu *et al.,* 2011; Leser *et al.,* 2002) which found *Clostridia* as the most abundant genus. The abundance of *Prevotella* in the ATG may be due to resistance strains from antibiotics usage on the farm. While *Prevotella* and *Bacteroides* were enriched in the ATG but only *Prevotella* was statistically significant (Kruskal-Wallis χ^2 = 3.9448, P = 0.04702; Kruskal-Wallis χ^2 =0.85359, P = 0.3555 respectively), the abundance of *Lactobacillus* was not significantly higher in the NAG (Kruskal-Wallis χ^2 = 2.6349, P = 0.1045). The results disagree with findings by (Kim *et al.,* 2012) who found increase in *Lactobacillus* concentration in Tylosin treated pigs. Similar studies related the increase in *Lactobacillus* concentration to weight again in antibiotics growth promoter treated animal models (Lin, 2011).

Differences in within-community dissimilarity of the different age groups was significant (ANOSIM; R=0.8043, P = 0.0001) (Figure 4.3). Differences between community composition of all the age groups in the antibiotics and non antibiotics groups were significant (PERMANOVA; F = 3.3037 P = 0.001). The difference between community composition in the two groups irrespective of age was also significant (PERMANOVA; F = 1.9068, P = 0.008). Pairwise comparisons at each growth stage between the ATG and NAG were significant for days 0- 35 (PERM MANOVA; P = 0.014, P=0.007, P = 0.034, P = 0.012, P = 0.026, P = 0.027 respectively) but not significant for days 56 and 70 (PERM MANOVA; P = 0.133 and P= 0.067 respectively). This showed that bacterial composition between the age groups in the ATG and the NAG are different at the younger stages but as the animal gets older composition becomes similar. This may suggest that the non antibiotics group might have acquired resistance strains probably from the environment since no antibiotics were administered directly. Differences in within-community dispersion for the age groups and for the treatment groups was not significant (ANOVA; F= 1.526, P =0.1327 and F= 0.5578, P = 0.453). This suggests that differences in

bacterial compositions between the age groups and treatment groups may be as a result of less abundant species unique to the different age groups and hence most of the shared species could suggest some resistance strains are present in the NAG. To visualize the bacterial composition and structure between the age groups, a non metric multi dimensional scaling (nMDS) and PCoA ordination plots was used. Days 0 for ATG and NAG were clustered separately, however day 5 to 35 were distributed evenly and only shifting with age, day 56 for both groups was clustered together (Figure 4.4). All this is suggesting that use of antibiotics in pig farms either for prophylaxis or metaphylaxis increases bacterial population in pig gut even in non treated pigs, which probably harbor resistant genes that could be transferred to humans or other animals along the food chain posing an important public health risk.


Antibiotics group
Non antibiotics group
966
2560
798


N0
A0
699
641
436


A5
N5
590
849
441

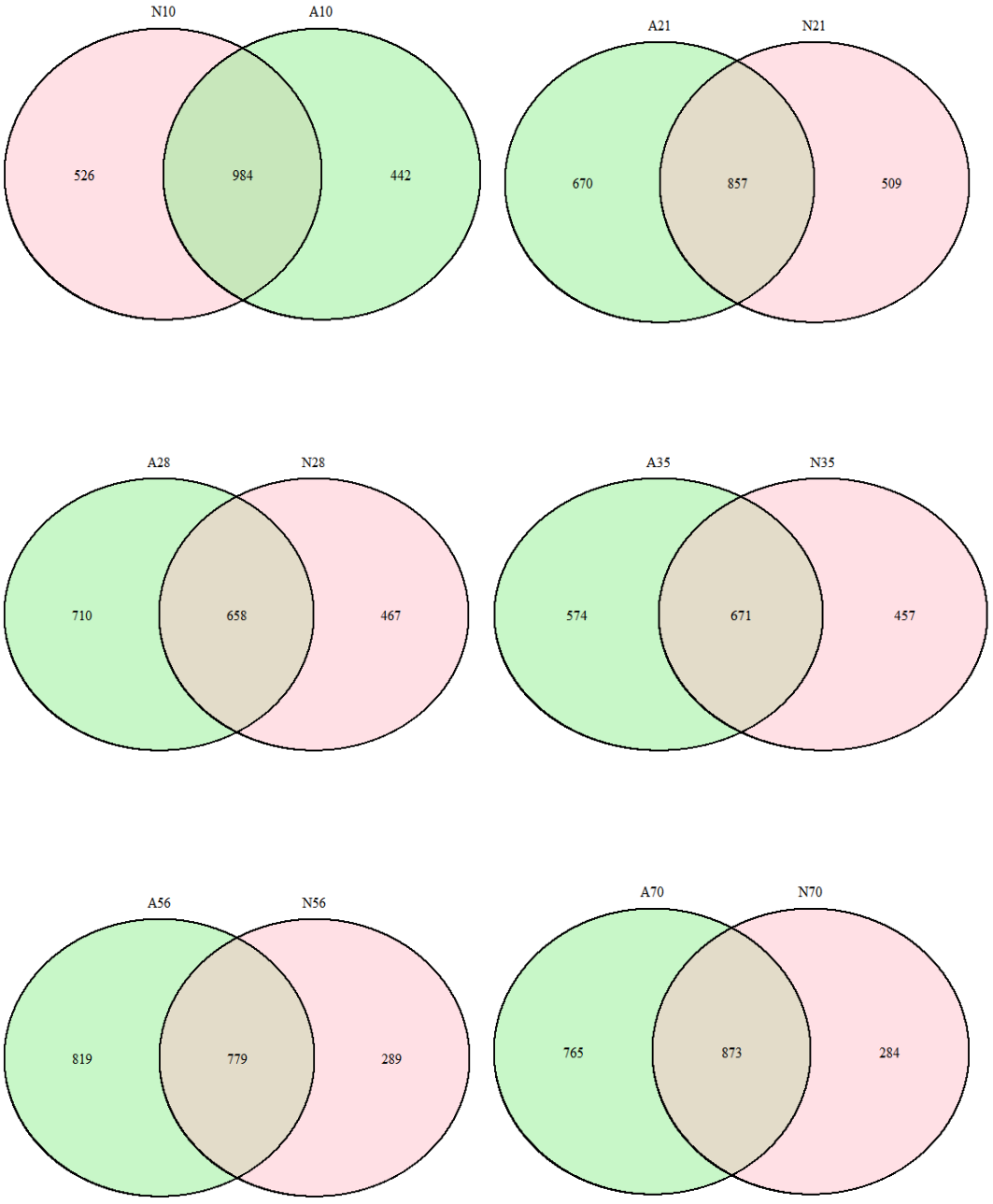


Figure 0.1: Venndiagrams showing bacterial compositional differences between the two groups.

Table 0.1: Diversity measures during the growing period

Diversity Measure	Group	Day 0	Day 5	Day 10	Day 21	Day 28	Day 35	Day 56	Day 70
Alpha	A	699.80	935.80	945.60	1042.60	954.75	870.00	1180.25	1346.25
	N	904.25	810.00	986.80	866.00	889.67	951.00	1089.50	1174.50
Gamma	A	1559.00	1988.00	1942.00	2129.00	1973.00	1744.00	2324.00	2297.00
	N	1802.00	1796.00	2060.00	1885.00	1656.00	1681.00	1558.00	1695.00
Beta	A	1.23	1.12	1.05	1.04	1.07	1.00	0.97	0.71
	N	0.99	1.22	1.09	1.18	0.86	0.77	0.43	0.44

Table 0.2: Mean and standard deviation of alpha diversity indices during the growing period.

age_days	m.richness	sd.richness	m.shannon	sd.shannon	m.invsimpson	sd.invsimpson	m.simpson	sd.simpson	m.pielou	sd.pielou
A0	451.200	37.372	2.767	0.204	7.299	1.886	0.855	0.040	0.453	0.033
N0	637.500	78.928	4.666	0.188	45.612	11.891	0.977	0.007	0.723	0.029
A5	637.000	66.287	4.177	0.223	23.831	8.708	0.954	0.016	0.647	0.028
N5	540.400	50.491	3.791	0.372	19.993	8.754	0.939	0.036	0.602	0.053
A10	642.000	93.582	4.250	0.518	26.026	10.694	0.955	0.022	0.657	0.068
N10	671.000	127.797	4.278	0.486	28.205	12.493	0.958	0.019	0.658	0.056
A21	699.600	76.618	4.259	0.427	25.709	10.215	0.953	0.028	0.651	0.063
N21	596.400	46.409	3.909	0.155	18.243	6.299	0.939	0.025	0.612	0.024
A28	629.500	101.448	3.971	0.614	19.173	13.693	0.930	0.036	0.616	0.082
N28	582.333	89.243	3.797	0.632	16.556	8.603	0.927	0.037	0.596	0.087
A35	589.750	118.874	3.869	0.712	18.240	13.203	0.923	0.047	0.606	0.094
N35	602.667	29.670	3.669	0.704	17.807	12.152	0.885	0.130	0.573	0.107
A56	773.250	42.906	4.212	0.388	17.955	12.273	0.920	0.053	0.633	0.059
N56	728.000	38.184	4.101	0.527	17.761	10.241	0.932	0.039	0.622	0.075
A70	908.750	88.729	4.695	0.421	35.914	18.098	0.957	0.041	0.689	0.056
N70	784.500	79.903	4.386	0.497	26.525	23.235	0.939	0.054	0.658	0.065

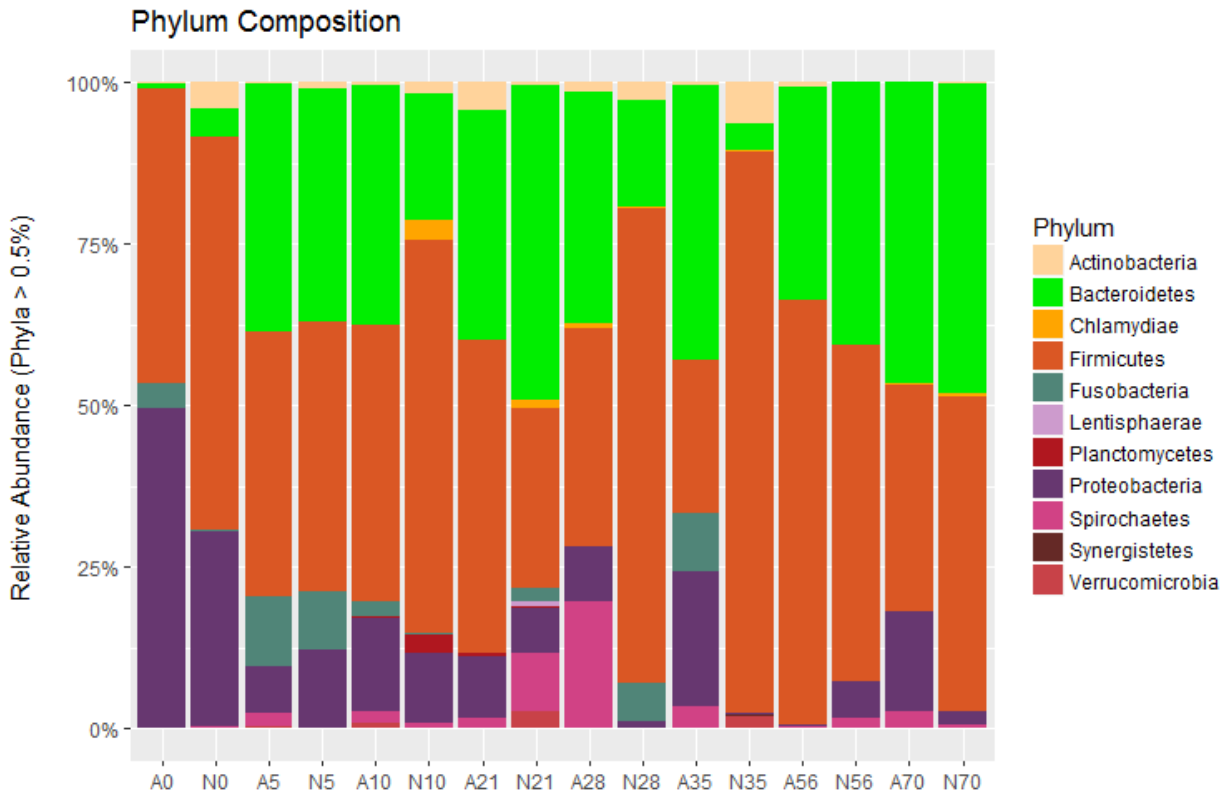


Figure4.2a

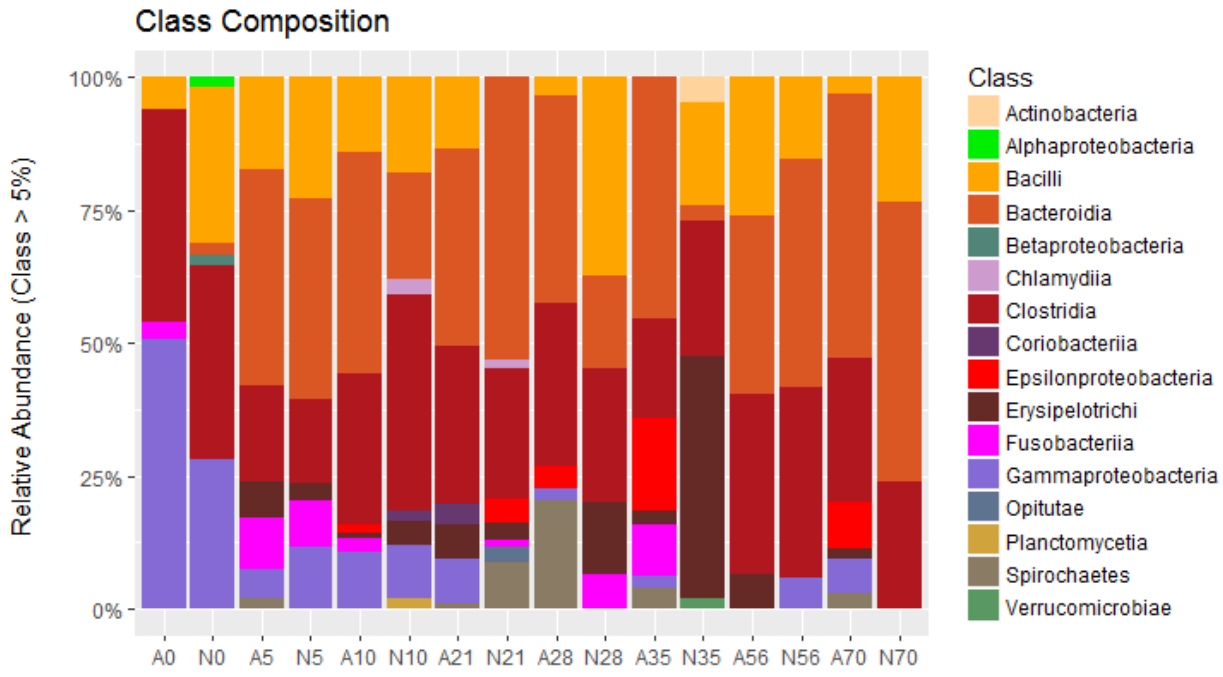


Figure 4.2b

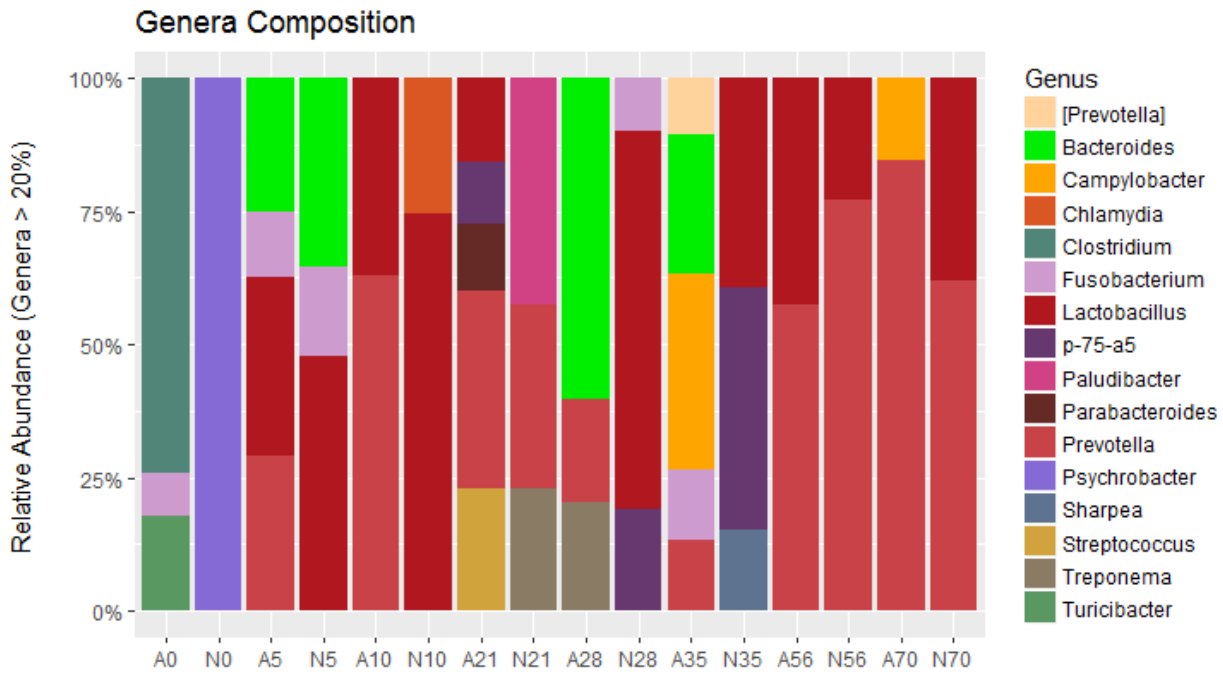


Figure 4.2c

Figure 0.2 a, b and c: Relative abundance of the most abundant phyla, classes and genera.

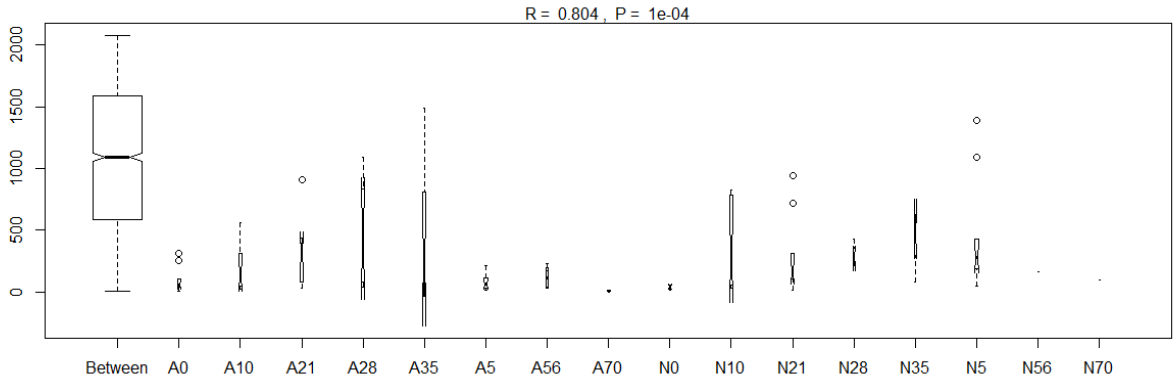


Figure 0.3: Anosim plot, showing the dissimilarities in community composition between the two groups and within the age groups

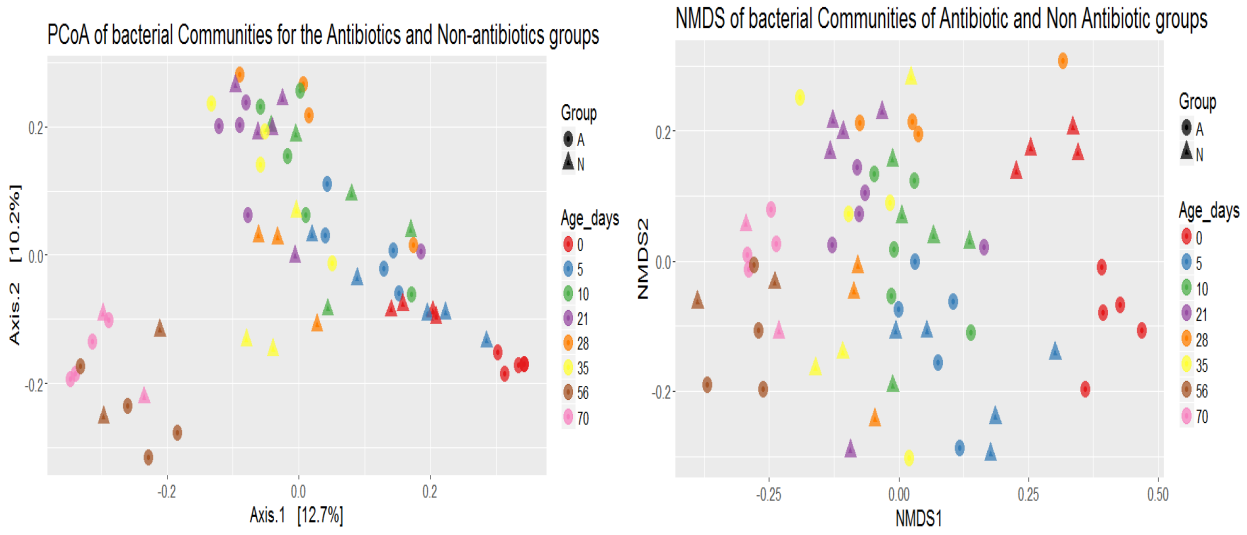


Figure 0.4: Ordination plots for the Antibiotics and Non – antibiotics groups

4.5 References

Alcaide, M., Messina, E., Richter, M., Bargiela, R., Peplies, J., Huws, S.A., Newbold, C.J., Golyshin, P.N., Simón, M.A. and López, G. (2012) 'Gene sets for utilization of primary and secondary nutrition supplies in the distal gut of endangered Iberian lynx', *PLoS One,* 7(12), pp. e51521.

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J. (2010) 'Call of the wild: antibiotic resistance genes in natural environments', *Nature Reviews Microbiology,* 8(4), pp. 251-259.

Allen, H.K., Looft, T., Bayles, D.O., Humphrey, S., Levine, U.Y., Alt, D. and Stanton, T.B. (2011) 'Antibiotics in feed induce prophages in swine fecal microbiomes', *mBio,* 2(6), pp. 10.1128

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. and Gordon, J.I. (2005) 'Host-bacterial mutualism in the human intestine', *Science (New York, N.Y.),* 307(5717), pp. 1915-1920.

Bray, J.R. and Curtis, J.T. (1957) 'An ordination of the upland forest communities of southern Wisconsin', *Ecological Monographs,* 27(4), pp. 325-349.

Brulc, J.M., Antonopoulos, D.A., Miller, M.E., Wilson, M.K., Yannarell, A.C., Dinsdale, E.A., Edwards, R.E., Frank, E.D., Emerson, J.B., Wacklin, P., Coutinho, P.M., Henrissat, B., Nelson, K.E. and White, B.A. (2009) 'Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases', *Proceedings of the National Academy of Sciences of the United States of America,* 106(6), pp. 1948-1953.

Buffie, C.G. and Pamer, E.G. (2013) 'Microbiota-mediated colonization resistance against intestinal pathogens', *Nature Reviews Immunology,* 13(11), pp. 790-801.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K. and Gordon, J.I. (2010) 'QIIME allows analysis of high-throughput community sequencing data', *Nature methods,* 7(5), pp. 335-336.

Coates, M.E., Fuller, R., Harrison, G., Lev, M. and Suffolk, S. (1963) 'A comparision of the growth of chicks in the Gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin', *British journal of nutrition,* 17(01), pp. 141-150.

Cromwell, G.L. (2002) 'Why and how antibiotics are used in swine production', *Animal Biotechnology,* 13(1), pp. 7-27.

Dai, X., Zhu, Y., Luo, Y., Song, L., Liu, D., Liu, L., Chen, F., Wang, M., Li, J. and Zeng, X. (2012) 'Metagenomic insights into the fibrolytic microbiome in yak rumen', *PLoS One,* 7(7), pp. e40430.

Dethlefsen, L., Huse, S., Sogin, M.L. and Relman, D.A. (2008) 'The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing', *PLoS biol,* 6(11), pp. e280.

Dibner, J.J. and Richards, J.D. (2005) 'Antibiotic growth promoters in agriculture: history and mode of action', *Poultry science,* 84(4), pp. 634-643.

Edgar, R.C. (2010) 'Search and clustering orders of magnitude faster than BLAST', *Bioinformatics (Oxford, England),* 26(19), pp. 2460-2461.

Gilliland, S.E. and Speck, M.L. (1977) 'Deconjugation of bile acids by intestinal lactobacilli', *Applied and Environmental Microbiology,* 33(1), pp. 15-18.

Gotz, A., Pukall, R., Smit, E., Tietze, E., Prager, R., Tschape, H., van Elsas, J.D. and Smalla, K. (1996) 'Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR', *Applied and Environmental Microbiology,* 62(7), pp. 2621-2628.

Greenblum, S., Turnbaugh, P.J. and Borenstein, E. (2012) 'Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease', *Proceedings of the National Academy of Sciences of the United States of America,* 109(2), pp. 594-599.

Hess, M., Sczyrba, A., Egan, R., Kim, T.W., Chokhawala, H., Schroth, G., Luo, S., Clark, D.S., Chen, F., Zhang, T., Mackie, R.I., Pennacchio, L.A., Tringe, S.G., Visel, A., Woyke, T., Wang, Z. and Rubin, E.M. (2011) 'Metagenomic discovery of biomass-degrading genes and genomes from cow rumen', *Science (New York, N.Y.),* 331(6016), pp. 463-467.

Ilmberger, N., Güllert, S., Dannenberg, J., Rabausch, U., Torres, J., Wemheuer, B., Alawi, M., Poehlein, A., Chow, J. and Turaev, D. (2014) 'A comparative metagenome survey of the fecal microbiota of a breast-and a plant-fed Asian elephant reveals an unexpectedly high diversity of glycoside hydrolase family enzymes', *PloS one,* 9(9), pp. e106707.

Isaacson, R. and Kim, H.B. (2012) 'The intestinal microbiome of the pig', *Animal Health Research Reviews,* 13(01), pp. 100-109.

Jacobs, J.P. and Braun, J. (2014) 'Immune and genetic gardening of the intestinal microbiome', *FEBS letters,* 588(22), pp. 4102-4111.

Karami, N., Martner, A., Enne, V.I., Swerkersson, S., Adlerberth, I. and Wold, A.E. (2007) 'Transfer of an ampicillin resistance gene between two *Escherichia coli* strains in the bowel microbiota of an infant treated with antibiotics', *The Journal of antimicrobial chemotherapy,* 60(5), pp. 1142-1145.

Kim, H.B., Borewicz, K., White, B.A., Singer, R.S., Sreevatsan, S., Tu, Z.J. and Isaacson, R.E. (2011) 'Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs', *Veterinary microbiology,* 153(1), pp. 124-133.

Kim, H.B., Borewicz, K., White, B.A., Singer, R.S., Sreevatsan, S., Tu, Z.J. and Isaacson, R.E. (2012) 'Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin', *Proceedings of the National Academy of Sciences of the United States of America,* 109(38), pp. 15485-15490.

Kurokawa, K., Itoh, T., Kuwahara, T., Oshima, K., Toh, H., Toyoda, A., Takami, H., Morita, H., Sharma, V.K., Srivastava, T.P., Taylor, T.D., Noguchi, H., Mori, H., Ogura, Y., Ehrlich, D.S., Itoh, K., Takagi, T., Sakaki, Y., Hayashi, T. and Hattori, M. (2007) 'Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes', *DNA research : an international journal for rapid publication of reports on genes and genomes,* 14(4), pp. 169-181.

Lamendella, R., Santo Domingo, J.W., Ghosh, S., Martinson, J. and Oerther, D.B. (2011) 'Comparative fecal metagenomics unveils unique functional capacity of the swine gut', *BMC microbiology,* 11(1), pp. 103.

Legendre, P. and Gallagher, E.D. (2001) 'Ecologically meaningful transformations for ordination of species data', *Oecologia,* 129(2), pp. 271-280.

Leser, T.D., Amenuvor, J.Z., Jensen, T.K., Lindecrona, R.H., Boye, M. and Moller, K. (2002) 'Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited', *Applied and Environmental Microbiology,* 68(2), pp. 673-690.

Ley, R.E., Peterson, D.A. and Gordon, J.I. (2006) 'Ecological and evolutionary forces shaping microbial diversity in the human intestine', *Cell,* 124(4), pp. 837-848.

Ley, R.E., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R., Bircher, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R. and Gordon, J.I. (2008) 'Evolution of mammals and their gut microbes', *Science (New York, N.Y.),* 320(5883), pp. 1647-1651.

Lin, J. (2011) 'Effect of antibiotic growth promoters on intestinal microbiot a in food animals: a novel model for studying the relationship between gut microbiota and human obesity?', *Human health and disease in a microbial world,* , pp. 57.

Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., Sul, W.J., Stedtfeld, T.M., Chai, B., Cole, J.R., Hashsham, S.A., Tiedje, J.M. and Stanton, T.B. (2012) 'In-feed antibiotic effects on the swine intestinal microbiome', *Proceedings of the National Academy of Sciences of the United States of America,* 109(5), pp. 1691-1696.

Lunney, J.K. (2007) 'Advances in swine biomedical model genomics', *International journal of biological sciences,* 3(3), pp. 179-184.

Martinez, J.L., Fajardo, A., Garmendia, L., Hernandez, A., Linares, J.F., Martinez-Solano, L. and Sanchez, M.B. (2009) 'A global view of antibiotic resistance', *FEMS microbiology reviews,* 33(1), pp. 44-65.

Oloo, F., Valverde, A., Quiroga, M.V., Vikram, S., Cowan, D. and Mataloni, G. (2016) 'Habitat heterogeneity and connectivity shape microbial communities in South American peatlands', *Scientific reports,* 6, pp. 25712.

Pope, P.B., Mackenzie, A.K., Gregor, I., Smith, W., Sundset, M.A., McHardy, A.C., Morrison, M. and Eijsink, V.G. (2012) 'Metagenomics of the Svalbard reindeer rumen microbiome reveals abundance of polysaccharide utilization loci', *PLoS One,* 7(6), pp. e38571.

Pope, P.B., Denman, S.E., Jones, M., Tringe, S.G., Barry, K., Malfatti, S.A., McHardy, A.C., Cheng, J.F., Hugenholtz, P., McSweeney, C.S. and Morrison, M. (2010) 'Adaptation to herbivory by the Tammar wallaby includes bacterial and glycoside hydrolase profiles different from other herbivores', *Proceedings of the National Academy of Sciences of the United States of America,* 107(33), pp. 14793-14798.

Ramotar, K., Conly, J.M., Chubb, H. and Louie, T.J. (1984) 'Production of menaquinones by intestinal anaerobes', *The Journal of infectious diseases,* 150(2), pp. 213-218.

Rettedal, E., Vilain, S., Lindblom, S., Lehnert, K., Scofield, C., George, S., Clay, S., Kaushik, R.S., Rosa, A.J., Francis, D. and Brozel, V.S. (2009) 'Alteration of the ileal microbiota of weanling piglets by the growth-promoting antibiotic chlortetracycline', *Applied and Environmental Microbiology,* 75(17), pp. 5489-5495.

Salyers, A.A. and Amabile-Cuevas, C.F. (1997) 'Why are antibiotic resistance genes so resistant to elimination?', *Antimicrobial Agents and Chemotherapy,* 41(11), pp. 2321-2325.

Shimada, K., Bricknell, K.S. and Finegold, S.M. (1969) 'Deconjugation of bile acids by intestinal bacteria: review of literature and additional studies', *The Journal of infectious diseases,* , pp. 273-281.

Shoemaker, N.B., Vlamakis, H., Hayes, K. and Salyers, A.A. (2001) 'Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon', *Applied and Environmental Microbiology,* 67(2), pp. 561-568.

Singh, K.M., Ahir, V.B., Tripathi, A.K., Ramani, U.V., Sajnani, M., Koringa, P.G., Jakhesara, S., Pandya, P.R., Rank, D.N. and Murty, D.S. (2012) 'Metagenomic analysis of Surti buffalo (Bubalus bubalis) rumen: a preliminary study', *Molecular biology reports,* 39(4), pp. 4841-4848.

Sommer, M.O., Dantas, G. and Church, G.M. (2009) 'Functional characterization of the antibiotic resistance reservoir in the human microflora', *Science (New York, N.Y.),* 325(5944), pp. 1128-1131.

Stanton, T.B. and Humphrey, S.B. (2011) 'Persistence of antibiotic resistance: evaluation of a probiotic approach using antibiotic-sensitive Megasphaera elsdenii strains to prevent colonization of swine by antibiotic-resistant strains', *Applied and Environmental Microbiology,* 77(20), pp. 7158-7166.

Stanton, T.B., Humphrey, S.B. and Stoffregen, W.C. (2011) 'Chlortetracycline-resistant intestinal bacteria in organically raised and feral Swine', *Applied and Environmental Microbiology,* 77(20), pp. 7167-7170.

Turnbaugh, P.J. and Gordon, J.I. (2008) 'An invitation to the marriage of metagenomics and metabolomics', *Cell,* 134(5), pp. 708-713.

Varel, V. (1987) 'Activity of fiber-degrading microorganisms in the pig large intestine', *Journal of animal science,* 65(2), pp. 488-496.

Warnecke, F., Luginbühl, P., Ivanova, N., Ghassemian, M., Richardson, T.H., Stege, J.T., Cayouette, M., McHardy, A.C., Djordjevic, G. and Aboushadi, N. (2007) 'Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite', *Nature,* 450(7169), pp. 560-565.

Xiao, L., Estellé, J., Kiilerich, P., Ramayo-Caldas, Y., Xia, Z., Feng, Q., Liang, S., Pedersen, A.Ø., Kjeldsen, N.J. and Liu, C. (2016) 'A reference gene catalogue of the pig gut microbiome', *Nature Microbiology,* 1, pp. 16161.

Yang, S., Ma, S., Chen, J., Mao, H., He, Y., Xi, D., Yang, L., He, T. and Deng, W. (2010) 'Bacterial diversity in the rumen of Gayals (Bos frontalis), Swamp buffaloes (Bubalus bubalis) and Holstein cow as revealed by cloned 16S rRNA gene sequences', *Molecular biology reports,* 37(4), pp. 2063-2073.

Yang, H., Huang, X., Fang, S., Xin, W., Huang, L. and Chen, C. (2016) 'Uncovering the composition of microbial community structure and metagenomics among three gut locations in pigs with distinct fatness', *Scientific reports,* 6, pp. 27427.

Yolton, D.P. and Savage, D.C. (1976) 'Influence of certain indigenous gastrointestinal microorganisms on duodenal alkaline phosphatase in mice', *Applied and Environmental Microbiology,* 31(6), pp. 880-888.

Zhu, L., Wu, Q., Dai, J., Zhang, S. and Wei, F. (2011) 'Evidence of cellulose metabolism by the giant panda gut microbiome', *Proceedings of the National Academy of Sciences of the United States of America,* 108(43), pp. 17714-17719.

Zoetendal, E.G., Collier, C.T., Koike, S., Mackie, R.I. and Gaskins, H.R. (2004) 'Molecular ecological analysis of the gastrointestinal microbiota: a review', *The Journal of nutrition,* 134(2), pp. 465-472.

Chapter Five

5.1 General discussion conclusion and recommendation.

A review on the prevalence of bacterial infections of pigs with particular reference to *Escherichia coli*; a bacterium that is regularly isolated with multiple infections in pigs showed that bacteria that affect pigs are diverse and vast. Detection of virulence factors in piglets (Suckling) and weaners from several countries around the world were closely related (57.93% and 57.9%). However, in porkers the overall prevalence of *E. coli* virulence factor detection was 36.45%. This shows that presence of *E.coli* virulence factors that may eventually result in disease is more prevalent in the young pigs, hence better control strategies should be targeted on piglets and weaners as low levels of pathogenic *E.coli* in the young pigs will translate to negligible presence of Pathogenic *E.coli* in the porkers which will ensure healthier, pathogen free pigs that will eventually get to the potential consumers.

The total of 241 *E. coli* strains isolated in the growing pigs from both groups between days 0 and 70 showed high presence of virulence genes: antibiotic group (24.8%) and non antibiotic group (43.5%). The study showed that usage/non usage of antibiotics in growing pigs does not prevent occurrence of disease causing virulence genes and other factors may be involved.

Resistance to oxytetracycline was most common. Amoxicillin and trimethoprim resistance were also high on most of the sampled days. Eight multidrug resistant phenotypes were observed. Tetracycline resistance genes were observed in 63.9% of isolates. *TetA and tetB* was the most commonly observed. Oxytetracycline, Amoxicillin and Trimethoprim have the highest level of resistance. Tetracycline resistance genes in pigs can be found at any point during the growth period with or without antibiotic usage.

Metagenomics analysis of fecal samples collected over the 70 days study period revealed differences between community composition and structure in the two groups irrespective of age. Pairwise comparisons at each growth stage between the antibiotics group and non-antibiotics were significant for days 0- 35 but not significant for days 56 and 70. Both groups were dominated by the phylum; *Firmicutes* (41.99% and 54.04%), *Bacteriotedes* (32.88% and 26.75%) and *Proteobacteria* (14.13% and 9.94%) in the antibiotics and non-antibiotic groups respectively. The most abundant classes were *Bacteroidia* (32.9% and 26.6%), *Clostridia* (26.4% and 26.2%), *Bacilli* (11.2% and 18.8%), *Gammaproteobacteria* (11.6% and 8.1%) for the antibiotics and non-antibiotics groups respectively. The most abundant genera were *Prevotella* (21.3% and 12.7%), *Lactobacillus* (11.6% and 21.1%) and *Bacteroides* (9.1% and 5.8) in the

antibiotics and non antibiotics groups respectively and were enriched in the antibiotics treated bacterial community but not significant. This has shown that antibiotics usage increases gut bacterial population in growing pigs when used for a period of time and non treated pigs could acquire resistance strains as they age which could be due to environmental factors.

In conclusion therefore, the study has shown that bacteria pathogens of pigs is more prevalent in young pigs in different parts of the world with South Africa inclusive, *E.coli* virulence genes are distributed through out the growing period and antibiotics resistance are present in pig that did not receive antibiotics, hence better control measures that will reduce use of antibiotics is required in the pig production industry.

I recommend further studies to include several farms and in different regions of South Africa to determine the prevalence of antibiotic resistance in non treated pigs also more research is needed in the area of bacteria vaccines for pigs.

Appendix 1

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Development, quantification and assessment of dynamics of enteric bacterial population in growing pigs**
PROJECT NUMBER	**V029-16**
RESEARCHER/PRINCIPAL INVESTIGATCR	**Dr RH Abubakar**

STUDENT NUMBER (where applicable)	**UP_14458242**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Porcine**	
NUMBER OF ANIMALS	**60**	
Approval period to use animals for research/testing purposes		**May 2016 – May 2017**
SUPERVISOR	**Prof. FO Fasina**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 30 May 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

V029-16

Appendix 2

Average relative abundance and standard deviation for Phyllum, Class and genus levels

S/No	Phylum	Group	Avg	sd	Class	Group	Avg	sd	Genus	Group	Avg	sd
1	Firmicutes	N	54.05	21.891	Bacteroidia	A	32.88	22.332	Prevotella	A	21.31	18.967
2	Firmicutes	A	42.00	18.518	Bacteroidia	N	26.63	20.095	Lactobacillus	N	21.12	21.863
3	Bacteroidetes	A	32.88	22.327	Clostridia	A	26.38	12.099	Prevotella	N	12.70	17.803
4	Bacteroidetes	N	26.75	19.969	Clostridia	N	26.22	11.899	Lactobacillus	A	11.62	14.197
5	Proteobacteria	A	16.13	20.399	Bacilli	N	18.80	16.160	Bacteroides	A	9.12	13.071
6	Proteobacteria	N	9.94	12.222	Gammaproteobacteria	A	11.64	18.819	p-75-a5	N	6.51	14.424
7	Spirochaetes	A	3.75	8.358	Bacilli	A	11.15	11.498	Bacteroides	N	5.77	10.627
8	Fusobacteria	A	3.45	6.369	Erysipelotrichi	N	9.03	13.413	Fusobacterium	A	4.67	7.788
9	Fusobacteria	N	2.73	6.222	Gammaproteobacteria	N	8.15	11.410	Campylobacter	A	4.39	10.251
10	Actinobacteria	N	2.19	3.407	Erysipelotrichi	A	4.47	3.588	Psychrobacter	N	3.99	8.167
11	Spirochaetes	N	1.85	4.455	Epsilonproteobacteria	A	3.96	9.165	Parabacteroides	A	3.93	5.905
12	Actinobacteria	A	1.23	2.992	Spirochaetes	A	3.74	8.362	Paludibacter	N	3.91	12.830
13	Chlamydiae	N	0.83	2.885	Fusobacteriia	A	3.45	6.369	Clostridium	A	3.41	11.137
14	Verrucomicrobia	N	0.76	2.489	Fusobacteriia	N	2.73	6.222	Fusobacterium	N	3.37	7.166
15	Planctomycetes	N	0.59	1.714	Spirochaetes	N	1.85	4.455	p-75-a5	A	3.03	4.184
16	Verrucomicrobia	A	0.21	0.617	Actinobacteria	N	1.15	2.695	[Prevotella]	A	2.75	4.259
17	Lentisphaerae	N	0.16	0.671	Coriobacteriia	N	1.04	1.887	Treponema	N	2.40	7.410
18	Planctomycetes	A	0.15	0.249	Epsilonproteobacteria	N	1.03	2.555	Treponema	A	2.33	6.176
19	Chlamydiae	A	0.14	0.307	Coriobacteriia	A	0.91	2.947	Streptococcus	A	2.22	7.173
20	Synergistetes	N	0.13	0.275	Chlamydiia	N	0.83	2.885	Sphaerochaeta	A	2.08	4.500
21	Synergistetes	A	0.02	0.036	Planctomycetia	N	0.59	1.714	Parabacteroides	N	1.99	2.740
22	Fibrobacteres	A	0.01	0.051	Betaproteobacteria	A	0.46	0.985	[Prevotella]	N	1.87	3.585
23	Deferribacteres	A	0.01	0.037	Opitutae	N	0.45	2.340	[Eubacterium]	N	1.76	3.177
24	Lentisphaerae	A	0.01	0.018	Betaproteobacteria	N	0.44	1.248	Oscillospira	N	1.75	2.771
25	[Thermi]	N	0.01	0.045	Actinobacteria	A	0.32	0.715	Turicibacter	A	1.69	8.584

26	Elusimicrobia	N	0.01	0.029	Verrucomicrobiae	N	0.27	0.988	Facklamia	N	1.64	3.610
27	Elusimicrobia	A	0.01	0.014	Alphaproteobacteria	N	0.27	1.207	[Ruminococcus]	N	1.64	1.792
28	Deferribacteres	N	0.00	0.006	[Lentisphaeria]	N	0.16	0.671	Sharpea	N	1.56	4.577
29	TM7	N	0.00	0.004	Planctomycetia	A	0.15	0.249	Chlamydia	N	1.52	5.975
30	[Thermi]	A	0.00	0.007	Opitutae	A	0.14	0.592	[Eubacterium]	A	1.50	2.403
31	TM7	A	0.00	0.003	Chlamydiia	A	0.14	0.307	Ruminococcus	A	1.49	2.785
32	Fibrobacteres	N	0.00	0.002	Synergistia	N	0.13	0.275	Oscillospira	A	1.47	1.682
33	Tenericutes	A	0.00	0.002	Flavobacteriia	N	0.08	0.295	Catenibacterium	N	1.34	3.994
34	Tenericutes	N	0.00	0.000	Deltaproteobacteria	A	0.07	0.085	[Ruminococcus]	A	1.33	1.545
35					Deltaproteobacteria	N	0.06	0.116	SMB53	N	1.27	2.336
36					Verrucomicrobiae	A	0.05	0.204	Megasphaera	A	1.27	2.471
37					Verruco-5	N	0.04	0.156	Streptococcus	N	1.12	1.414
38					Sphingobacteriia	N	0.03	0.080	Campylobacter	N	1.11	2.871
39					Synergistia	A	0.02	0.036	Enterococcus	N	1.03	3.917
40					Fibrobacteria	A	0.01	0.051	Aerococcus	N	0.98	2.527
41					Deferribacteres	A	0.01	0.037	Blautia	A	0.95	1.433
42					[Lentisphaeria]	A	0.01	0.018	Sphaerochaeta	N	0.92	1.764
43					Verruco-5	A	0.01	0.020	Succinivibrio	A	0.88	3.331
44					[Brachyspirae]	A	0.01	0.055	Phascolarctobacterium	N	0.88	1.193
45					Cytophagia	N	0.01	0.049	Enterococcus	A	0.86	2.460
46					Deinococci	N	0.01	0.045	CF231	N	0.84	2.051
47					Elusimicrobia	N	0.01	0.029	Sutterella	A	0.84	1.864
48					Alphaproteobacteria	A	0.01	0.037	Corynebacterium	N	0.82	1.886
49					[Saprospirae]	N	0.01	0.029	RFN20	N	0.80	2.890
50					Flavobacteriia	A	0.01	0.021	Ruminococcus	N	0.79	1.148
51					Elusimicrobia	A	0.01	0.014	Psychrobacter	A	0.75	1.771
52					Deferribacteres	N	0.00	0.006	Paludibacter	A	0.74	1.644
53					TM7-3	N	0.00	0.004	SMB53	A	0.73	0.922
54					Deinococci	A	0.00	0.007	Turicibacter	N	0.70	1.632
55					TM7-3	A	0.00	0.003	Clostridium	N	0.68	1.105

56					Thermoleophilia	A	0.00	0.005	CF231	A	0.67	1.125
57					Fibrobacteria	N	0.00	0.002	Blautia	N	0.65	0.913
58					Mollicutes	A	0.00	0.002	Dorea	N	0.63	0.688
59					[Spartobacteria]	A	0.00	0.002	Rahnella	N	0.62	3.173
60					[Saprospirae]	A	0.00	0.001	Bifidobacterium	N	0.57	2.790
61					Cytophagia	A	0.00	0.001	Helicobacter	A	0.51	1.517
62					[Brachyspirae]	N	0.00	0.000	Phascolarctobacterium	A	0.50	0.506
63					[Spartobacteria]	N	0.00	0.000	Roseburia	A	0.49	1.109
64					Mollicutes	N	0.00	0.000	Peptostreptococcus	N	0.47	1.146
65					Sphingobacteriia	A	0.00	0.000	Pseudoramibacter_Eubacterium	A	0.45	1.252
66					Thermoleophilia	N	0.00	0.000	Anaerococcus	N	0.45	1.036
67									Sharpea	A	0.41	1.078
68									Collinsella	N	0.40	0.655
69									Dorea	A	0.40	0.557
70									Actinobacillus	A	0.40	0.605
71									Butyricimonas	N	0.39	0.993
72									Akkermansia	N	0.37	1.239
73									Flexispira	A	0.37	1.079
74									Faecalibacterium	A	0.37	0.583
75									Aerococcus	A	0.37	1.338
76									Shuttleworthia	N	0.36	1.790
77									Sarcina	A	0.36	1.967
78									Corynebacterium	A	0.35	0.795
79									Mogibacterium	N	0.35	0.968
80									Faecalibacterium	N	0.34	0.968
81									Porphyromonas	N	0.34	0.858
82									Helcococcus	N	0.34	0.932
83									Roseomonas	N	0.33	1.791
84									Catenibacterium	A	0.33	0.734
85									Megasphaera	N	0.33	0.706

86									Collinsella	A	0.31	0.524
87									Coprococcus	A	0.30	0.452
88									Epulopiscium	A	0.30	1.225
89									Bulleidia	A	0.29	0.531
90									Mitsuokella	A	0.29	1.344
91									Facklamia	A	0.28	0.670
92									Anaerobiospirillum	A	0.27	1.431
93									Anaerococcus	A	0.27	0.917
94									Bulleidia	N	0.26	0.482
95									Anaerovibrio	N	0.25	0.685
96									Pseudoramibacter_Eubacterium	N	0.24	0.470
97									Jeotgalicoccus	N	0.22	0.604
98									Chlamydia	A	0.21	0.421
99									Achromobacter	N	0.21	0.795
100									RFN20	A	0.20	0.345
101									Acidaminococcus	A	0.20	0.665
102									Shuttleworthia	A	0.19	0.441
103									Roseburia	N	0.18	0.456
104									Coprococcus	N	0.17	0.262
105									Helicobacter	N	0.17	0.388
106									GW-34	N	0.17	0.440
107									Mitsuokella	N	0.16	0.632
108									Victivallis	N	0.16	0.817
109									Flexispira	N	0.16	0.555
110									Pseudomonas	N	0.15	0.505
111									Odoribacter	A	0.15	0.532
112									Acinetobacter	N	0.14	0.335
113									Butyricimonas	A	0.14	0.318
114									Dialister	A	0.14	0.316
115									Peptostreptococcus	A	0.14	0.209

116									Mogibacterium	A	0.13	0.501
117									Actinomyces	N	0.13	0.503
118									Acidaminococcus	N	0.13	0.416
119									Erwinia	N	0.12	0.656
120									Actinobacillus	N	0.12	0.235
121									Peptococcus	N	0.12	0.164
122									Peptoniphilus	N	0.11	0.251
123									Veillonella	N	0.11	0.216
124									Sutterella	N	0.11	0.172
125									Proteiniclasticum	N	0.10	0.304
126									Desulfovibrio	A	0.10	0.121
127									Veillonella	A	0.09	0.136
128									Desulfovibrio	N	0.09	0.152
129									Weissella	N	0.09	0.247
130									L7A_E11	N	0.09	0.185
131									Succinivibrio	N	0.08	0.191
132									vadinCA02	N	0.08	0.186
133									Anaerovibrio	A	0.08	0.123
134									Peptococcus	A	0.07	0.105
135									Wautersiella	N	0.07	0.195
136									Staphylococcus	N	0.07	0.221
137									Burkholderia	N	0.07	0.359
138									Bifidobacterium	A	0.07	0.108
139									Akkermansia	A	0.07	0.255
140									L7A_E11	A	0.06	0.086
141									Trichococcus	N	0.06	0.147
142									Synergistes	N	0.06	0.263
143									Actinomyces	A	0.06	0.191
144									Peptoniphilus	A	0.06	0.152
145									Jeotgalicoccus	A	0.06	0.175

146									Odoribacter	N	0.05	0.141
147									Flavobacterium	N	0.05	0.270
148									YRC22	N	0.05	0.104
149									Pasteurella	A	0.05	0.134
150									Ochrobactrum	N	0.05	0.250
151									Sphingobacterium	N	0.05	0.130
152									Butyrivibrio	A	0.04	0.098
153									#####	N	0.04	0.122
154									Delftia	N	0.04	0.119
155									Asteroleplasma	A	0.04	0.083
156									Parvimonas	A	0.03	0.098
157									Pyramidobacter	N	0.03	0.113
158									Butyrivibrio	N	0.03	0.107
159									Parvimonas	N	0.03	0.118
160									ph2	N	0.03	0.117
161									Weissella	A	0.03	0.117
162									Anaerobiospirillum	N	0.03	0.027
163									Finegoldia	N	0.03	0.081
164									Yaniella	N	0.03	0.129
165									Epulopiscium	N	0.03	0.052
166									Succiniclasticum	N	0.03	0.070
167									vadinCA02	A	0.03	0.052
168									Sarcina	N	0.02	0.045
169									Dialister	N	0.02	0.071
170									Pasteurella	N	0.02	0.052
171									#####	A	0.02	0.071
172									Staphylococcus	A	0.02	0.068
173									Propionibacterium	N	0.02	0.094
174									Bilophila	N	0.02	0.078
175									YRC22	A	0.02	0.036

176									Moraxella	N	0.02	0.043
177									Gallicola	N	0.02	0.062
178									Pseudomonas	A	0.02	0.069
179									Dehalobacterium	N	0.02	0.039
180									Mucispirillum	A	0.02	0.047
181									Rahnella	A	0.02	0.036
182									Acinetobacter	A	0.02	0.054
183									Fibrobacter	A	0.02	0.058
184									Alkalibacterium	N	0.02	0.057
185									Anoxybacillus	N	0.02	0.069
186									Lachnospira	A	0.02	0.045
187									Brevibacterium	N	0.02	0.049
188									Propionibacterium	A	0.02	0.072
189									Sporosarcina	N	0.02	0.044
190									Finegoldia	A	0.02	0.034
191									Trueperella	A	0.01	0.042
192									Stenotrophomonas	N	0.01	0.076
193									Bacillus	N	0.01	0.047
194									rc4-4	A	0.01	0.039
195									Trichococcus	A	0.01	0.051
196									Janthinobacterium	N	0.01	0.073
197									Brevibacterium	A	0.01	0.056
198									Anaerofustis	N	0.01	0.034
199									Carnobacterium	N	0.01	0.058
200									Porphyromonas	A	0.01	0.039
201									Enterobacter	N	0.01	0.047
202									Brachyspira	A	0.01	0.064
203									02d06	N	0.01	0.053
204									GW-34	A	0.01	0.019
205									Succiniclasticum	A	0.01	0.032

206									Lachnobacterium	A	0.01	0.028
207									Slackia	N	0.01	0.024
208									Bacillus	A	0.01	0.044
209									Deinococcus	N	0.01	0.050
210									Helcococcus	A	0.01	0.017
211									rc4-4	N	0.01	0.034
212									Erwinia	A	0.01	0.035
213									Gallicola	A	0.01	0.030
214									Asteroleplasma	N	0.01	0.017
215									Trueperella	N	0.01	0.020
216									Selenomonas	A	0.01	0.040
217									Microbacterium	N	0.01	0.031
218									Anaerostipes	A	0.01	0.020
219									Pseudochrobactrum	N	0.01	0.031
220									Dehalobacterium	A	0.01	0.014
221									Lachnospira	N	0.01	0.023
222									Sporosarcina	A	0.01	0.035
223									Slackia	A	0.01	0.018
224									Elusimicrobium	N	0.01	0.032
225									Segetibacter	N	0.01	0.039
226									Micrococcus	N	0.01	0.026
227									Atopobium	N	0.01	0.025
228									Enhydrobacter	N	0.01	0.023
229									Brachybacterium	A	0.01	0.024
230									Leuconostoc	N	0.01	0.021
231									Aequorivita	N	0.01	0.031
232									Moraxella	A	0.01	0.019
233									Luteococcus	N	0.01	0.029
234									Mobiluncus	N	0.01	0.020
235									Alloiococcus	N	0.01	0.023

236									Butyricicoccus	A	0.00	0.024
237									Natronobacillus	N	0.00	0.026
238									Bilophila	A	0.00	0.018
239									Atopobium	A	0.00	0.024
240									Proteiniclasticum	A	0.00	0.011
241									Oxalobacter	N	0.00	0.012
242									WAL_1855D	N	0.00	0.015
243									Mucispirillum	N	0.00	0.010
244									Leucobacter	N	0.00	0.013
245									Varibaculum	A	0.00	0.016
246									Victivallis	A	0.00	0.007
247									Arcobacter	N	0.00	0.020
248									Agrobacterium	A	0.00	0.018
249									Moryella	N	0.00	0.019
250									Macrococcus	N	0.00	0.019
251									Thermus	N	0.00	0.017
252									Alkalibacterium	A	0.00	0.016
253									Comamonas	A	0.00	0.012
254									Vagococcus	A	0.00	0.011
255									Shigella	A	0.00	0.016
256									Ornithobacterium	N	0.00	0.011
257									Klebsiella	A	0.00	0.009
258									Brachybacterium	N	0.00	0.010
259									Coprobacillus	A	0.00	0.007
260									Yaniella	A	0.00	0.011
261									Pediococcus	N	0.00	0.012
262									Deinococcus	A	0.00	0.011
263									Sphingobium	A	0.00	0.014
264									Streptomyces	A	0.00	0.008
265									Delftia	A	0.00	0.012

266									Lactococcus	N	0.00	0.010
267									Rothia	N	0.00	0.006
268									Kocuria	N	0.00	0.010
269									Kocuria	A	0.00	0.011
270									Desemzia	N	0.00	0.007
271									Thermoactinomyces	A	0.00	0.006
272									Erysipelothrix	A	0.00	0.009
273									Aggregatibacter	A	0.00	0.008
274									Geobacillus	N	0.00	0.007
275									Vagococcus	N	0.00	0.006
276									Agrococcus	N	0.00	0.008
277									Synergistes	A	0.00	0.004
278									Cohnella	N	0.00	0.009
279									Elusimicrobium	A	0.00	0.007
280									Achromobacter	A	0.00	0.006
281									Shigella	N	0.00	0.006
282									Anaerofustis	A	0.00	0.005
283									Coprobacillus	N	0.00	0.004
284									Agrobacterium	N	0.00	0.004
285									Methylobacterium	A	0.00	0.009
286									Stenotrophomonas	A	0.00	0.006
287									Halomonas	N	0.00	0.007
288									Arcanobacterium	N	0.00	0.005
289									02d06	A	0.00	0.005
290									Ellin506	A	0.00	0.008
291									Wautersiella	A	0.00	0.005
292									Burkholderia	A	0.00	0.008
293									Streptomyces	N	0.00	0.006
294									Williamsia	A	0.00	0.007
295									Rothia	A	0.00	0.003

296									Saccharomonospora	A	0.00	0.004
297									Flavobacterium	A	0.00	0.003
298									Fibrobacter	N	0.00	0.004
299									Enterobacter	A	0.00	0.006
300									Anaerostipes	N	0.00	0.005
301									Pyramidobacter	A	0.00	0.003
302									Leucobacter	A	0.00	0.004
303									Geobacillus	A	0.00	0.005
304									Novosphingobium	A	0.00	0.005
305									Tessaracoccus	A	0.00	0.005
306									Arthrobacter	N	0.00	0.004
307									Tissierella_Soehngenia	A	0.00	0.004
308									Cloacibacterium	A	0.00	0.005
309									Ignatzschineria	N	0.00	0.003
310									Selenomonas	N	0.00	0.003
311									Enhydrobacter	A	0.00	0.003
312									Myroides	A	0.00	0.004
313									Micrococcus	A	0.00	0.003
314									Roseomonas	A	0.00	0.003
315									Microbacterium	A	0.00	0.003
316									Ochrobactrum	A	0.00	0.003
317									Actinomycetospora	A	0.00	0.004
318									Devosia	A	0.00	0.004
319									Mycobacterium	A	0.00	0.004
320									Buchnera	N	0.00	0.004
321									Agromyces	A	0.00	0.004
322									Ornithobacterium	A	0.00	0.003
323									Janibacter	N	0.00	0.004
324									Sinomonas	N	0.00	0.004
325									ph2	A	0.00	0.002

326									Guggenheimella	A	0.00	0.004
327									Proteus	A	0.00	0.003
328									Pseudoclavibacter	N	0.00	0.003
329									Aggregatibacter	N	0.00	0.002
330									Desemzia	A	0.00	0.002
331									Klebsiella	N	0.00	0.003
332									Cloacibacterium	N	0.00	0.002
333									Butyricicoccus	N	0.00	0.003
334									Lachnobacterium	N	0.00	0.002
335									Sphingobium	N	0.00	0.002
336									Natronobacillus	A	0.00	0.003
337									Arcanobacterium	A	0.00	0.002
338									Limnohabitans	A	0.00	0.003
339									Mycoplasma	A	0.00	0.002
340									Salmonella	N	0.00	0.002
341									Curtobacterium	A	0.00	0.003
342									Gemella	A	0.00	0.002
343									Oligella	A	0.00	0.002
344									Ruminobacter	N	0.00	0.002
345									Kaistobacter	A	0.00	0.002
346									WAL_1855D	A	0.00	0.002
347									Saccharomonospora	N	0.00	0.002
348									Rickettsiella	N	0.00	0.002
349									Arthrobacter	A	0.00	0.001
350									Gemella	N	0.00	0.002
351									Propionimicrobium	N	0.00	0.002
352									Prauseria	A	0.00	0.002
353									Pediococcus	A	0.00	0.002
354									Actinoplanes	N	0.00	0.001
355									Geodermatophilus	N	0.00	0.001

356									Tessaracoccus	N	0.00	0.001
357									Christensenella	A	0.00	0.001
358									Candidatus Rhodoluna	N	0.00	0.001
359									Skermanella	N	0.00	0.001
360									Anoxybacillus	A	0.00	0.001
361									Thermoactinomyces	N	0.00	0.001
362									Christensenella	N	0.00	0.001
363									Pseudochrobactrum	A	0.00	0.001
364									Oxalobacter	A	0.00	0.001
365									Salmonella	A	0.00	0.001
366									Mobiluncus	A	0.00	0.001
367									Sediminihabitans	A	0.00	0.001
368									Devosia	N	0.00	0.001
369									Rhizobium	N	0.00	0.001
370									Xenophilus	N	0.00	0.001
371									Aurantimonas	N	0.00	0.001
372									A17	A	0.00	0.001
373									Pontibacter	A	0.00	0.001
374									Leuconostoc	A	0.00	0.001
375									Paracoccus	A	0.00	0.001
376									Ruminobacter	A	0.00	0.001
377									Morganella	A	0.00	0.001
378									A17	N	0.00	0.000
379									Actinomycetospora	N	0.00	0.000
380									Actinoplanes	A	0.00	0.000
381									Aequorivita	A	0.00	0.000
382									Agrococcus	A	0.00	0.000
383									Agromyces	N	0.00	0.000
384									Alloiococcus	A	0.00	0.000
385									Arcobacter	A	0.00	0.000

386									Aurantimonas	A	0.00	0.000
387									Brachyspira	N	0.00	0.000
388									Buchnera	A	0.00	0.000
389									Candidatus Rhodoluna	A	0.00	0.000
390									Carnobacterium	A	0.00	0.000
391									Cohnella	A	0.00	0.000
392									Comamonas	N	0.00	0.000
393									Curtobacterium	N	0.00	0.000
394									Ellin506	N	0.00	0.000
395									Erysipelothrix	N	0.00	0.000
396									Geodermatophilus	A	0.00	0.000
397									Guggenheimella	N	0.00	0.000
398									Halomonas	A	0.00	0.000
399									Ignatzschineria	A	0.00	0.000
400									Janibacter	A	0.00	0.000
401									Janthinobacterium	A	0.00	0.000
402									Kaistobacter	N	0.00	0.000
403									Lactococcus	A	0.00	0.000
404									Limnohabitans	N	0.00	0.000
405									Luteococcus	A	0.00	0.000
406									Macrococcus	A	0.00	0.000
407									Methylobacterium	N	0.00	0.000
408									Morganella	N	0.00	0.000
409									Moryella	A	0.00	0.000
410									Mycobacterium	N	0.00	0.000
411									Mycoplasma	N	0.00	0.000
412									Myroides	N	0.00	0.000
413									Novosphingobium	N	0.00	0.000
414									Oligella	N	0.00	0.000
415									Paracoccus	N	0.00	0.000

416									Pontibacter	N	0.00	0.000
417									Prauseria	N	0.00	0.000
418									Propionimicrobium	A	0.00	0.000
419									Proteus	N	0.00	0.000
420									Pseudoclavibacter	A	0.00	0.000
421									Rhizobium	A	0.00	0.000
422									Rickettsiella	A	0.00	0.000
423									Sediminihabitans	N	0.00	0.000
424									Segetibacter	A	0.00	0.000
425									Sinomonas	A	0.00	0.000
426									Skermanella	A	0.00	0.000
427									Sphingobacterium	A	0.00	0.000
428									Thermus	A	0.00	0.000
429									Tissierella_Soehngenia	N	0.00	0.000
430									Varibaculum	N	0.00	0.000
431									Williamsia	N	0.00	0.000
432									Xenophilus	A	0.00	0.000