MONASH University

Molecular Epidemiology, Virulence Determinants, Antibiotic Resistance Characteristics and Genome Sequencing of *Enterococcus faecalis* Isolated from Various Sources

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Abstract

Enterococcus faecalis ranks as one of the leading causes of nosocomial infections, such as urinary tract infection (UTI), surgical wound infection and endocarditis in humans. Enterococci inhabiting nonhuman reservoirs appear to play a critical role in the acquisition and dissemination of antibiotic resistance determinants. In addition, enterococci can have multiple virulence factors and may also be able to produce biofilms. These issues have become a major concern in both human and veterinary medicine, especially in countries such as Malaysia where there are poor laws and regulations to control the supply and excessive use of antimicrobials. The overall objective of this study is to gain understanding of the variation among *E. faecalis* strains between different reservoirs (farm animals, water sources and hospital patients) and its possible impact on epidemiology and disease severity.

In this thesis, I employ 250 *E. faecalis* isolates to study intra-specific genetic variation across various sources (water sources, farm animal feces and UTI patients) in Malaysia. High levels of genetic diversity were found in all sources (Simpson's Diversity Index = ≥0.901). No pulsotype was common to all the three sources. Each patient room also had its own unique PFGE pattern which persisted after six months, suggesting the patients picked up *E. faecalis* from the individual patient rooms, i.e. hospital bedding, shared bathroom within the room, as a result of infection. Most of the antibiotics used in this study were categorized by the World Health Organization as Rank I, i.e. critically important to human health. Therefore, the high percentage of resistant isolates (80%) observed among *E. faecalis* isolates are of concern for both clinical treatments as well as for the ecological implications for the transmission of this opportunistic pathogen. Resistance to Tetracycline was the most prevalent particularly in isolates from farm animals (62%). Distribution of 9 virulence markers tested in the study varied between sources, with the *gelE* gene, the product of which is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other peptides, having the highest prevalence (75.6%) in this study.

The influence of various factors on biofilm formation among *E. faecalis* strains isolated from different reservoirs was also investigated. The results indicated that cells supplemented with 1% glucose had a greater ability to form biofilms ($p<0.05$). Positive correlations were observed between cell surface hydrophilicity and attachment and biofilm forming ability of 63 *E. faecalis* strains on all abiotic surfaces tested (stainless steel, polyurethane and silicone tubing). In addition, clinical strains exhibited higher cell attachment and biofilm formation compared to strains from environmental sources, notably on polyurethane (10.02 – 15.71 log $CFUcm^{-2}$ and 10.23 – 15.72 log $CFUcm^{-2}$ respectively). This suggests that cell surface hydrophilicity plays a major role in the degree of attachment of *E. faecalis* on abiotic surfaces.

The influence of the genetic makeup of *E. faecalis* strains was investigated through whole genome sequencing of six *E. faecalis* strains. In general, the genomes from six *E. faecalis* strains shared great similarity with each other as well as with the reference genome *E. faecalis* ATCC 19433. Despite the distant phylogenetic relationship of strains S16 and S17 based on the PFGE analysis (only ~80% similarity), this study shows that the strains S16 and S17 share more functional groups, suggesting a closer phylogenetic relationship of both strains and/or possibly the occurrence of some cases of lateral gene transfer. All strains possessed multiple adhesin and biofilm-associated genes regardless of the biofilm and attachment properties exhibited.

This study contributes to a better understanding of the characteristics and genetic variation among *E. faecalis* strains from different sources including farm animal feces, water sources and UTI patients. The collective findings of my work and that of recent studies that showed epidemiological links between various reservoirs, could be useful for future studies to analyze the persistence of *E. faecalis* in the environment or to develop more specific methods in examining the health significance of potentially virulent strains in environmental and clinical sources.

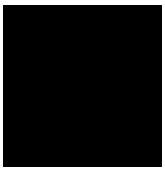
General declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers published in peer reviewed journals, one genome announcement paper and one unpublished manuscript. The core theme of the thesis is the molecular epidemiology, virulence determinants, antibiotic resistance characteristics and genome sequencing of *Enterococcus faecalis* isolated from various sources. The ideas, development and writing up of all the papers in this thesis were the principal responsibility of myself, the student, working within the School of Science under the supervision of Professor Sadequr Rahman; with contributions from Dr. Lee Sui Mae, Dr. Gan Han Ming and Prof. Gary Dykes.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researches and acknowledges input unto team-based research. In the case of Chapter 1, Chapter 2, Chapter 3 and Chapter 4 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Publication Status	Nature and % of student contribution
1	Public Health Risks of Multiple-Drug-Resistant *Enterococcus* spp. in Southeast Asia	Published as: Daniel et al., 2015, *AEM*, 81(18): 6090–6097.	Planned and conducted literature review search, wrote original manuscript and coordinated subsequent manuscript revisions (80% contribution)
2	Genetic diversity of *Enterococcus faecalis* isolated from environmental, animal and clinical sources in Malaysia	Published as: Daniel et al., 2017, *J Infect Pub Health*, doi: 10.1016/j.jiph.2017.02.006	Participated in sample collection, planned and conducted the majority of analyses, wrote original manuscript and coordinated subsequent manuscript revisions (80% contribution)
3	Biofilm Formation and Cell Surface Hydrophobicity of *Enterococcus faecalis* from Clinical and Environmental Origins	Under review as: Daniel et al., 2017, *J Infect Pub Health*, Manuscript ID: JIPH-D-17-00445	Planned and conducted the majority of analyses, wrote original manuscript and coordinated subsequent manuscript revisions (80% contribution)
4	Draft Genome Sequences of Six *Enterococcus faecalis* Strains Isolated from Malaysian Clinical and Environmental Origins	Published as: Daniel et al., 2017, *Genome Announc*	Assembled and annotated the genome, performed analysis, wrote original manuscript and coordinated subsequent manuscript revisions (80% contribution)

Student's signature:  **Date:** 15/03/2018

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor's signature:  **Date:** 15/03/2018

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I thank Ms. Yin Peng Lee from the Monash University Malaysia Tropical Medicine and Biology Multidisciplinary Platform for her assistance in next-generation sequencing. Funding for this study was provided by the Monash University Malaysia, School of Science and Monash University Malaysia, and Tropical Medicine Biology Multidisciplinary Platform.

I would like to direct my thanks to the former lab members of Professor Gary Dykes' research team for their kind assistance, support and accompaniment during the span of my research. Last but not least, I would like to express my special thanks to Dominic Soloman George, I could not ask for a more loving and supportive partner and friend.

List of Tables

List of Figures

Table of Contents

Monash University

Declaration for Thesis Chapter 1

Declaration by candidate

In the case of Chapter 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Planned and conducted literature review search, wrote original manuscript and coordinated subsequent manuscript revisions	80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Sadequr Rahman	Participated in the development of ideas and commented on draft manuscripts.
Gary A. Dykes	Participated in the development of ideas and commented on draft manuscripts.
Lee Sui Mae	Participated in the development of ideas and commented on draft manuscripts.
Gan Han Ming	Participated in the development of ideas and commented on draft manuscripts.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature  **Date: 15/03/2018**

Main Supervisor's Signature  **Date: 15/03/2018**

CHAPTER 1

Literature review

Part of the review presented in this chapter represents the following peer reviewed publication:

Daniel, D.S., Lee, S.M., Gan, H.M., Dykes, G.A., and Rahman, S. (2015). Public Health Risks of Multiple-Drug-Resistant *Enterococcus* spp. in Southeast Asia. *Applied and Environmental Microbiology* **81**: 6090-6097.

Chapter 1 – Literature Review

1.1 Enterococci

Enterococcus is a widely distributed group of gram-positive lactic acid bacteria of the phylum Firmicutes that are capable of causing infections in humans (Sherman, 1937, Maccallum & Hastings, 1899). In recent decades enterococci have emerged as important nosocomial pathogens, largely due to their intrinsic antimicrobial resistance and their capacity to acquire further antimicrobial resistance (Moellering, 1992, Arias & Murray, 2012). Their genomic plasticity has also contributed to their adaptation to hospital environments. In the early 1980s, *Enterococcus faecalis* accounted for 90% of enterococcal infections, and *E. faecium* accounted for 10% of enterococcal infections (Murray, 1990). In 1986 transferable high-level vancomycin resistant enterococci (VRE) were discovered (Leclercq et al., 1988, Uttley et al., 1988). Since then, a gradual increase in enterococcal infections has been seen. *E. faecalis* accounted for 60% of all enterococcal infections and *E. faecium* accounted for 40% of all enterococcal infections as reviewed by Dahlen et al. (2012). *E. faecalis* has also displayed increased virulence and demonstrated intrinsic antimicrobial resistance, particularly to clinically achievable concentrations of aminoglycosides (Sharifi et al., 2012). Enterococci are now the third and fourth most frequent class of microorganism isolated from hospital associated infections in the US and Europe, respectively (Top et al., 2007, Hidron et al., 2008). Also, enterococci are recognized as the second-most common cause of urinary tract infections and the third-most common cause of nosocomial bacteraemia worldwide (Lindenstrau et al., 2011).

1.2 General characteristics of *Enterococcus faecalis*

Enterococci are commensals of the human and animal intestinal flora (Sghir et al., 2000). They are also commonly used in food fermentation and easily detectable in environmental sources such as in water, plants and soil (Gelsomino et al., 2001). Enterococci were historically regarded as streptococci and not allocated a separate genus until the mid 1980s, although their unique characteristics were recognized among the streptococci. With the serological Lancefield's classification and the discovery of the group D antigen, enterococci were classified as salt-tolerant group D streptococci prior to 1984. However, the group D antigen is a lipoteichoic acid (LTA), one of the class of compounds that is found in virtually all Gram-positive bacteria and that is very different from the carbohydrate group antigen of other streptococci (Malanovic & Lohner, 2016). In 1984, DNA–DNA and DNA–RNA hybridization studies demonstrated a distant relationship of Gram-positive cocci with the streptococci, and two new genera, *Lactococcus* and *Enterococcus* were established thus giving enterococci a formal genus status. By 2012 there were 47 species in the *Enterococcus* genus registered in the Taxonomy browser in GenBank (http://www.ncbi.nlm.nih.gov/taxonomy/?term=enterococcus).

Enterococci are Gram positive facultative anaerobic organisms that are catalase negative, with the ability to hydrolyse esculin (a hydroxycoumarin glucoside) in the presence of bile (Malanovic & Lohner, 2016). They can grow under harsh conditions, including temperatures as low as 10°C and high as 45°C, high levels of salt (6.5% NaCl), and even alkaline conditions (pH 9.6). In addition, enterococci survive for 30 minutes at 60°C (Top et al., 2007). The overall GC-content in the enterococci is low (36-40%), but can vary within the genome (Lam et al., 2012).

Sequencing of *E. faecalis* genomes have shown that it has an open pan genome but with a limit approaching 3.3 Mb (Bakshi et al., 2016). Sequencing also revealed that the genome is very plastic due to, at least in part, the high numbers of Insertion Sequence (IS) - and other mobile genetic elements present in these genomes (Leavis et al., 2004, Palmer et al., 2012).

1.3 Clinical significance of *Enterococcus faecalis*

1.3.1 Epidemiology

Enterococci are a common cause of hospital acquired infections worldwide. In Europe, the prevalence of enterococcal hospital acquired infections is around 8% of all hospital acquired infections and enterococci infections are only outnumbered by *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (ECDC, 2008). Although enterococci do not reach the top-ten list of nosocomial outbreak pathogens (Werner et al., 2008), the European Centre for Disease Prevention and Control (ECDC) has placed them on the list of pathogens posing a major threat to healthcare systems (ECDC, 2008). This is in large part a result of the increasing antimicrobial resistance in enterococci. In Europe the prevalence of VRE has traditionally been low, and in the Scandinavian countries prevalence is still below 1%. However, increasing rates of VRE have been reported from many European countries, and in Greece and Ireland the prevalence is even >30% (Werner et al., 2008). VRE prevalence among clinical isolates has been estimated to range from 12% to 21% in Korea, and similar estimates have been made in Taiwan (Palmer et al., 2012). The prevalence of non-duplicated blood VRE isolates in a Taiwanese hospital increased significantly from 3.9% in 2003 to 18.9% in 2010 (Palmer et al., 2012). In Chinese hospitals, the prevalence of VRE increased from 0 in 2005 to 4.9% in 2010, and among the VRE isolates, the *van*A gene was the most prevalent gene (Fukuda et al., 2011).

In Norway enterococci are the 5th most common aetiological agent causing bacteraemia (NORM/NORM-VET, 2010). In parallel to the increase in enterococcal infections in Norway, an increase of high-level gentamicin resistance (HLGR) has been observed. The increased microbial resistance to vancomycin and gentamicin is part of an international trend occurring worldwide (Araoka et al., 2011).

1.3.2 Disease and risk factors

Enterococci are considered opportunistic pathogens. As commensals of the human gut flora they do not normally cause infections in healthy people, with the exception of occasional urinary tract infections. However, as indicated earlier, enterococci frequently cause opportunistic infections in hospitalized patients, particularly in debilitated hosts (Yip et al., 2011). It has been shown that exposure to antimicrobials promotes colonisation by enterococci which leads to changes in the intestinal microbiota increased density of enterococci in the intestines and subsequent bloodstream infection (Ubeda et al., 2010). Other risk factors for colonization and subsequent infections with enterococci include admission to a critical care unit, co-morbidity, exposure to other patients with hospital adapted enterococci, long periods of hospitalization, haemodialysis, and solid organ and bone marrow transplantation (Sydnor & Perl, 2011).

Most studies investigating risk factors focus on Vancomycin resistant enterococci (Munita et al., 2014, Chow et al., 2016). However, the crucial determinant giving enterococci the ability to colonize and infect a host is the presence of virulence elements which are involved in the attachment to host cells, the attachment to extracellular matrix protein, or implicated in cell and tissue damage (Semedo et al., 2003). Hence, one could assume the risk factors for acquiring enterococcal infection should be similar between Vancomycin resistant (VR) and Vancomycin susceptible enterococci.

Enterococci can cause a variety of infections, most of them facilitated Urinary tract infections (UTI) being the most common enterococcal infection, and often associated with urinary catheters (Chen & Zervos, 2009). If not accompanied by bacteraemia, it generally only requires single-drug therapy, although seriously ill patients with pyelonephritis may benefit from combination therapy (Chen & Zervos, 2009, Heintz et al., 2010). Intra-abdominal and pelvic infections are also common but often polymicrobial in origin. Although enterococci are detected in 20% of these (Dupont, 2007), it is debatable to what extent they contribute to the infections (Harbarth & Uckay, 2004). Enterococci account for 5-20% of cases of endocarditis as a result of enterococcal bacteraemia, and are thus the 2nd -3rd most common cause of endocarditis (Pintado et al., 2003). Enterococcal meningitis is rare accounting for about 0.3% to 4% of meningitis cases (Pintado et al., 2003). Severe enterococcal infection generally requires combination therapy for its cure (Arias et al., 2010).

1.3.3 Antibiotics used to treat enterococcal infections

Enterococci are traditionally treated with a combination of cell wall active antimicrobials such as β-lactams or glycopeptides, and aminoglycosides (Arias et al., 2010). Aminoglycoside antibiotics were one of the early discovered classes of antibiotics and have been in use for over 60 years. They bind to the 30S ribosomal subunit (Recht & Puglisi, 2001), rendering the ribosome unavailable for translation and thereby resulting in cell death (Kotra et al., 2000). Aminoglycosides have a broad antimicrobial spectrum covering a wide variety of aerobe Gram negatives and some Gram positives (Ebert & Craig, 1990). They display concentration-dependent bactericidal activity and can be effective even when the bacterial inoculum is large (Vakulenko & Mobashery, 2003). The aminoglycosides are seldom drugs of first choice for monotherapy of infections, except for some cases of

uncomplicated urinary tract infections (Vidal et al., 2007). Gentamicin is the aminoglycoside most often used, because of its low cost and reliable activity against Gram negative aerobes (Rougier et al., 2004). The major limitations of aminoglycosides are a relatively low therapeutic index with both nephrotoxicity and ototoxicity, and that they are not absorbed orally due to their cationic nature and thus must be given by either an intravenous or intramuscular route (Rougier et al., 2004).

Cell wall active antimicrobials such as β-lactams and glycopeptides act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls (Jana & Deb, 2006). Natural penicillins are considered bacteriostatic against enterococci, and are the most widely used antimicrobials in the world (Jana & Deb, 2006). Glycopeptides only work on Gram positive bacteria and is considered bacteriostatic against enterococci (Moellering, 1992). An example of a glycopeptide is Vancomycin.

In the last decade several additional antimicrobials with effect on enterococci have emerged. They all exhibited around 70% clinical success (Wang & Hsueh, 2009). Clinical success is defined as resolution or improvement of clinical symptoms and signs of infection and discontinuation of the antibiotics (Rao et al., 2006). To improve their efficacy and reduce the development of resistance, it is preferable to employ them as part of a combination regimen (Wang & Hsueh, 2009). Linezolid inhibits protein synthesis and is active against all clinically important Gram-positive bacteria, although it only displays a bacteriostatic effect (Leach et al., 2011). Daptomycin interferes with the cytoplasmic membrane causing depolarization and cessation of protein-, DNA and RNA-synthesis (Enoch et al., 2007). It has concentration-dependent bactericidal activity against enterococci (Critchley et al., 2003). Tigecycline is a broad-spectrum antibiotic that inhibits protein synthesis. A recent review

showed that it was more effective against enterococci than other Gram-positive bacteria, but infections included were mostly skin and soft tissue infections and intra-abdominal infections (Tasina et al., 2011, Yahav et al., 2011).

1.4 Antibiotic resistant *Enterococcus faecalis*

The discovery of antibiotics is considered one of the most significant health related events of modern times and antibiotic therapy is one of the cornerstones in modern medicine. Use and misuse of antibiotics in human medicine and animal husbandry over the past 70 years have caused an unremitting selection pressure that has given rise to microorganisms resistant to these medicines. The use of antibiotics is positively correlated to the emergence of resistant bacteria (Davies & Davies, 2010). Several bacterial strains in the hospital setting in many countries worldwide are now multi-resistant (Hidron et al., 2008), leaving few treatment options. Hence, the development of antibiotic resistance by bacteria constitutes a major threat to human health.

Enterococci are intrinsically or naturally resistant to various antimicrobials including beta-lactams (cephalosporins and semisynthetic penicillinase-resistant penicillins), clindamycin, low concentrations of aminoglycosides and fluroquinolones. Aminopenicillins (i.e. Ampicillin) have a slighter greater activity against Enterococci. They are naturally sensitive to vancomycin, but can acquire resistance to this antibiotic after exposure. They are able to develop resistance to tetracyclines, macrolides, glycopeptides (vancomycin and teicoplanin), choloramphenicol and to high concentrations of beta-lactams as well as aminoglycosides.

The acquisition of antibiotic resistance occurs mostly through the acquisition of resistance genes on plasmids or transposons from other bacteria. Transposons may be

acquired by natural transformation (Domingues et al., 2012). Enterococci can secrete pheromones (Sghir et al., 2000), which stimulate the synthesis of the surface aggregation substance (Magauran & Salgado, 2010). This facilitates the contact between the cells and the formation of the mating aggregate, which finally will lead to the exchange of plasmids carrying resistance. Infrequently, DNA coding for resistance genes can be directly taken up from the environment (Domingues et al., 2012) or be transferred through transduction by phages (Davies & Davies, 2010).

In the last few years, enterococci have received increasing attention because of the development of resistance to multiple antimicrobial drugs. Studies have found examples of acquired resistance such as resistance to Chloramphenicol, Erythromycin, Tetracycline, as well as resistance to high levels of Clindamycin, Aminoglycosides, Beta-lactams, Fluoroquinolones, and glycopeptides (Murray, 1990). This may be one explanation for the dominance of enterococci in nosocomial infections. Vancomycin-resistant enterococci (VRE) probably represent the most serious challenge among many microbes with antibiotic resistance, as a source of human clinical infections in the past decades. Two distinct phenotypes of transferable VRE have been described: the VanA phenotype, associated with a high level of inducible resistance to vancomycin and cross-resistance to teicoplanin; and the VanB phenotype, which usually has variable levels of inducible resistance only to vancomycin. Vancomycin resistance is not due to the acquisition of only one gene. Each resistance phenotype is associated with a complex cluster of genes. These genes are physically grouped in operons and are located on plasmids or in the chromosomes, and they can be easily transferred to other species and even between the same species. The mechanism of resistance has been best characterized for the *van*A cluster of nine genes carried on transposon *Tn1546f*. This mobile genetic element is 10,851 base pairs long and encodes two genes responsible for transposition functions (*orf*1 and *orf*2); five genes responsible for the regulation and expression of resistance (vanR, vanS, vanH,

vanA and vanX); and two genes with auxiliary roles (vanY and vanZ). Similar gene clusters are found in the remaining resistance phenotypes. Phenotypes VanA, VanB, and VanD, found in *E. faecalis*, *E. faecium* and, to a much lesser extent, in other enterococci, are associated with high-level resistance. The VanA phenotype is characterized by high level resistance to both Vancomycin and Teicoplanin. It has been found in a number of enterococcal species. The VanB phenotype has low to moderate level resistance to Vancomycin with preserved Teicoplanin susceptibility. VanD phenotype is characterized by low to moderate level resistance to both Vancomycin and Teicoplanin (Chetinkaya et al., 2000). Due to the ability of enterococci to transfer plasmids to streptococci and staphylococci there is the possibility of spread of penicillin- and vancomycin-resistance to these and other Gram-positive species as well (Matar et al., 2006).

Although there is a large amount of data about the emergence of antimicrobial-resistant enterococci in Southeast Asian countries, most of this information is fragmented since it has been published in different papers in different countries over several decades (Teale and Moulin, 2012). However, several studies show the extent of unregulated and inappropriate use of antimicrobials in food animals in developing Southeast Asian countries such as Malaysia (reviewed in Daniel et al., 2015). The results from this review emphasize the need for stronger regulations to be implemented in terms of unregulated and inappropriate use of antibiotics in food-animals and clinical settings.

1.5 Virulence factors of *Enterococcus faecalis*

Virulence is the degree of pathogenicity caused by an organism. This ability represents a genetic component or genetic factors, which are defined as virulence determinants or virulence associated genes, which contribute to the ability of enterococci to survive and cause infection in a host environment. Some of these factors are part of the core genome, while others

are traits that can be acquired and shared. This ability to easily acquire new virulence traits enables *E. faecalis* to colonize new areas in the host and cause infection. Much work has been done over the last 20 years to identify these virulence determinants, and to characterize their mechanism of action (Garsin et al., 2014).

Virulence associated genes are often found on pathogenicity islands, which are horizontally transmitted elements that usually range from 10-200 kb and often have base compositions different from the core genome (McBride et al., 2007). The virulence associated genes in human pathogenic *E. faecalis* encode among others, a collagen-binding protein (*ace*) (Rich et al., 1999), an aggregation substance (*asa*1) (Vankerckhoven et al., 2004), a haemolysin activator (*cylA*) (Vankerckhoven et al., 2004), an endocarditis antigen (*efaA*) (Templer et al., 2008), a surface protein (*esp*) (Vankerckhoven et al., 1998) and gelatinase (*gelE*) (Qin et al., 2012). However, none of the virulence genes has been exclusively associated with or proven indispensable for disease manifestation. For example, a study by Poulsen et al. (2012) in Vietnam revealed that isolates from urine in UTI patients and poultry showed identical virulence gene profiles. Another study by Seputiene et al. (2012) in Lithuania revealed the presence of clinical *E. faecalis* isolates harbouring genes coding for virulence factors *agg*, *esp*, *fsr* and *gelE*, with a high prevalence of the *esp* gene in isolates from cattle (63%) and pigs (79%). The same study also revealed resistance of the isolates to aminoglycosides, tetracycline and erythromycin.

1.6 Biofilm formation

Not only are enterococci resistant to many antibiotics, and have multiple virulence factors, but they are also able to produce biofilms. Biofilms are communities of organisms that are attached to a range of biotic and abiotic surfaces and are encased in exopolymeric

substances (EPS) (Mohamed & Huang, 2007). Such a community can contain a single species or multiple species of micro-organisms (O'Toole et al., 2000). Biofilms accelerate the transfer of DNA between bacteria, through eDNA released by cells, conjugation or other means of horizontal transfer (Donlan, 2002).

Bacteria, when they are not attached to a surface, are planktonic and free-floating (Hall-Stoodley et al., 2004). These planktonic cells may attach to a surface and form microcolonies, but that is dependent on several key elements, such as the properties of the cell, the properties of the substratum and the environment. Initial attachment of bacterial cells may require electrostatic, Lewis acid-base interactions, Lifshitz-van der Waals forces and hydrophobic forces to overcome the repulsion of the usually net negative charge surfaces, and some of the above interactions are helped by the cell surface proteins (van Merode et al., 2006). This attachment is initially reversible but eventually it becomes irreversible. There are also genetic changes occurring, due to possibly the sensing of a change in environment, which triggers a shift in expression of genes resulting in products which further stimulate attachment (Beloin & Ghigo, 2005, Monds & O'Toole, 2009).

Bacteria also have other surface structures that are important to initial attachment, which include fimbriae, lipoproteins, lipopolysaccharides, enzymes, and adhesins (Lejeune, 2003, Latasa et al., 2006). The properties of the substratum also has a role, as bacteria have been shown to attach better to rougher surfaces as well as those that are more hydrophobic (Donlan, 2002). The environmental factors that can affect initial attachment include flow velocity, pH, temperature, cations and the presence of antimicrobials agents. All of these may affect attachment, which in some cases coincides with changes in gene expression as mentioned above (Donlan, 2002, Beloin & Ghigo, 2005).

When microcolonies form on the surface there is also an increase in the production of EPS (Hall-Stoodley & Stoodley, 2002), which is essential for the production of a biofilm as it

holds cells closely together and further helps attachment to the surface. Components of EPS include polysaccharides, proteins (enzymes and structural proteins), extracellular DNA (eDNA), lipids, and biopolymers. The amount of EPS varies between biofilms, due to temperature, shear force, nutrients available and the organisms within the biofilm having the ability to form components of the EPS. These combined factors mean that even the composition of EPS produced by identical bacterial species may vary considerably (Sutherland, 2001, Allison, 2003, Flemming & Wingender, 2010). Within the biofilm structure conditions can vary, for instance, some areas may have less oxygen or nutrients than in others. These differences in local conditions will not be advantageous for all the cells (Flemming & Wingender, 2010).

Horizontal gene transfer is extremely rare in planktonic cultures. The persistent biofilm growth provides not only a favourable environment for increased spontaneous mutation, but also an increased frequency of horizontal gene transfer. The probability of plasmid transfer is increased greatly in biofilms compared to planktonic cells (Monds & O'Toole, 2009). For instance, in *Staphylococcus aureus* biofilms the frequency of horizontal gene transfer is increased by almost 16 000-fold compared to the frequency in planktonic cultures (Savage et al., 2013). It was also shown another study that the copy number of pBR32, a plasmid carrying resistance genes against Ampicillin and Tetracycline, was increased approximately two-fold in *E. coli* cells growing in a biofilm compared to planktonic copy numbers (Cook and Dunny, 2014).

One of the main clinical problems with biofilms is that the cells in biofilms tend to be resistant to several host defence systems. Studies have shown that phagocytes are unable to attack bacteria in biofilms due to the protective layer of the EPS. This also prevents proper interaction of antibodies with cells as the antibodies can only interact with the biofilm surface. Furthermore, bacteria within the biofilms have been shown to produce toxins that kill

polymorphic neutrophils, preventing biofilm clearance (Hall-Stoodley & Stoodley, 2009). Additionally, adhesins and secretory proteins produced by bacteria in biofilms further strengthen attachment to abiotic and biotic surfaces, further facilitating the invasion of host tissues. Biofilm-producing enterococcal isolates are characterized by the quantity of biofilm produced (i.e. strong, medium, weak or non-biofilm producer) with an optical density (OD570) classification (Toledo-Arana et al., 2001, Mohamed et al., 2004). Not all enterococcal isolates can produce biofilms. In Okayama, Japan, Seno et al. (2005) reported that all of 352 *E. faecalis* isolates derived from urinary tract infections were capable of producing biofilms. In Poland, 59 % of *E. faecalis* isolates collected from clinical specimens produced biofilms (Dworniczek et al., 2005). A study from a tertiary care hospital in India showed that 44 of the 171 isolates (26 %) of *E. faecalis* and none of the 25 *E. faecium* isolates produced biofilms (Prakash, 2005). In Rome, Italy, among a collection of 52 *E. faecalis* isolates from orthopaedic infections 96 % produced biofilms (Baldassarri et al., 2006). Collectively, these data suggest that biofilm formation may be an important factor in the pathogenesis of enterococcal infection. These studies, however, are limited to predominantly clinical settings of a limited geographical area and do not provide information about biofilm production of *E. faecalis* from environmental sources (Dworniczek et al., 2005, Prakash, 2005, Seno et al., 2005, Baldassarri et al., 2006, Di Rosa et al., 2006). The current study investigates and compares the biofilm producing ability and attachment properties of *E. faecalis* from both clinical and environmental sources on abiotic surfaces commonly used in healthcare settings.

1.7 Reservoirs and transmission

Due to the prevalence of *E. faecalis* in nosocomial infections, many have suggested that hospital settings serve as the reservoir for antimicrobial-resistant strains (Ruiz-Garbajosa et al., 2006). Additional studies suggest environmental sources of the bacteria, including

animals, can also serve as important reservoirs for antimicrobial resistant *E. faecalis* strains (Sørensen et al., 2001, Mallon et al., 2002). Human populations, animal populations, and the environment are all interconnected, (Chomel, 1998). Figure 1 shows the complex epidemiology of enterococci and its ecological relationship between different reservoirs. The interaction between the different reservoirs contributes to the increasing distribution of MDR enterococci (modified from Gilmore, 2002). The amount of antibiotics used on food animals plays a major role in the propagation of antibiotic resistant enterococci in animal reservoirs. Transmission of resistance can take place through food animals or directly through contact between animals and humans. Treated sewage sludge, a by-product from treated sewage waste water containing the faecal contents of animals and humans, can be used as fertilizers which potentially pass on MDR strains to the food supply.

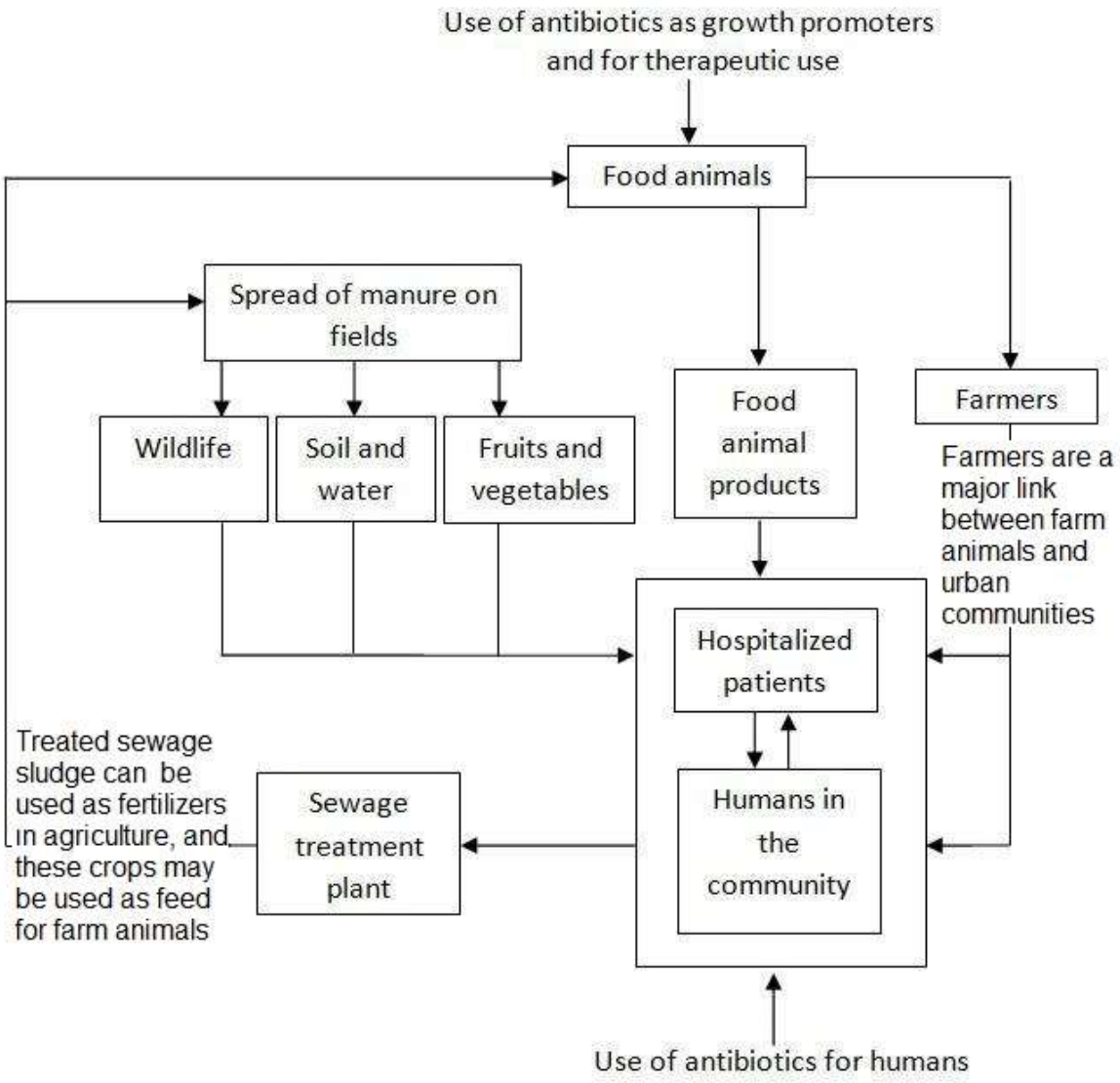


Figure 1. Ecological relationships between different reservoirs (modified from Gilmore, 2002).

The sources of enterococcal infections in humans are not clear, but animal reservoirs have been suggested (Jarvis & Martone, 1992, Donabedian et al., 2003, Hammerum et al., 2010, Larsen et al., 2010, Freitas et al., 2011). A number of phenotypic or genotypic typing methods (including biochemical typing, serotyping, multilocus enzyme electrophoresis [MLEE], phage typing, insertion sequence element-based typing, pulsed-field gel electrophoresis [PFGE], restriction fragment length polymorphism [RFLP] analysis,

ribotyping, repetitive sequence-based PCR, arbitrary primed PCR, and random amplification of polymorphic DNA) have been applied to the epidemiological investigations of *E. faecalis* (Gordillo et al., 1993, Kuhn et al., 1995, Descheemaeker et al., 1997, Malathum et al., 1998, Murray et al., 1999). A study comparing enterococcal isolates from 4 European countries and the United States demonstrated that *E. faecalis* isolated from pigs in Portugal had pulsed-field gel electrophoresis (PFGE) patterns identical to those of multidrug-resistant isolates at hospitals in Spain, Italy, and Portugal, all of which were shown by multilocus sequence typing (MLST) to belong to sequence type (ST) 6 (Freitas et al., 2011). In Denmark, high-level gentamicin-resistant *E. faecalis* of ST16 with an identical PFGE pattern was isolated from pigs and from humans with endocarditis (Larsen et al., 2010). Identical and closely related PFGE patterns were demonstrated by isolates from humans and from pork and chicken meat in the United States, all of which contained high-level gentamicin-resistance genes (Donabedian et al., 2003). Poulsen et al. (2012) investigated whether poultry might be a reservoir for *E. faecalis*–associated urinary tract infections (UTIs) in humans. They characterized *E. faecalis* isolates from patients in Vietnam with UTIs during January 2008–January 2010 and poultry living in close contact with them by MLST, pulsed-field gel electrophoresis, analysis of antimicrobial drug susceptibility patterns, and sequencing of virulence genes. In 7 (23%) of 31 UTI cases, they detected identical MLST, indistinguishable or closely related pulsed-field gel electrophoresis patterns, and similar antimicrobial drug susceptibility patterns. Isolates from urine and poultry showed identical virulence gene profiles, except for one variation, and individual genes showed identical sequences. It is a possibility that the *E. faealis* pathotypes found in poultry might represent transmission from humans, e.g., from UTI patients, although the route of *E. faecalis* transmission wasn't thouroughly investigated in this study. However, poultry as carriers of ST16 has been documented (Gregesen et al., 2010), and it seems more likely that humans are exposed to poultry litter than that poultry are exposed to human feces.

However, the route could also be colonization of the human intestine and subsequently ascending the urethra. Humans with endocarditis in Denmark have been shown to harbor identical *E. faecalis* to pigs through the MLST technique (Larsen et al., 2010). Clearly therefore, multidrug resistance can be transmitted either from the food animals to humans or from humans to animals. Further studies are required to explain routes of transmission, but the emergence of *E. faecalis* as a cause of human infections and their resistance to antibiotics used for human treatment emphasizes the need to elucidate transmission routes and reservoirs for *E. faecalis* and their resistance genes. While studies (Donabedian et al., 2003, Gregesen et al., 2010, Larsen et al., 2010, Poulsen et al., 2012) report a potential link between food-animal and humans, there is no definitive proof of the route of transmission. These previous studies also failed to investigate the persistence of *E. faecalis* strains over a period of time, and a relatively small sample size (approximately 30 to 60 samples) was also noted. The current study aims to determine an epidemiological link between clinical and environmental *E. faecalis* strains as well investigate its persistence over a period of 6 months in specific locations.

1.8 Statement of the problem

The development of antibiotic resistance among bacteria is a point of concern in both human and animal medicine. *Enterococcus faecalis* has a remarkable ability to acquire new genetic traits (Coburn et al., 2007) and has been found to be increasingly resistant to multiple antibiotics in last few years (Tremblay et al., 2011, Al-Gheethi et al., 2013). *E. faecalis* are of particular concern in human and animal medicine because some strains have constitutive antimicrobial resistance traits, and others carry inducible resistance traits (Eisner et al., 2005). Another concern is that these organisms can transfer resistance genes to other bacterial species including pathogens (Moubareck et al., 2003, Lester et al., 2006). Additionally, *E. faecalis* is

usually found in large numbers in farm animals such as pigs, cattle and poultry (Hammerum et al., 2010, Tremblay et al., 2011) and this may be one source of the antimicrobial resistant *E. faecalis* found in clinical settings.

There is extensive use of antimicrobials in animal production, in Malaysia. Unfortunately, most of the antimicrobials used are on WHO's list of critically important antimicrobials. As a result, the microbial flora of food animals in Malaysia frequently carries resistance to a range of antimicrobials including some that are used to treat human infections (Getachew et al., 2012, Getachew et al., 2013). Studies have demonstrated a link between human sources of resistant strains of *Enterococcus* isolates and the environment, and some have suggested that animals and their products are contaminated secondary to interaction with humans and the environment (Aarestrup et al., 2001, Iversen et al., 2004). Antibiotics enter waste streams through faeces and urine, thus making sewage effluent from sewage treatment plants an important source for antibiotic resistant bacteria in the environment (Al-Gheethi et al., 2013). The sewage treatment plant effluent must meet regulatory limits for fecal indicator bacteria such as total coliforms, however regulatory limits in Malaysia are poorly enforced and have not been developed for antibiotic agents and the effect of low antibiotic concentrations in the environment might lead to the development of bacterial resistance of antibiotics (Reinthaler et al., 2003). The role of sewage treatment plants in the spread of antibiotic resistance to the natural environment is an important key to the ecological impact of human discharges (Garcia-Armisen et al., 2011).

The characterisation of *E. faecalis* is important in studying their population structures, particularly in environmental samples. Studies focusing on isolation and characterization of *E. faecalis* from various host groups could help to evaluate which species, virulence determinants, and antibiotic resistances are prevalent in each group, and data generated by these studies is useful for public health risk assessment analyses. Comparison of virulence gene profiles and antibiotic resistant properties between *E. faecalis* of human and animal origin would add to

the knowledge of the zoonotic risk associated with *E. faecalis*. Evaluation of biofilm formation in different conditions is a complementary approach to better understand the mechanisms by which bacteria adapt to environmental stresses and colonize different niches (Sarjit et al., 2015). Although a number of studies have investigated the biofilm properties of enterococci, such studies are typically limited to food products and clinical settings, and studies of a limited geographical area (Diani et al., 2014, De Silva et al., 2015). The results of this thesis help to extend our understanding of the factors that influence attachment and biofilm formation in clinical and environmental *E. faecalis* strains. Acquistion of genome data has become increasingly affordable and provides a higher level of resolution of relationships between different isolates. Comparison of genomic data from *E. faecalis* may improve our understanding of the virulence factors and pathogenesis present in *Enterococcus*.

1.9 Objectives of research

The overall aim of this project is to gain understanding of the variation among *E. faecalis* strains between different reservoirs (farm animals, water sources and hospital patients) and the possible impact of this variation on epidemiology and disease severity. The broad working hypothesis is that the *E. faecalis* in the different reservoirs are all interconnected but there are reservoir-specific adaptations. This leads to the following predictions that can be tested.

1. There is a high prevalence (60%) of antibiotic resistance and putative virulence genes in *E. faecalis* isolated from both clinical and environmental samples. This prediction was tested by determining the antibiotic resistance and virulence factors of *E. faecalis* from farm animals, water, and UTI patients in Selangor (Peninsular Malaysia) and Sabah (East Malaysia).

2. There is a close genetic relationship (as determined by identical or near identical PFGE profiles) between clinical and environmental samples and in both cases constant pulsotypes are observed. The genetic relatedness of *E. faecalis* strains between and

within the different reservoirs was determined using Pulsed Field Gel Electrophoresis (PFGE) from Selangor (Peninsular Malaysia) and Sabah (East Malaysia). These results help determine if there is an epidemiological link between clinical and environmental sources and if the strains are persisting after a period of time. These findings are important in deciding whether an extensive strengthening of infection control in Malaysian farms, wastewater treatment plants and hospitals is needed. Previous studies have reported a possible link between human and animal sources, as well as a high genetic diversity of *E. faecalis.*

3. *E. faecalis* that have more hydrophilic surface determinants will attach more strongly to hydrophilic abiotic surfaces and form better biofilms. The cell hydrophilicity as well as biofilm and attachment properties of the above mentioned *E. faecalis* strains was determined on materials commonly used in hospitals (stainless steel, silicone tubes and polyurethane) to test the hypothesis. The results may help guide researchers and healthcare providers to develop effective biofilm management strategies and aid in the monitoring of treatment progress.

4. Whole genome sequencing will reveal previously unknown genetic relationships and genotypes. This hypothesis was tested by comparing and analyzing genomic sequence data from *E. faecalis* strains obtained from farm animals, water and UTI patients. This analysis will help provide detailed genomic information of *E. faecalis* strains from different sources and a comparison of results obtained by two different genotyping methods (PFGE vs Whole Genome Sequencing). This analysis may also improve our understanding of the virulence factors and pathogenesis present in Enterococci and may ultimately be useful in curbing enterococcal infections. The first hypothesis of this objective states that whole genome sequencing shows different relationships between

strains compared to PFGE. The second hypothesis for this objective states that the presence of biofilm/pili associated genes can be identified through whole genome sequencing.

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Participated in sample collection, planned and conducted the majority of analyses, wrote original manuscript and coordinated subsequent manuscript revisions	80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Sadequr Rahman	Participated in the development of ideas and commented on draft manuscripts.
Gary A. Dykes	Participated in the development of ideas and commented on draft manuscripts.
Lee Sui Mae	Participated in the development of ideas and commented on draft manuscripts.
Gan Han Ming	Participated in the development of ideas and commented on draft manuscripts.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature  **Date: 15/03/2018**

Main Supervisor's Signature  **Date: 15/03/2018**

CHAPTER 2

Genetic diversity of *Enterococcus faecalis* isolated from environmental, animal and clinical sources in Malaysia

The work presented in this chapter represents the following peer reviewed publication:

Daniel, D.S., Lee, S.M., Gan, H.M., Dykes, G.A., and Rahman, S. (2017). Genetic Diversity of *Enterococcus faecalis* Isolated from Environmental, Animal and Clinical Sources in Malaysia. *Journal of Infection and Public Health* **10**: 617-623.

Chapter 2 - Genetic diversity of *Enterococcus faecalis* isolated from environmental, animal and clinical sources in Malaysia

2.1 Introduction

Enterococcus faecalis is found in a variety of environments, such as soil and water, and in association with plants, and animals (Mallon et al., 2002). In humans, as well as in other mammals, these microbes are mainly found in the gastrointestinal tract as commensals. However, *E. faecalis* may become an opportunistic pathogen in individuals whose immune systems are compromised (Texeira & Merquior, 2012). The virulence associated genes in human pathogenic *E. faecalis* may encode among others a collagen-binding protein (*ace*) (Rich et al., 1999), an aggregation substance (*asa1*) (Vankerckhoven et al., 2004), a haemolysin activator (*cylA*) (Vankerckhoven et al., 2004), an endocarditis antigen (*efaA*) (Templer et al., 2008), a surface protein (*esp*) (Vankerckhoven et al., 2004), gelatinase (*gelE*) (Qin et al., 2000) and two putative surface antigens, *EF0591* and *EF3314* (Creti et al., 2004). *E. faecalis* has also been shown to have the ability to acquire resistance to a wide range of antibiotics (Comerlato et al., 2013). As a result, enterococcal infections such as urinary tract infections (UTI) have emerged as a therapeutic challenge (Texeira & Merquior, 2012). Around the world *E. faecalis* remains one of the most frequently isolated species from enterococcal infections in humans (Giridhara et al., 2010).

Selection and persistence of antimicrobial resistance is primarily due to the misuse or overuse of antibiotics in humans and animals (Roca et al., 2015). The resistance can be spread in the environment due to horizontal transfer of resistance genes among bacteria and environmental contamination through livestock slurry and plant wastewater. The spread of resistance appears to have accelerated in the past decade and today, bacteria resistant to multiple

antimicrobials constitute a global problem (Roca et al., 2015). Although a number of studies have investigated the prevalence and characteristics of antimicrobial resistance among enterococci in clinical and environmental settings in Malaysia, such studies are typically limited to vancomycin-resistant enterococci and/or studies of a limited geographical area (Hamzah et al., 2011, Praveena et al., 2011, Dada et al., 2012, Getachew et al., 2012, Getachew et al., 2013, Weng et al., 2013). In this study, the virulence determinants and antibiotic susceptibilities found in clinical and environmental *E. faecalis* isolates were assessed in this chapter, with the hypothesis that there is a high prevalence (>60%) of putative virulence genes and antibiotic resistance in *E. faecalis* isolated from both clinical and environmental sources. This hypothesis was tested by running PCR to determine putative virulence markers, and performing standard antibiotic susceptibility testing according to the Clinical and Laboratory Standards Institute. In addition, water samples from sewage works and the river the treated sewage flowed unto in Selangor were also investigated; this was not possible in Sabah due to the absence of sewage works.

Due to the prevalence of *E. faecalis* in nosocomial infections, studies have suggested hospital settings as a source for antibiotic-resistant strains (Ruiz-Garbajosa et al., 2006). Additional studies suggest environmental sources including animals and water can serve as important sources for antibiotic resistant *E. faecalis* strains (Mallon et al., 2002) as human populations, animal populations, and the environment are all interconnected (Mallon et al., 2002). It is important to investigate the genetic relationships between microbes, such as *E. faecalis*, that are found in both the environment and hospitals, as a possible relationship between the different sources may be established. As such, the second hypothesis of this study is that there is an epidemiological link between clinical and environmental *E. faecalis* isolates. In addition, the persistence of each type of isolate in each source from the same location after a period of six months was assessed, hypothesizing that strains would not likely persist after

the six-month period due to a high genetic diversity of *E. faecalis* as seen in previous reports (Praveena et al., 2011, Getachew et al., 2013). The pulsed-field gel electrophoresis method was used to test these hypotheses by comparing PFGE fingerprints and constructing a dendrogram based on pulsotypes.

This chapter tested the following predictions based on the hypotheses of the thesis.

1. There is a high prevalance (60%) of antibiotic and virulence genes in isolates from different reservoirs tested.

2. Identical resistance and virulence profiles will be observed for isolates from environmental and clinical sources and the pulsotypes obtained from *E. faecalis* from hospitals will be identical or near identical to those from surrounding environmental sources such as farms and river water.

2.2 Materials and Methods

2.2.1 Study Site and Sample Collection

Sampling was carried out in two states representing different geographical regions in Malaysia; Selangor (West Malaysia) and Sabah (East Malaysia). Study sites comprised of chicken and cattle farms, and hospitals in both Selangor and Sabah. Additionally, wastewater treatment plants and the Klang river were sampled as indicated. All farms and water sources were located within a 15 km radius of the hospitals in Selangor and Sabah respectively, as shown in Figure 2.1. The sampling areas in Sabah comprised of small to medium residential communities surrounded by rural agricultural regions as opposed to Selangor which included sampling areas around semi-urban development constituting smallholder farms. Sampling was conducted at two different sampling times, June and December 2014.

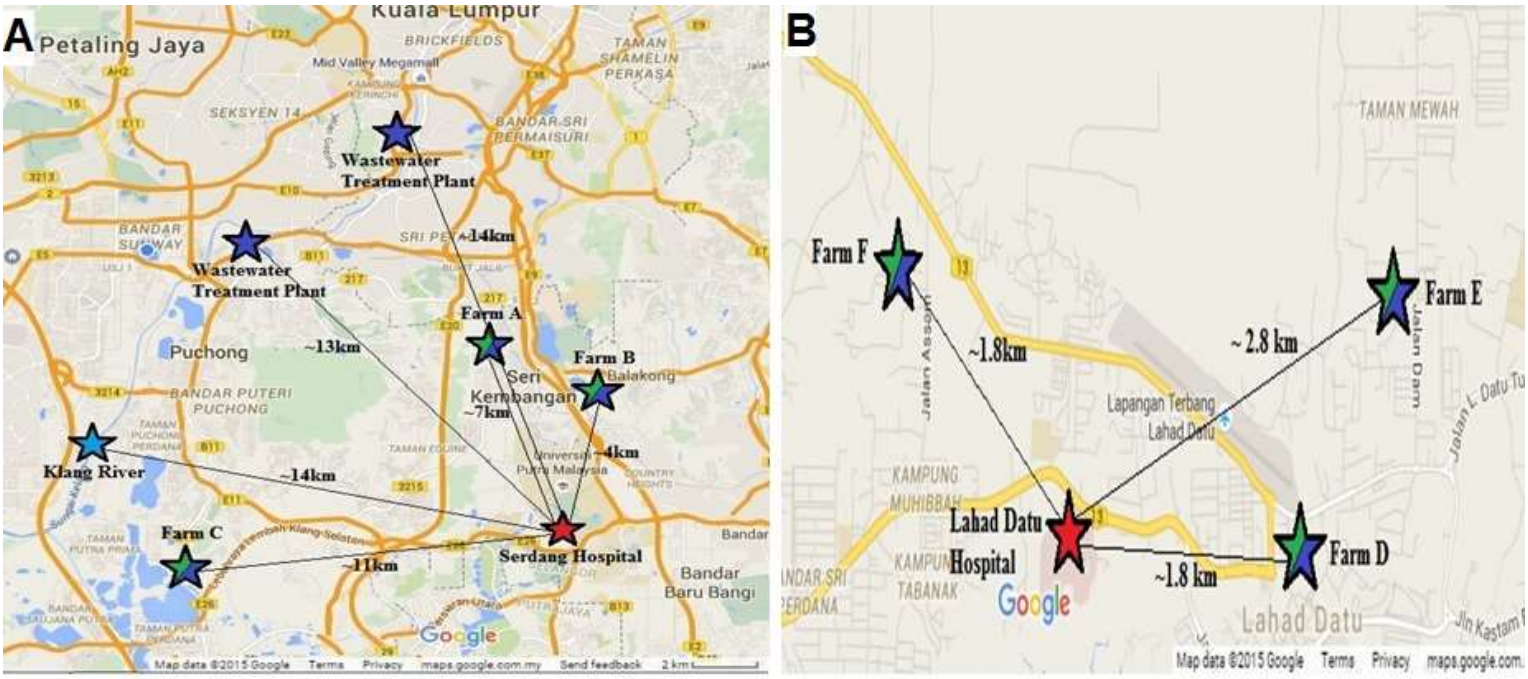


Figure 2.1. Sampling sites in Selangor (A) and Sabah (B).Red star: clinical samples, Light blue star: river water samples, Dark blue star: wastewater samples.

Green/blue star: farm animal fecal samples and farm animal drinking water samples.

2.2.1a. Farm Animal Feces

Sampling was conducted at one chicken farm and two cattle farms each in Selangor and Sabah. One fecal sample per animal was obtained and clinically ill animals were excluded from this study. The fecal samples obtained after the 6-month period were from a different set of animals. Using a sterile swab, approximately 20 g of freshly deposited animal feces was collected and stored in sterile universal bottles. All samples were labeled accordingly and kept on ice for a maximum of 10 h before inoculating unto enterococci selective media. All fecal samples collected from farm animals were divided into a 10 g portion for long term storage in 25% glycerol stocks with tryptic soy broth (TSB; Merck) at -80°C, and a 10 g portion for culturing by dissolving in 100 ml phosphate-buffered saline (PBS) (pH 7.2) followed by thorough homogenization by vortexing. Using a sterile swab, the suspension was then

inoculated onto Bile Aesculin Azide (BAA) agar (Merck), a selective agar medium for the isolation of enterococci, and incubated at 37ºC for 24 h. One isolate per sample was randomly picked for analysis.

2.2.1b. Water sources

Water samples were collected from a distributary channel leading from the Klang river as shown in Figure 2.1 and two sewage wastewater treatment plants in Selangor. Wastewater and river samples were collected from the same site after the 6-month period. No water samples were collected from Sabah because there were no sewerage treatment plants and rivers within the 15km vicinity. Animal drinking water was also collected from the six previously mentioned farms in both Selangor and Sabah. Animal drinking water was collected directly from the drinking troughs in the farms. Water samples were collected according to the Standard Methods for the Examination of Water and Wastewater protocol.

All water samples obtained were collected in sterile receptacles, transported on ice to the laboratory and analyzed within six to 12 h. A volume of 100 ml of each water sample was analyzed. Each sample was filtered through a sterile 0.45-µm-pore-size membrane (Merck), which was placed on BAA agar and incubated at 37ºC for 24 h. One isolate per sample was randomly picked for analysis. The colonies were then individually transferred to BAA agar and incubated at 37ºC for 24 h.

2.2.1c. Clinical isolates

Bacterial samples were provided by Hospital Serdang, located in Selangor and Hospital Lahad Datu, located in Sabah. The *E. faecalis* isolates were obtained from UTI urine cultures

using standard clinical microbiology tests by authorized clinical personnel. The bacterial samples obtained after the 6-month period were from different patients. A total of 30 *E. faecalis* isolates, from 30 individual patients, was collected and transported to the lab within eight hours in bacteria culture tubes. Cultures were then transferred to BAA agar plates and incubated at 37ºC for 24 h. There was limited clinical information on hospital patients. It was noted however, that these patients were suffering from urinary tract infections and were receiving antibiotic treatment including beta-lactams such as Penicillin and Cephalosporins. Patient rooms had between two to four beds separated by a curtain system, and bathrooms were shared by all occupants of the room.

The distribution of all samples collected from farm animal feces, water sources and hospital patients in Selangor and Sabah, Malaysia is stated in Table 2.1.

Table 2.1: Distribution of samples from farm animal feces, water sources and hospital patients at two different sampling times (June and December 2014) in Selangor and Sabah, Malaysia.

Location	Region	June 2014		December 2014		Total Samples
		Number of animals		Number of animals		
Farm A	Selangor	Chicken	10	Chicken	16	26
		Cattle	-	Cattle	-	
Farm B	Selangor	Chicken	10	Chicken	14	24
		Cattle	-	Cattle	-	
Farm C	Selangor	Chicken	-	Chicken	-	20
		Cattle	10	Cattle	10	
Farm D	Sabah	Chicken	-	Chicken	-	20
		Cattle	10	Cattle	10	
Farm E	Sabah	Chicken	-	Chicken	-	20
		Cattle	10	Cattle	10	
Farm F	Sabah	Chicken	-	Chicken	-	10
		Cattle	-	Cattle	10	
		Number of bottles (100ml each)		Number of bottles (100ml each)		
Klang River	Selangor	20		10		30
Sewage wastewater treatment plant A	Selangor	10		5		15
Sewage wastewater treatment plant B	Selangor	10		5		15
Farm A	Selangor	-		7		7
Farm B	Selangor	-		7		7
Farm C	Selangor	-		7		7
Farm D	Sabah	-		7		7
Farm E	Sabah	-		7		7
Farm F	Sabah	-		5		5

Table 2.1 (continued): Distribution of samples from farm animal feces, water sources and hospital patients at two different sampling times (June and December 2014) in Selangor and Sabah, Malaysia.

		Number of patients		**Number of patients**		
Serdang Hospital (Room A)	Selangor	Female	1	Female	1	3
		Male	-	Male	1	
Serdang Hospital (Room B)	Selangor	Female	2	Female	1	5
		Male	1	Male	1	
Serdang Hospital (Room C)	Selangor	Female	1	Female	1	3
		Male	1	Male	-	
Serdang Hospital (Room D)	Selangor	Female	1	Female	2	4
		Male	-	Male	1	
Serdang Hospital (Room E)	Selangor	Female	1	Female	1	3
		Male	-	Male	1	
Serdang Hospital (Room F)	Selangor	Female	1	Female	2	4
		Male	1	Male	-	
Lahad Datu Hospital (Room G)	Sabah	Female	-	Female	3	4
		Male	-	Male	1	
Lahad Datu Hospital (Room H)	Sabah	Female	-	Female	2	4
		Male	-	Male	2	
						250

2.2.2 Isolation and Identification of *E. faecalis*

Suspected *E. faecalis* appearing as typical black to brown colonies on BAA agar, indicating esculin hydrolysis, were transferred on Slanetz and Bartley (SlaBa) agar (Oxoid, UK) and identified by growth and biochemical reactions as described by Olutiola et al. (2000).

2.2.3 Confirmation of *E. faecalis* Identity by Sequencing of 16S Ribosomal DNA

All presumptive *E. faecalis* isolates, including the clinical *E. faecalis* isolates obtained from hospital patients, were further characterized by 16S rDNA sequencing to confirm their identity as proposed by Marchesi et al. (1998). The primers used for the 16S rDNA sequencing were 8F, 5′-AGAGTTTGATCCTGGCTCAG-3′, and 787R, 5′-CGACTACCAGGGTATCTAAT-3′ (Ryu et al., 2013). Total DNA was extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis, Malaysia). Primers were obtained from First BASE Laboratories, Malaysia. Species identification was determined from the best-scoring reference sequence of the BLAST output and whether the best-scoring reference sequence in the database had a sequence identity of 98% with e-values 10^{-5} and at least 96% query coverage.

2.2.4 Antibiotic Susceptibility Testing

The minimum inhibitory concentration (MIC) was determined for all *E. faecalis* isolates against a range of antibiotics using the broth microdilution technique according to standard recommendations (Clinical and Laboratory Standards Institute, 2012). The list of antibiotics tested in this study is provided in Appendix I. These antibiotics were chosen because they are

either used in both human medicine and animal husbandry or because previous studies have reported *E. faecalis* resistance to them (Arias, 2010). All antibiotics were purchased from Oxoid (UK) and Nacalai Tesque (Japan). The results were interpreted according to the cut-off levels proposed by CLSI guidelines (Clinical and Laboratory Standards Institute, 2012).

2.2.5 Screening for *vanA* and *vanB* Genes

All isolates were subjected to PCR for *vanA* and *vanB* genes according to Dutka-Malen et al. (1995). Primers were obtained from First BASE Laboratories, Malaysia.

2.2.6 Putative Virulence Markers

All primers for testing the presence of putative virulence markers were selected according to Creti et al. (2004). Primers for all virulence markers tested in this study are listed in Appendix II. Primers were obtained from First BASE Laboratories, Malaysia.

2.2.7 Pulsed-Field Gel Electrophoresis (PFGE) Analysis

PFGE was performed (3 replicates per isolate) subsequent to DNA digestion with *SmaI* (Promega, USA) as described by Weng et al. (2013). The PFGE marker (Promega, USA) containing lambda concatemers and lambda-digested *Hind*III fragments was used as a size standard. Comparison of the PFGE fingerprints was analyzed with Cliqs 1D Pro software (Cliqs 1D Pro, USA).

2.2.8 Statistical Analysis

The prevalence of resistance to each antibiotic among *E. faecalis* isolates from all sources was compared using the chi-squared test. A P-value of <0.05 was considered to be statistically significant. Simpson's index of diversity (D) was calculated (Hunter & Gaston, 1988) to assess the differentiation of *E. faecalis* pulsotypes by PFGE. PFGE analysis was based on Dice similarity coefficient and unweighted pair group method using arithmetic averages (UPGMA) clustering with position tolerance and optimization coefficient of 1.5% (Weng et al., 2013).

2.3 Results

2.3.1 Sample Collection

In this study, one isolate per sample was randomly picked for analysis. This was done to ensure that one isolate represents one sample of the population. A total of 250 *E. faecalis* isolates were obtained throughout this study; 120 from farm animal feces, 100 from water sources and 30 from hospital patients.

2.3.2 Antibiotic Susceptibility Test

Antibiotic resistance patterns of all *E. faecalis* isolates are presented in Figure 2.2 and Figure 2.3. Additional data on the antibiotic resistance profile of *E. faecalis* from all sources tested is available in Appendix III. Of the total isolated *E. faecalis* in this study, 80% were resistant to at least one of the antibiotics tested. Comparison of the prevalence of antibiotic resistance of *E. faecalis* between Sabah and Selangor revealed variable differences in the

proportion of antibiotic resistant *E. faecalis*, depending on the antibiotic tested (Table 2.2).

Isolates from farm animal feces and water sources were most commonly resistant to Tetracycline (Figure 2.2). In contrast, 7 out of 30 clinical *E. faecalis* isolates were found to be resistant to Penicillin (2 isolates), Levofloxacin (2 isolates), Ciprofloxacin (1 isolate), Tetracycline (1 isolate) and Nitrofurantoin (1 isolate). The highest frequency of resistance in this study, (except to Vancomycin and Nitrofurantoin), was found among isolates from farm animal feces. Multi-resistance (≥2 antibiotics) was common amongst isolates from water sources (74%) and farm animal feces (73%) (Figure 2.3). River water held a higher percentage (83%) of multi-resistant *E. faecalis* isolates compared to wastewater (60%). None of the clinical isolates in this study demonstrated multi-resistance.

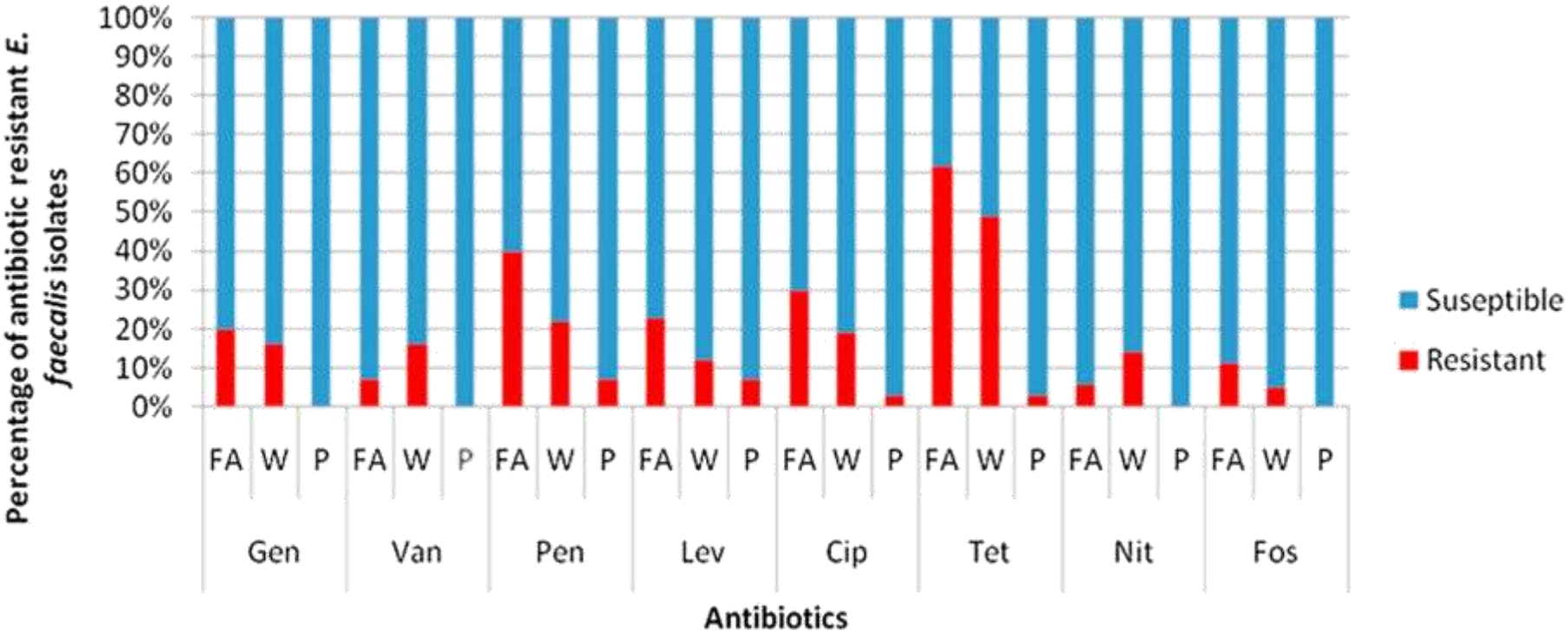


Figure 2.2 Distribution of antibiotics for *Enterococcus faecalis* isolated from farm animal feces, water sources and hospital patients.

Note: GEN = Gentamicin, VAN = Vancomycin, PEN = Penicillin, LEV = Levofloxacin, CIP = Ciprofloxacin, TET = Tetracycline, NIT = Nitrofuratoin, FOS = Fosfomycin

FA: Farm animals, W: Water, P: Patients

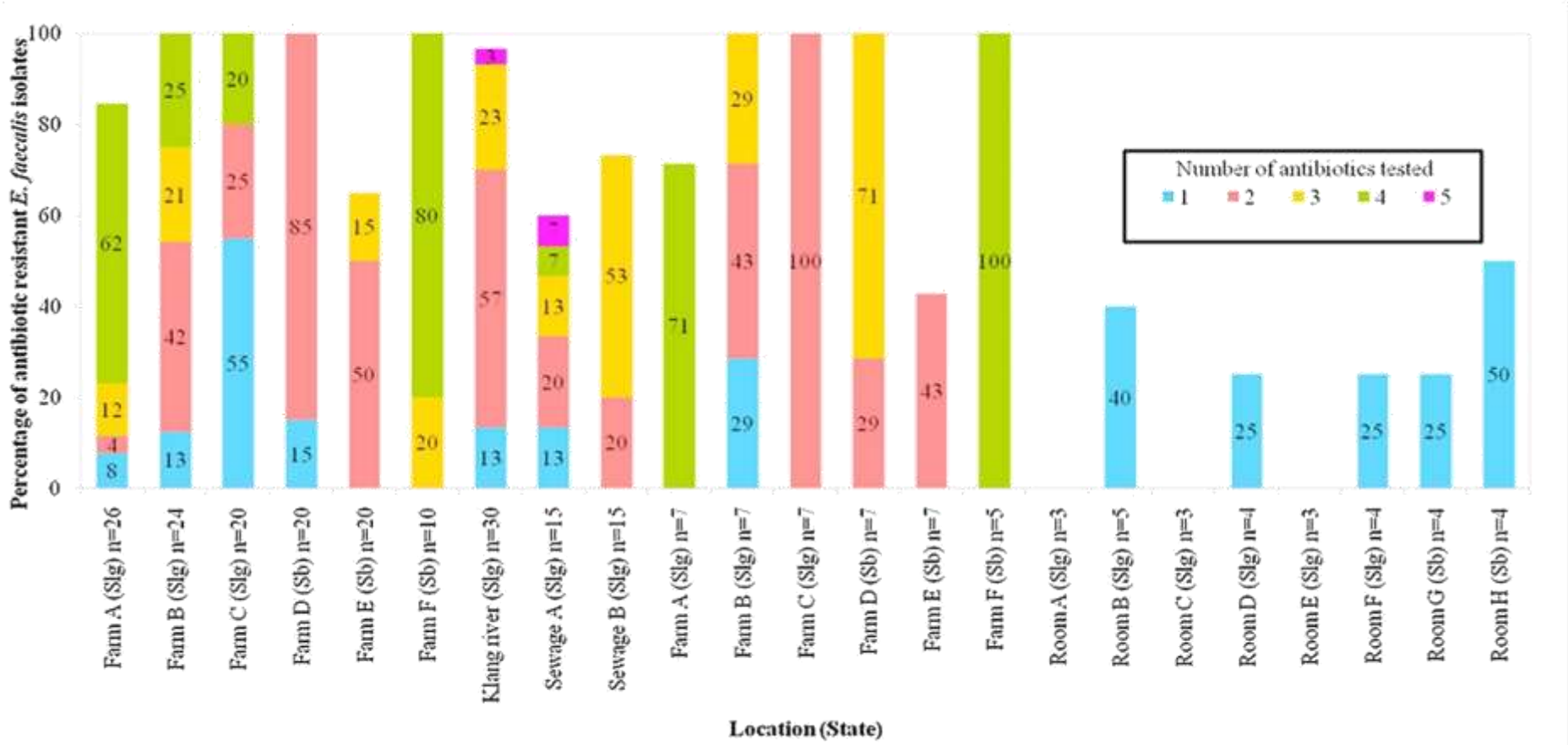


Figure 2.3. Prevalence of single and multi resistant antibiotic (≥2 antibiotics) *Enterococcus faecalis* isolates from farm animal feces, water sources and hospital patients

Note: Slg: Selangor, Sb: Sabah

Table 2.2. Prevalence of antibiotic resistant *Enterococcus faecalis* isolates in Sabah (n=77) and Selangor (113) in percentage (number of isolates).

	VAN	**HL-GEN**	**TET**	**PEN**	**CIP**	**LEV**	**NIT**	**FOS**
Sabah	2.59% (2)	35.06% (27)	67.53% (52)	32.46% (25)	45.45% (35)	5.19% (4)	5.19% (4)	3.89% (3)
Selangor	9.73% (11)	24.78% (28)	49.56% (56)	44.25% (50)	23% (26)	28.31% (32)	7.08% (8)	11.50% (13)

Note: VAN = Vancomycin, HL-GEN = High-Level Gentamicin, TET = Tetracycline, PEN = Penicillin, CIP = Ciprofloxacin, LEV = Levofloxacin, NIT = Nitrofuratoin, FOS = Fosfomycin.

Twenty-four out of 250 isolates (9.6%) in the present study that possessed *van*A were resistant to high levels of Vancomycin (MIC 32 µg/ml to 128 µg/ml) with the exception of one isolate from river water that possessed the *van*A gene but didn't express Vancomycin resistance. There was no specific correlation observed between antibiogram patterns and the groupings obtained by PFGE (Appendix IV).

2.3.3 Prevalence of Virulence Markers

Distribution of nine virulence markers tested in the study varied between sources. All isolates carried at least one of the virulence genes tested, except for one isolate from cattle feces. Virulence gene *gelE* was found to be the most common factor (75.6%) in *E. faecalis* isolates in this study (Table 2.3). Water isolates had a statistically ($P<0.05$) higher prevalence of the *asa1* gene than the other two sources as shown in Table 2.3. A high proportion of isolates from river water were found to have the *asa1*gene (93%), whereas isolates from wastewater

had an equally high prevalence of both *asa1* (83%) and *ace* (83%) genes. Clinical isolates revealed high prevalence of the *esp* (87%) and *gelE* (83%) genes. However, the *EF3314* gene was not present in any of the clinical isolates tested. Isolates with the same PFGE pattern showed different virulence profiles in a few cases in this study (Appendix IV).

Table 2.3. Prevalence of virulence genes among *Enterococcus faecalis* isolates from all sources sampled.

Source	**Location (State)**	**Number of isolates with virulence gene present**								
		esp	***gel*E**	***cyl*A**	***asa*373**	***asa*1**	***ace***	***efa*A**	***EF0591***	***EF3314***
Chicken	Farm A (Selangor) (n=26)	23	15	12	13	14	19	23	7	8
(n=50)	Farm B (Selangor) (n=24)	22	21	0	3	13	13	13	6	5
Cattle	Farm C (Selangor) (n=20)	17	19	7	8	13	12	14	1	1
(n=70)	Farm D (Sabah) (n=20)	16	15	4	8	15	15	13	1	8
	Farm E (Sabah) (n=20)	13	16	6	0	12	12	12	5	5
	Farm F (Sabah) (n=10)	2	7	9	4	6	2	7	10	4
River (n=30)	Klang river (Selangor) (n=30)	9	18	2	9	28	14	16	1	3
Treated sewage wastewater	A (Selangor) (n=15)	1	10	1	2	12	12	10	1	3
(n=30)	B (Selangor) (n=15)	0	9	2	7	13	13	12	2	2

Table 2.3. (contd.) Prevalence of virulence genes among *Enterococcus faecalis* isolates from all sources sampled.

Animal drinking water	Farm A (Selangor) (n=7)	7	5	4	2	5	6	7	3	1
(n=40)	Farm B (Selangor) (n=7)	7	7	0	0	7	7	7	0	0
	Farm C (Selangor) (n=7)	5	7	2	1	4	4	6	0	0
	Farm D (Sabah) (n=7)	2	6	1	0	6	7	1	0	1
	Farm E (Sabah) (n=7)	5	4	4	0	6	6	6	0	0
	Farm F (Sabah) (n=5)	1	5	5	0	5	1	4	5	0
Hospital Serdang	Room A (Selangor) (n=3)	3	3	0	0	3	0	3	3	0
(n=22)	Room B (Selangor) (n=5)	5	5	0	1	3	5	1	0	0
	Room C (Selangor) (n=3)	0	3	0	3	3	3	0	0	0
	Room D (Selangor) (n=4)	3	3	0	0	1	0	3	0	0
	Room E (Selangor) (n=3)	3	0	3	0	0	0	0	0	0
	Room F (Selangor) (n=4)	4	3	0	0	4	0	3	0	0
Hospital LahadDatu	Room G (Sabah) (n=4)	4	4	0	0	0	4	0	4	0
(n=8)	Room H (Sabah) (n=4)	4	4	0	4	4	0	0	0	0
Total		**156**	**189**	**62**	**65**	**177**	**155**	**161**	**49**	**41**

2.3.4 Diversity of *Enterococcus faecalis* isolates by PFGE

The analysis based on the dendrogram generated from the PFGE profiles grouped the *E. faecalis* isolates into 63 pulsotypes (with ≥90% similarity) with 44 clonal populations and 19 isolates that were treated as unique. The PFGE patterns of samples from Selangor and Sabah showed distinct differences. The complete dendrogram is shown in Figure 2.4.

A total of 27 pulsotypes for isolates from farm animal feces, 47 for isolates from water sources and 8 for clinical isolates were obtained. Isolates from the same farm clustered together, with the exception of four isolates in pulsotypes XLII and XLVIII which displayed identical PFGE patterns between Farm A and Farm B, as shown in Figure 2.4. There was no overlapping of PFGE patterns between isolates from chicken and cattle feces. All isolates from animal drinking water showed similar PFGE patterns to those from farm animals with respect to the farms sampled. Isolates from river water and wastewater showed large genetic variability. *E. faecalis* from wastewater did not cluster according to the two wastewater treatment plants that were sampled, although farm samples did cluster according to the source farm. In addition, this study found identical PFGE patterns between two pulsotypes consisting both wastewater and river water isolates as shown in clusters XXIII and XLVI in Figure 2.4. Clinical strains isolated from patients occupying the same room had the same PFGE pattern, which differed from one room to another (Figure 2.4). There was no overlapping of PFGE patterns between the three sources. The PFGE patterns obtained were highly variable for pooled isolates from each of the three sources (Simpson's diversity index; river and sewage wastewater D=0.975, farm animals D=0.951 and hospital patients D=0.901).

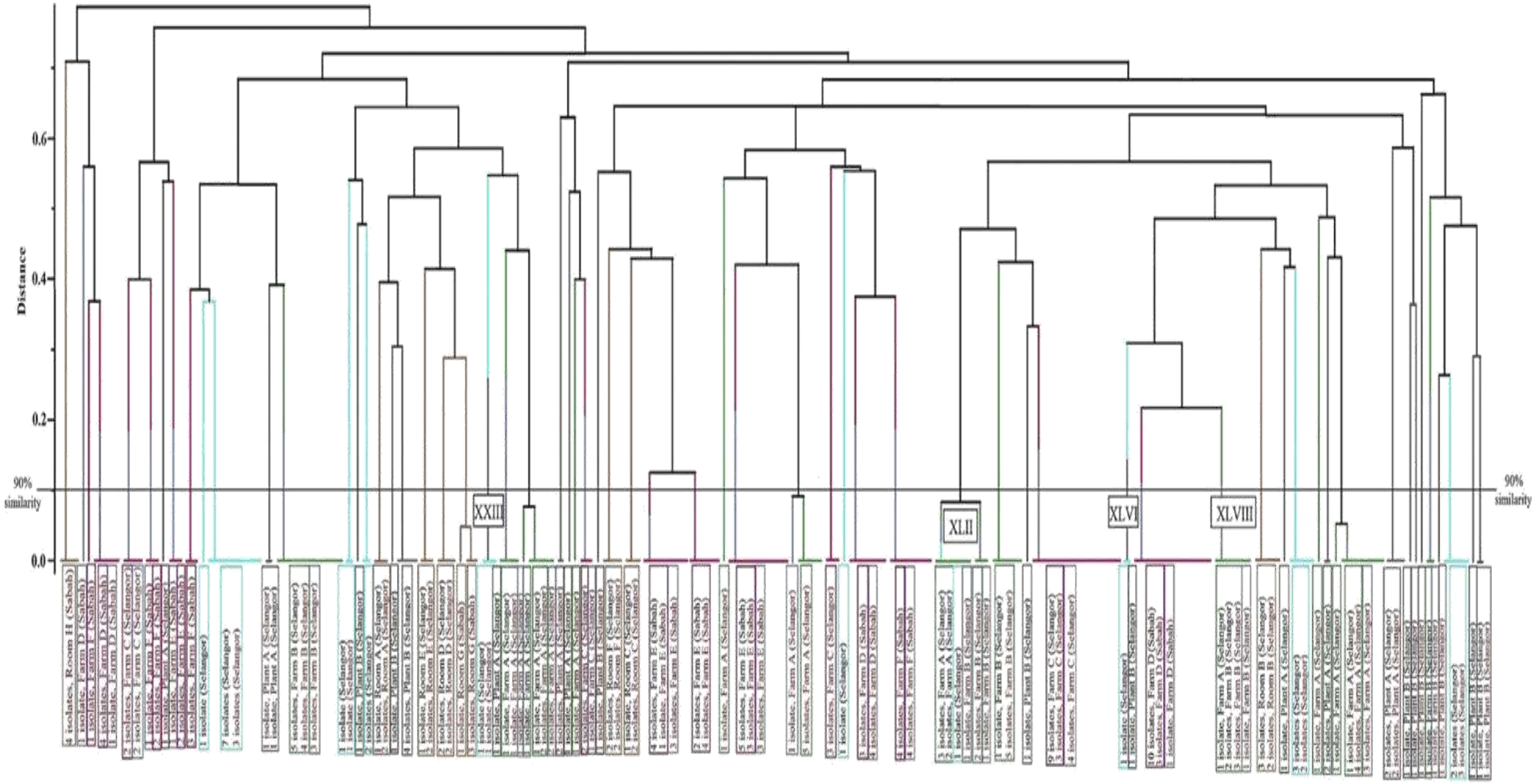


Figure 2.4. Dendrogram of similarity among the observed PFGE macrorestriction patterns of *SmaI*-digested DNA from 250 *Enterococcus faecalis* isolates from farm animal feces, water sources and hospital patients at two different sampling times (July and December 2014). Dendrogram was generated using Dice similarity coefficient and UPGMA clustering with position tolerance and optimization coefficient of 1.5%. A total of 63 pulsotypes were identified at 90% similarity.

Note: Black text denotes samples collected in July 2014; Red text denotes samples collected in December 2014

Cattle Chicken Water from troughs Wastewater River water Hospital patients

2.3.5 Persistence of *Enterococcus faecalis* pulsotypes

All the pulsotypes obtained for the clinical strains from each of the rooms in Selangor persisted after six months (Figure 2.4). Similarly, previously observed PFGE patterns were recovered in all farms after a follow-up period of at least six months (Figure 2.4); in addition, some variant pulsotypes were observed after the six-month sampling period. In contrast, pulsotypes for samples from river water and wastewater after a period of six months showed considerable genetic transience and diversity among *E. faecalis* isolates. The complete dendrogram and correlation between the pulsotypes, antibiogram and virulence genes are presented in Appendix IV.

2.4 Discussion

2.4.1 Antibiotic Susceptibility Patterns of *E. faecalis* and Prevalence of Virulence Markers

A number of studies in Malaysia have reported antibiotic resistant *E. faecalis* from farm animals (Getachew et al., 2012, Getachew et al., 2013), water sources (Hamzah et al., 2011, Praveena et al., 2011, Dada et al., 2012) and clinical sources (Weng et al., 2013). However, so far in Malaysia, little emphasis has been given to the prevalence and diversity of MAR (multiple antibiotic resistant) *E. faecalis* and it was of interest to assess this as the prevalence of MAR is associated with increasing health-care cost and risk of medical complications. Most of the antibiotics used in this study were categorized by the World Health Organization as Rank I, i.e. critically important to human health (Appendix III). Therefore, the high percentage of resistant isolates (80%) observed among *E. faecalis* isolates are of concern for both clinical treatments as well as for the ecological implications of this opportunistic pathogen.

Antibiotic resistant enterococci have been detected previously in livestock in Malaysia

(Getachew et al., 2012, Getachew et al., 2013), and has led to suggestions of an epidemiological link between livestock and human infections (Getachew et al., 2012, Getachew et al., 2013). Tetracycline is one of the classes of antibiotics that are commonly and currently used in animal husbandry and human medicine in the Southeast Asian region (Neela et al., 2013). A high level of Tetracycline resistance in *E. faecalis* isolated from farm animals (62%) in this study was similar to results by Butaye et al. (2001) in Belgium, which reported Tetracycline-resistant *E. faecalis* in almost all isolates (79%) from broilers/chickens. As intestinal inhabitants, enterococci are under selective pressure due to the routine supplement of antibiotics in livestock feed. In Malaysia, there are currently 97 antimicrobials registered for use according to the National Pharmaceutical Control Bureau (NPCB) of the Ministry of Health, Malaysia. Unfortunately, more than half of the antibiotics registered with the Ministry of Health for food animals in Malaysia are not recommended for veterinary use by the World Health Organization (WHO) (Neela et al., 2013). A high percentage of multi-antibiotic resistant *E. faecalis* isolates was obtained from both chicken (82%) and cattle (70%). On farms, many antibiotics are used routinely for disease prevention or for the treatment of avoidable outbreaks of disease. Increasing use of antibiotics that are critically important in human medicine is also a serious concern. Unfortunately, the intensive livestock industries are reluctant to reduce antibiotic use significantly, because this might increase production costs and government officials appear unable to enforce reduction. This calls for a greater awareness of this impending issue and a necessity to curb unnecessary or inappropriate antibiotic use on farm animals.

In previous reports, core issues affecting the bacteriological quality of rivers in Malaysia have been highlighted (Hamzah et al., 2011, Praveena et al., 2011, Dada et al., 2012). The current study, found comparable rates of antibiotic resistant *E. faecalis* isolates from river water (83%) as compared to sewage wastewater (60%). The river sampled in this study flows downstream from the sewage outfalls. It is therefore clear that a more integrated water management and monitoring system is vital for the community. Currently, the disinfection process involved in wastewater treatment in Malaysia

excludes the screening for antibiotic resistant pathogens and antibiotic resistant genes (transposons/intergrons). Therefore, a more stringent assessment of uality of the disinfection process and the effect of usage of such treated water on vegetable/crop sanitation and soil microbiome is needed. Fertilizers made from treated sewage wastewater may possess antibiotics and antibiotic resistant genes, and these fertilizers are commonly used on plantations to produce for food-animals. The results from this study emphasizes the need for further work to be conducted; on the effect of treatment of the wastewater on the content of antibiotic resistant bacteria and their genes.

Only 23% of clinical *E. faecalis* isolates were observed as antibiotic resistant in this study. However, reports in Turkey and Japan have demonstrated that underlying urinary tract diseases predispose patients to repeated UTIs and exposure to antibiotics such as Fluroquinolones, leading to the selection of resistant *E. faecalis* isolates and the development of UTIs which may be caused by Quinolone resistant *E. faecalis* (Yildirim et al., 2007, Yasufuku et al., 2011). Eventhough it is unusual for *E. faecalis* to be Penicillin resistant (Hollenbeck and Rice, 2012), the current study reports a total of 32% Penicillin-resistant *E. faecalis* in Sabah and 44% in Selangor. However, the vast majority of these Penicillin resistant *E. faecalis* were isolated from farm animals and water sources; only two out of 30 clinical isolates were found to be Penicillin resistant. Thus, Ampicillin and Penicillin may still be used to treat enterococcal infections, however, other antibiotic treatments are needed in cases of Penicillin resistance. Although clinical strains of patients in the same room had the same PFGE pattern, the antibiotic resistant profiles were not identical in all the strains from the same patient room (Appendix IV). No correlation was observed between antibiotic treatment and resistance of isolates for specific patients with antibiotic resistant *E. faecalis*. This suggests diversity and an exchange of antibiotic resistant genes among the population in a particular clinical setting. Although this study reports a low number (23%) of antibiotic resistant *E. faecalis* isolates from UTI patients, as compared to farm animals and water sources, health care professionals need to be cautious about prescribing

antibiotics to patients. On top of having strict regulations on antibiotic prescription, good hygiene and suitable infection control procedures need to be reestablished in hospitals and other health care facilities as well.

Although this study reports no multi-resistance isolates from UTI patients, the high numbers of these 'superbugs' in water sources and farm animals are a point of concern. Increasing resistance levels are driven by antibiotic use in all sectors: in humans in the community, on farms and in companion animals. This fact has been established by decades of research and is now fully accepted by organizations like the World Health Organization. The rise of these 'superbugs' can be controlled by implementing surveillance programs and strategies to educate the community of the misuse of antibiotics.

In terms of Vancomycin resistance, a total of 9.5% of isolates from this study were found to be resistant. *van*A and *van*B resistance have been linked with outbreaks of VRE and may be transferred to other organisms (Donabedian et al., 2003). The *van*A phenotype is related to a high level of inducible resistance to Vancomycin and cross-resistance to Teicoplanin, whereas and the *van*B phenotype has variable levels of inducible resistance only to Vancomycin (Yip et al., 2011). The absence of resistant behavior even when the *van*A gene is present, displayed by one of the isolates in this study, was also observed by Ribeiro et al. (2007). The lack of a phenotype may be due to undetected mutations in the gene. Studies have suggested that the occurrence of *van*A in feces of animals may be of risk to humans through direct contact or ingestion of contaminated products as this phenotype is related to a high level of inducible resistance to Vancomycin and Teicoplanin (Song et al., 2009). The risk factors for VRE infection in humans are hospitalization and antibiotic treatment (Song et al., 2009). While only 9.5% of isolates from this study were found to be Vancomycin resistant, there is still a need to curb this impending issue as Vancomycin is used as a drug of last resort. Vancomycin is commonly used for treatment of infections with multiple drug-resistant pathogens and all cases of Vancomycin resistance need to be taken seriously (Yip et al., 2011). It is

therefore necessary to implement control strategies to limit the inappropriate use of antibiotics.

Besides antibiotic resistance, a number of genes suggested to play a role in the virulence properties of *E. faecalis* were assessed in this study as well. The *gelE* gene, which is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other peptides, was found to be the most common marker (75.6%) in *E. faecalis* isolates in this study. Similar frequencies were seen by other researches in Japan (Kanemitsu et al., 2001) and Turkey (Gulhan et al., 2006) from clinical samples.

A high frequency of the presense of the *esp* gene was found in both farm animals (87%) and clinical (78%) *E. faecalis* isolates in this study. The *esp* gene encodes a surface exposed protein and is important for the initial adherence during biofilm formation and urinary tract colonization. The *asa1* gene, which encodes for aggregation substance, was found to be more common in river water (93%) and wastewater (83%) isolates as compared to the other sources. This suggests that *E. faecalis* may localize virulence-related genes to specific reservoirs. The *ace* gene (83%) was also a common virulence marker found in isolates from wastewater. A study by Sidhu et al. (2014) in Brisbane, Australia reported high prevalence of *ace* gene (74%) in fresh water collected after storm events. The *asa* gene was found in 47% of *E. faecalis* isolates in that study compared to 93% reported in this current study. This may be due to substantial geographical differences. The *EF3314* gene reported in this study was not present in any clinical isolates. The EF3312 gene encodes for a putative surface-exposed antigen.

No correlation was apparent between PFGE pulostypes and the virulence profiles of the strains. This observation is in agreement with the findings of Comerlato et al. (2013) who observed that clonal relationship among *E. faecalis* isolates did not influence the distribution of virulence determinants. This suggests that virulence genes in *E. faecalis* strains of same pulsotype patterns may differ. The natural ability of enterococci to readily acquire, accumulate, and share extra-chromosomal elements encoding virulence traits or antibiotic resistance genes lends advantages to their survival under unsusual

environmental stresses and in part explains their increasing importance as nosocomial pathogens. With the exception of clinical *E. faecalis* isolates, these results support the first hypothesis of this chapter, which states there is a high prevalence of putative virulence genes and antibiotic resistance profile in *E. faecalis*. Of all the isolates, 80% displayed resistance to at least one of the antimicrobials tested and all contained virulence genes as detected by PCR.

2.4.2 Genetic Variability of *E. faecalis*

The genetic relationship between *E. faecalis* isolates from the different sources mentioned was analyzed by genotyping using PFGE which has previously been used to identify clonal relationships among isolates (Teixeira et al., 2007).

The clustering of PFGE patterns according to Selangor and Sabah suggests geographical localization. The high diversity observed in each of the three sources (water, farm animals, hospital patients) (D=≥0.901) is not unexpected. Evolutionary process such as mutation, selection and recombination might have played a role in the development of environmental stress tolerance and resulted in the observed high diversity (Baureder et al., 2012). *E. faecalis* is also a ubiquitous colonizer in the gut of mammals and sauropods (Mallon et al., 2002). These results show the adaptive nature of *E. faeacalis* in the three reservoirs. A high genetic variation allows for evolution in response to changing environmental variables, and as a result, surviving in harsh conditions.

This study reports overlapping pulsotypes between Farm A and Farm B (Figure 2.4) which are both chicken farms. The two farms are approximately 5 km distance from each other. Farms traditionally do not operate in isolation and often share resources such as delivery trucks, and this type of interaction probably explains the shared pulsotypes (Kuhn et al., 2003).

All isolates from animal drinking water in this study showed identical PFGE patterns to those from farm animal feces at the same farm. These results may indicate that *E. faecalis* is disseminated or maintained within a herd by contaminated water. This study also reports identical PFGE patterns of isolates from wastewater and river water that were approximately 8 km from each other (Figure

2.1). Waste from hospitals and farms in the areas investigated are discharged into the sewer system. The treated sewage effluent from both treatment plants is discharged into the Klang river (Dada et al., 2012). The results are likely to indicate that some *E. faecalis* cells can survive the sewage treatment process. For each patient room, clinical strains had the same PFGE pattern, which was different from other rooms. This suggests probable hospital to patient transfer, possibly via contact with fixed materials within the specific patient room. However, there was no overlapping pulsoypes of *E. faecalis* between clinical and environmental sources therefore disproving for these samples the second prediction of this chapter which states there are identical pulsotypes in the clinical and environmental *E. faecalis* isolates. This inability to find identical pulsotypes in these samples is not particularly surprising given the wide diversity of pulsotypes available in the environment and the hospitals. It is possible that a far more extensive survey of pulsotypes would have yielded identity between clinical samples and environmental samples.

2.4.3 Genomic Persistence of *E. faecalis*

Mostly identical PFGE pattern was recovered in all farms after at least six months of follow-up although some variant pulsotypes were recovered as well (Figure 2.4). Consequently, the farm animals examined appeared to be sources of *E. faecalis*, whose persistence over time may be a function of survival and proliferation of some resident population. Proper cleaning and disinfection plans should be implemented in farms across Malaysia, with particular emphasis on appropriate drainage and waste disposal. In contrast, a great diversity was observed among *E. faecalis* isolated from water sources after a six-month interval, consistent with the high genetic diversity of *E. faecalis* as seen in previous reports (Praveena et al., 2011, Getachew et al., 2013). Isolates from river and wastewater appear to be transient populations that fluctuate (Kuhn et al., 2003). However, all clinical strains persisted after 6 months in each of the rooms tested. This suggests the patients picked up *E. faecalis* from the individual patient rooms, i.e. hospital bedding, shared bathroom within the room, as a result of infection; this confirms the nosocomial nature of *E. faecalis*.

Similar results were seen by Papaparaskevas et al. (2000) which found persisting clusters of *E. faecalis* PFGE patterns within a specific ward over a period of seven months. This highlights the need for better and more regular cleaning or sanitization of the hospital rooms including changing of bedding and disinfecting shared toilets within the rooms. The persistence of *E. faecalis* strains isolated from farm animals and UTI patients indicates an extensive strengthening of infection control in Malaysian farms and hospitals is needed. A systematic evaluation of the effectiveness of this measures need to be undertaken as well to ensure the policy has been followed thoroughly.

2.5 Conclusion

To the best of our knowledge, this report remains to be the first to describe phenotypic and genotypic characteristics of *E. faecalis* isolates from farm animals, water and patients in East and West Malaysia. Although the study design of this experiment is insufficient to fully address the transmission of *E. faecalis* from farms and environmental sources to hospitals, due to insufficient time and great diversity of *E. faecalis* strains, the present investigation gives insight into the genetic diversity of *E. faecalis* isolates recovered from different sources in Sabah and Selangor, Malaysia. The high antibiotic resistance level with MAR patterns among the strains from environmental samples should be of concern for public health. More encouragingly, hospital isolates showed lower levels of antibiotic resistance. Their relationship to environmental samples could not be delineated. However, if the genotypes found in the environmental samples make their way into hospital then clearly there will be massive problems in treatment. In terms of the predictions set out at the start of the chapter, the findings of high prevalence of antibiotic and virulence determinants were consistent with the first prediction. The inability to find identical pulsotypes between the reservoirs is likely to be a reflection of the number of samples taken relative to the diversity present in *E. faecalis* in Malaysia. A better knowledge of genotypic traits of *E. faecalis* might help in the design of strategies for the prevention and treatment of *E. faecalis* infections. The results of this study also emphasized the need for strict regulations on antibiotic prescription, good hygiene and suitable infection control procedures to be reestablished in farms, wastewater treatment plants, hospitals and other health care facilities.

Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Planned and conducted the majority of analyses, wrote original manuscript and coordinated subsequent manuscript revisions.	80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**
Sadequr Rahman	Participated in the development of ideas and commented on draft manuscripts.
Gary A. Dykes	Participated in the development of ideas and commented on draft manuscripts.
Lee Sui Mae	Participated in the development of ideas and commented on draft manuscripts.
Gan Han Ming	Participated in the development of ideas and commented on draft manuscripts.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature  **Date: 15/03/2018**

Main Supervisor's Signature  **Date: 15/03/2018**

CHAPTER 3

Biofilm Forming Ability and Cell Surface Hydrophobicity Properties of *Enterococcus faecalis* from Clinical and Environmental Origins

The work presented in this chapter represents the following manuscript submitted for peer review:

Daniel, D.S., Lee, S.M., Gan, H.M., Dykes, G.A., and Rahman, S. (2017). Biofilm Forming Ability and Cell Surface Hydrophobicity Properties of *Enterococcus faecalis* from Clinical and Environmental Origins. *Journal of Infection and Public Health*.

Chapter 3 - Biofilm Forming Ability and Cell Surface Hydrophobicity Properties of *Enterococcus faecalis* from Clinical and Environmental Origins

3.1 Introduction

Enterococcus faecalis is found in a variety of environments including soil, water, plants, and animals (Mallon et al., 2002). These bacteria are also members of the commensal human flora and are present in the colon in numbers as high as 10^8 CFU per g of feces (Huycke et al., 1998). *E. faecalis* may become an opportunistic pathogen in individuals whose immune systems are compromised (Texeira & Merquior, 2012). *E. faecalis* infection is also associated with the use of urinary and intravascular catheters. It ranks among the most common nosocomial agents infecting the bloodstream, surgical sites, and urinary tract (Diani et al., 2014).

Bacterial biofilms are either monospecific or multispecific bacterial communities attached to a biotic or an abiotic substrate and encased in a self produced matrix such as an extracellular polymeric substance (EPS) matrix that may be composed mostly of carbohydrates and protein as well as smaller amounts of extracellular DNA (eDNA) and other extracellular components (Donlan & Costerton, 2002, Whitchurch et al., 2002, Teh et al., 2014, Sarjit et al., 2015). Not only do biofilms play an important role in the pathogenesis of several chronic infections, such as bacterial endocarditis, infectious kidney stones, and cystic fibrosis (Parsek & Singh, 2003), but they are also central to nosocomial infections related to indwelling medical devices (Donlan & Costerton, 2002). *E. faecalis* has been reported to produce biofilms on various kinds of indwelling medical devices, such as artificial hip prostheses, intrauterine devices, prosthetic heart valves, central venous catheters, and urinary catheters (Donlan, 2002).

The formation of biofilm depends on multiple genetic elements and is controlled by environmental factors such as nutrient levels and temperature. Evaluation of biofilm formation in different conditions should help us to better understand the mechanisms by which bacteria adapt to environmental stresses and colonize different niches (Sarjit et al., 2015). Although a few studies have investigated the biofilm properties of enterococci, such studies are typically limited to food products and clinical settings, and studies of a limited geographical area (Diani et al., 2014, De Silva et al., 2015). As such, this study was carried out to determine the influence of prior modes of growth and environmental conditions by varying energy availability (via glucose supplementation), incubation temperature and incubation duration on biofilm formation among *E. faecalis* strains isolated in Malaysia belonging to different reservoirs (water sources, farm animal feces and hospitalized UTI patients). Biofilm formation was assayed using the crystal violet staining method and measuring the optical density (O.D.) of each well containing *E. faecalis* strains that produced biofilm under different growth conditions.

An essential step of biofilm formation is the attachment of microbial cells to surfaces. It is therefore important to understand the factors that contribute to microbial adhesion. Microbial adhesion is affected by the interaction between the abiotic surfaces and bacterial cell surfaces (Goulter et al., 2010). Successful adhesion is achieved when hydrogen bonding, ionic and dipole interactions, electrostatic interactions, hydrophobic and hydrophilic interactions between a cell surface and an abiotic surface are strong and the distance between the cell and the surface is less than 5 mm (Bazaka et al., 2011). Hydrophobic interactions are thought to occur between the cell surface and conditioning film, increasing microbial adhesion (Shi & Zhu, 2009). Adhered cells will proliferate, form EPS and establish themselves by forming a multi-layered community (Bazaka et al., 2011). Therefore, the ability of the various strains to

attach to, and form biofilm on different abiotic surfaces was also investigated. In addition, the relationship between cell surface hydrophobicity, attachment and biofilm formation of these strains were examined. The hydrophilic nature of *E. faecalis* has been reported previously (Van Merode et al. 2006). The experiments involve attachment and biofilm formation assays on abiotic surfaces commonly used in healthcare settings (stainless steel, polyurathene and silicone tube) by using the plate count method, and the hydrophobicity of the bacterial surfaces was measured using the sessile drop method and a goniometer.

To summarize, the predictions being tested in this chapter are

1. *E. faecalis* isolates from diverse environments show similar abilities to form biofilms and relative influence of growth conditions on the thickness of the biofilm produced is similar.

2. *E. faecalis* isolated from diverse environments show similar relative attachment abilities to diverse abiotic surfaces. The attachment ability depends on the hydrophobicity/hydrophilicity of the isolates and there will be little difference in the hydrophobicity/hydrophilicity of the isolates from different reservoirs.

Analysis of the data obtained will allow a more refined hypothesis and predictions to be put forward for further testing in the future.

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions

E. faecalis strains (n = 63) recovered from water sources (n = 29), farm animal feces (n = 26) and UTI patients (n = 8) in Selangor (Peninsular Malaysia) and Sabah (East Malaysia) were examined in this study. The strains used in this study were selected on the basis of different pulsed-field gel electrophoresis (PFGE) pulsotypes reported in the previous chapter of this thesis (Section 2.3.4) (Daniel et al., 2017). The antibiotic resistance profile and virulence genes of these strains are also included in the above mentioned published data shown in

Appendix V. Out of the 63 strains tested, 49 were found to be resistant to at least one antibiotic. The demographic distribution of the strains is mentioned in Appendix V. All *E. faecalis* strains were phenotypically identified by growth and biochemical reactions as described by Olutiola et al. (2000) and further characterized by 16S rDNA sequencing as a confirmation from phenotypic testing (Marchesi et al., 1998). For long term storage, strains were cryopreserved at -80°C in nutrient broth (Oxoid, UK) and 40% glycerol (R&M Chemical, Malaysia).

3.2.2 Biofilm formation assay under different growth conditions

Biofilm formation of *E. faecalis* strains was quantified using the microtiter plate assay as described by Marinho et al. (2013) with slight modifications. Briefly, all the strains were grown in Tryptic Soy Broth (TSB; Oxoid, UK) for 18 h at 37°C. After incubation, the bacterial cultures to be tested were diluted 1:10 in TSB. The concentration of cells in the suspension was adjusted to estimated 10^8 cfu/mL using a spectrophotometer at 600nm (OD600). Aliquots of 20 µL of each dilution was transferred (in triplicate) to the wells of sterile 96-well flat bottom polystyrene microtiter plates (TPP®, Swizerland) filled with 180 µL of TSB or TSB supplemented with 1.0% glucose (TSBG; Becton, Dickinson and Company, USA). The negative control wells (in triplicate) contained 200 µL of broth only. The inoculated plates were then incubated aerobically for 24 h, 48 h and 72 h at 28°C and 37°C. After incubation, the plates were turned over to dispose the contents of the plates, and the wells were thoroughly washed three times with sterile Phosphate Buffered Saline (PBS; First Base, Singapore) (pH 7.2). The adherent bacterial cells were then stained with 200 µL of 0.5% (w/v) crystal violet (Becton, Dickinson and Company, USA) stain per well for 10 min. After staining, the plates were washed three times with 300 µL/well of sterile distilled water. The plates were air dried and the stained adherent bacterial cells from each well were then resolved with 250 µL 33%

glacial acetic acid. The optical density (O.D.) of each well was measured at 590 nm using an automated ELISA reader (Tecan, Malaysia). The cutoff O.D. (ODc) was defined as three standard deviations above the mean OD of the negative controls. Thus, based on ODc, the *E. faecalis* isolates were classified into four categories: (i) non-biofilm producers: O.D. of test isolate ≤ODc; (ii) weak biofilm producers: O.D. of test isolate ≤(2 x ODc); (iii) moderate biofilm producers: O.D. of test isolate ≤(4 x ODc); (iv) strong biofilm producers: O.D. of test isolate N (4 x ODc). Experiments for biofilm formation in TSB and TSBG media at different temperatures and duration of incubation period were performed at least three times for each strain. *Staphylococcus epidermidis* American Type Culture Collection 35984 was used as the positive control, as it is classified as a strong adherent and has been used successfully in research studies of biofilm formation by enterococci (Hufnagel et al., 2004, Marinho et al., 2013).

3.2.3 Attachment and biofilm formation assays on different abiotic surfaces

The ability of *E. faecalis* strains to attach to, and form biofilm on different abiotic surfaces (stainless steel, polyurethane and silicone tube) was determined as described by De Silva et al. (2015) with slight modifications. Before being used in the attachment and biofilm assay, stainless steel slides (75 x 25 mm; type 302, #4 finish; Fuji, Malaysia), polyurethane coupons (75 x 25 mm; Oriken, Malaysia) and silicone tubing (30 cm length, 0.33 mm internal diameter; Yureka Sdn. Bhd., Malaysia) were soaked in acetone for 30 min and rinsed in distilled water and finally sterilized by autoclaving. Stainless steel slides and polyurethane coupons were incubated with 20 mL of bacterial cell suspension, with a cell density of approximately 10^8 cfu/ml, for a period of 1 h for the attachment assay and 6 days for the biofilm assay. With regards to *E. faecalis* attachment and biofilm formation on silicon tubing, a

continuous flow method established by Levering et al. (2016) was used with slight modification to accommodate an artificial bladder in a vertical orientation. Briefly, 100 mL of TSB cultures containing individual *E. faecalis* strains were pumped through a 20 cm section of the silicone tube that has an internal volume of 1 ml. The bacteria were left for 1 h to allow attachment to the silicone tube. The model was then run continuously at a flow rate of 0.5 mL/min supplied via peristaltic pumping for 6 days. Silicone tubes were then dissected in the middle section (75 mm long sections) and filleted in half. All biofilm growth was conducted in a sterile biosafety cabinet. The sterility of the prototype was confirmed by control runs without bacterial inoculation; no deposition was visually observed and microscopic examination confirmed no biofilm was formed on the control samples.

The evaluation of the adhesion and biofilm formation was performed using the plate count technique (De Silva et al., 2015). After incubation, the stainless steel slides, polyurethane coupons and filleted silicone tube sections were rinsed twice with PBS to remove planktonic cells, and thoroughly swabbed using sterile cotton swabs (Puritan, UK). The swabs were then immersed in 5 mL of PBS followed by vortexing (Copens Scientific Sdn. Bhd., Malaysia) for 2 min to remove the sessile cells. The resulting solution was serially diluted in PBS and plated on Tryptic Soy agar (TSA; Oxoid, UK); the plates were incubated at 37°C for 24 h. The experiment was repeated three times for each strain.

3.2.4 Bacterial surface hydrophobicity assay

The hydrophobicity of the bacterial surfaces was measured using contact angle measurement (CAM) using the sessile drop method and a goniometer (Model 250, Rame-Hart Inc., USA) as previously described by Lopes et al. (2012) and Teh et al. (2016) with slight modifications. A bacterial lawn was prepared by filtering 10 ml of bacterial suspension in

sterile distilled water that was adjusted to an OD_{550} of 1 ± 0.2 The suspension was then filtred through a membrane filer (0.45 µm pore diameter, 25 mm filter diameter; Millipore). The bacterial cell-filters were air-dried for 30 min, then attached to glass slides using double sided adhesive tape and dried in desiccators overnight (dessicant from Sigma-Aldrich, USA). A drop of sterile distilled water was placed on the filter using a 10 µl syringe fitted with a needle gage (Rame-Hart Inc., USA) and the contact angles were analyzed by a curve fitting method using the tangent approximation through a goniometer (Model 250, Rame-Hart Inc., USA) with the aid of DROPimage software (Rame-Hart Inc., USA). For each bacterial strain, at least 3 drops of sterile distilled water were deposited onto each of three independently prepared filters and the mean values were reported in this study. Contact angles of more than 50 were regarded as indicating hydrophobicity and hydrophilicity is used to denote the opposite.

3.2.5 Statistical analysis

All experiments were performed in triplicate. Before applying the statistical analysis, the average of each replicate was calculated. This average corresponds to an independent experiment.

All investigated variables were subjected to an analysis of variance (ANOVA), and pairwise comparisons of the means were conducted using Tukey's post hoc test at a 95% confidence level. Statistical significance was defined as $p<0.05$. The statistical analyses were performed using the SPSS 18 software.

3.3 Results

3.3.1 Effect of glucose, temperature and incubation duration on biofilm formation

Results from this study showed that biofilm formation of all *E. faecalis* strains were significantly higher ($p<0.05$) in TSB supplemented with 1% glucose (TSBG) as compared to TSB without glucose (Figure 3.1) in all tested conditions (28°C and 37°C for 24 h, 48 h and 72 h). In addition, this study also reports a higher percentage of strong biofilm producing *E. faecalis* isolated from UTI patients and farm animal feces as compared to strains from water sources when supplemented with 1% glucose. The effect of different growth temperatures on biofilm formation of *E. faecalis*, which is relevant to the reservoir of interest [humans (37°C), farm animals (~37 - 42°C) and surface water (~28°C)], was studied by assaying biofilm formation at 28°C and 37°C. However, no significant difference was seen in biofilm formation at different temperatures. In terms of incubation duration, a significant increase in the number of biofilm-producing *E. faecalis* strains was seen when assayed at 48 h as compared to 24 h ($p<0.05$). However, there was no significant difference observed in the number of biofilm producing strains between 48 h and 72 h.

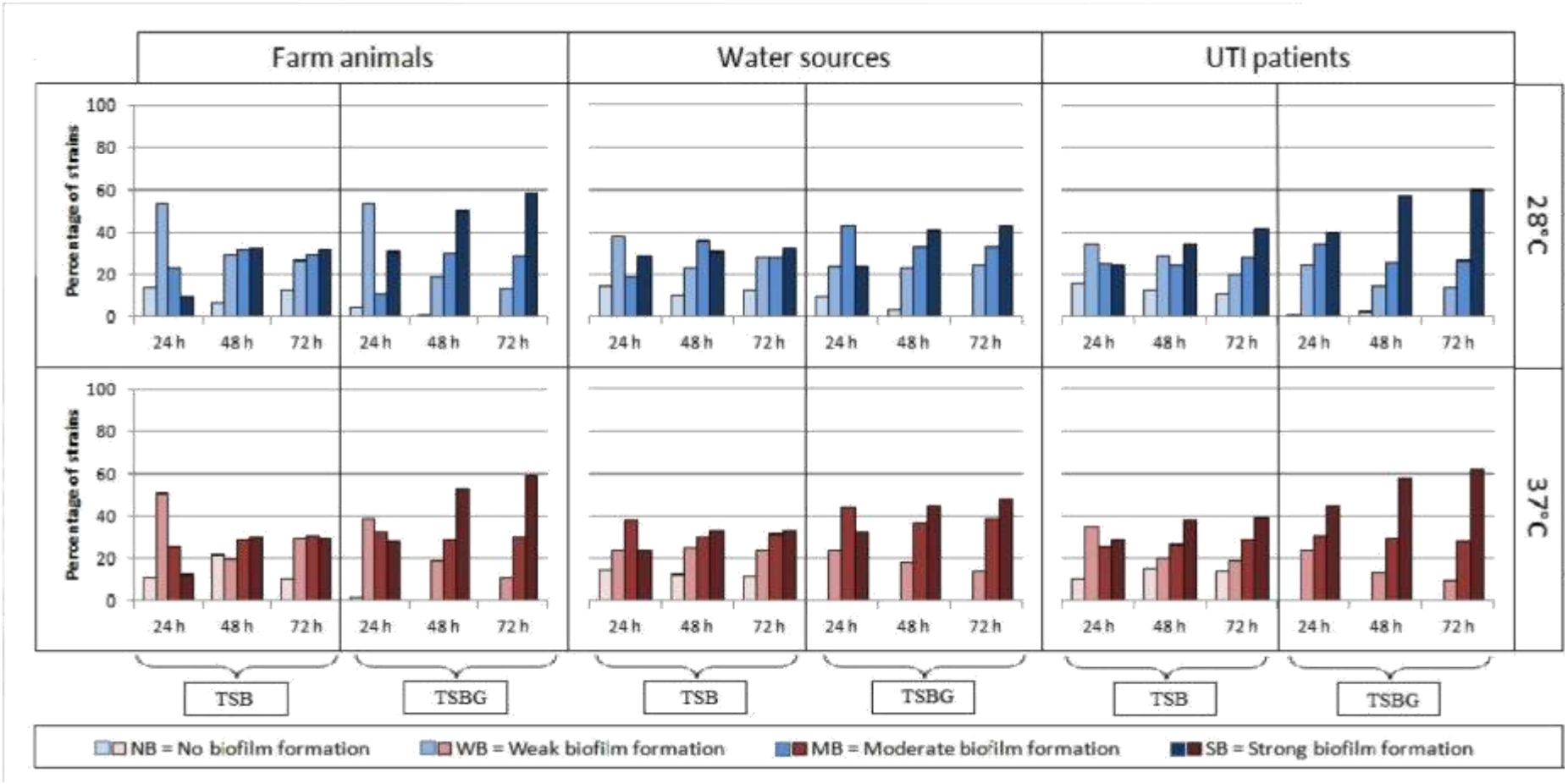


Figure 3.1. The capacity of biofilm producing *E. faecalis* strains isolated from farm animal feces, water sources and hospitalized UTI patients, at different incubation temperatures (28°C and 37°C) in the absence or presence of 1% glucose for varying incubation durations (24 h, 48 h and 72 h) at an optical density (OD) measurement of 590 nm. Strains were classified into four categories: (i) non-biofilm producers: O.D. of test isolate ≤ODc; (ii) weak biofilm producers: O.D. of test isolate ≤(2 x ODc); (iii) moderate biofilm producers: O.D. of test isolate ≤(4 x ODc); (iv) strong biofilm producers: O.D. of test isolate N (4 x ODc). The cutoff O.D. (ODc) was defined as three standard deviations above the mean OD of the negative controls.

3.3.2 Attachment and biofilm formation on different abiotic surfaces

In the present study, all the *E. faecalis* strains tested attached to and formed biofilm on all the three abiotic surfaces used (Figure 3.2). There was a positive correlation in the bacterial count between attachment and biofilm formation on each of the three abiotic surfaces tested. The complete table of the correlation between cell surface hydrophobicity, attachment and biofilm production is reported in Table 3.1 together with details on source of the strains.

The values obtained for all *E. faecalis* strains tested in this study ranged from contact angles of 30.11° to 35.75°, indicating that the strains are mostly hydrophilic in nature. Contact angle measurements for the abiotic surfaces tested in this study varied; 74.6° for silicone tube (hydrophobic), 72° for stainless steel (hydrophobic) and 50° for polyurethane (hydrophilic). In the present study, a significant positive correlation ($p<0.05$) was observed between cell surface hydrophilicity and attachment as well as biofilm forming ability of *E. faecalis* strains on all abiotic surfaces tested.

Figure 3.2 presents results on the correlation between the degree of attachment, biofilm formation and hydrophobicity of all *E. faecalis* strains in this study on the three abiotic surfaces tested. Our study revealed that strains from UTI patients were slightly more hydrophilic in nature (30.11° – 31.73°) when compared to strains from water sources and farm animal feces (30.24° - 35.75°), however it was not a statistically significant difference.

In addition, it is evident from Figure 3.2 that strains from UTI patients have a higher degree of attachment and biofilm formation particularly on polyurethane (10.02 – 15.71 log CFU/cm^2 and 10.23 – 15.72 log CFU/cm^2, respectively), which is more hydrophilic compared to the other two abiotic surfaces.

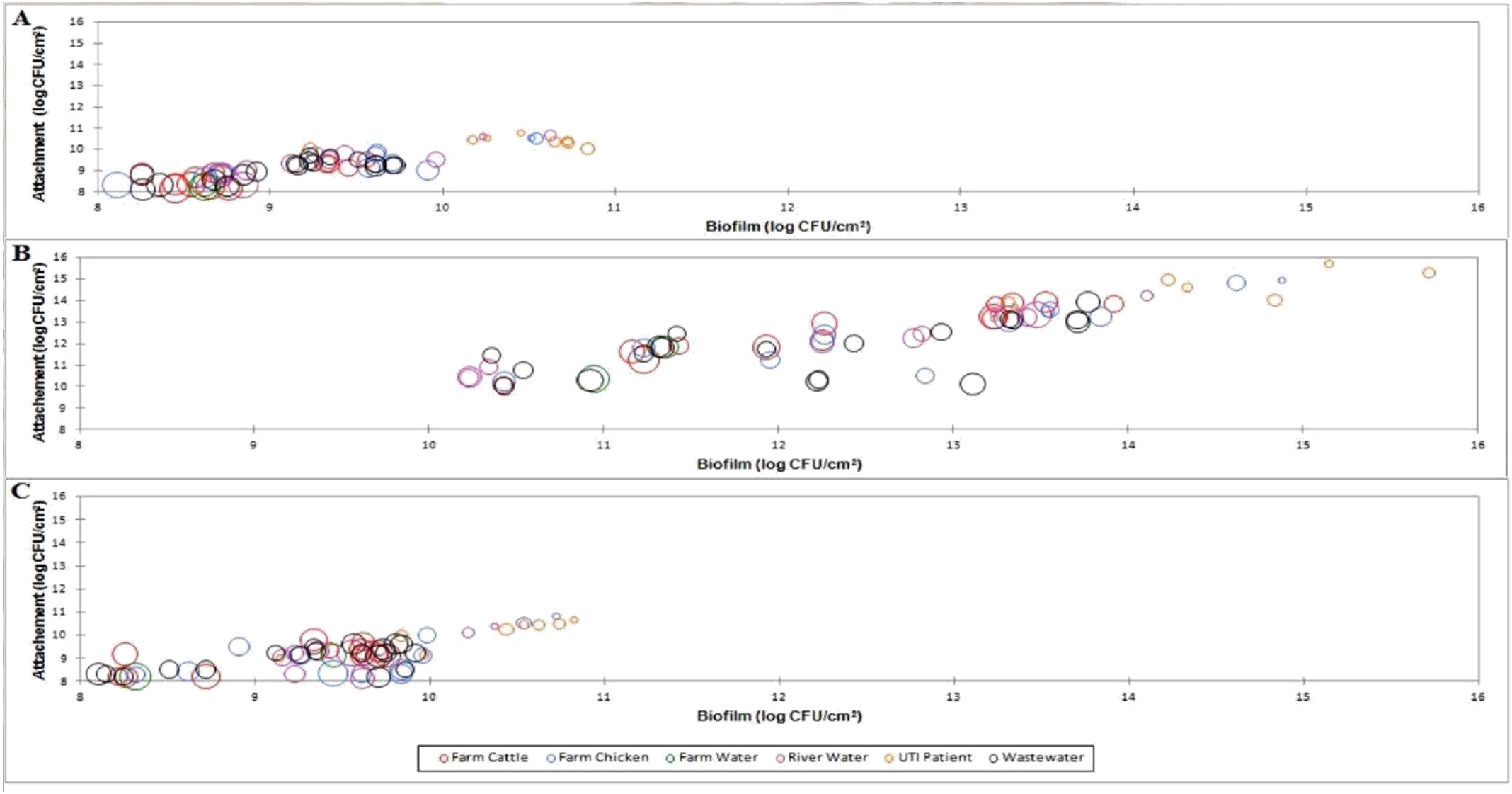


Figure 3.2. Correlation between the degree of attachment, biofilm formation and cell surface hydrophobicity of all *E. faecalis* strains isolated from farm animal feces, water sources and hospitalized UTI patients, on three abiotic surfaces: A) Stainless steel, B) Polyurethene, C) Silicone tube. Note: Cell surface hydrophobicity is denoted by the ring sizes; the larger the ring, the more hydrophobic the cell surface.

Table 3.1. Bacterial surface hydrophobicity, attachement and biofilm formation of 63 *Enterococcus faecalis* strains from various sources (Water sources, farm animal feces and UTI patients). Note: Strain ID's in black font indicate sampling was conducted June 2014 and red font indicate sampling was conducted in December 2014.

E. faecalis strain	Source	Site	Location	Bacterial surface hydrophobicity (°)	Attachment (log CFU/cm^2)			Biofilm formation (log CFU/cm^2)		
					Stainless steel	Polyurethene	Silicone tube	Stainless steel	Polyurethene	Silicone tube
R6	River	Selangor		30.24 ± 0.35	10.63 ± 1.96	13.46 ± 0.07	10.43 ± 0.45	10.23 ± 0.81	13.23 ± 0.95	10.37 ± 0.72
R15				31.12 ± 4.99	10.69 ± 0.14	14.21 ± 0.82	10.13 ± 0.26	10.62 ± 0.81	14.11 ± 0.22	10.22 ± 1.73
R13				32.67 ± 0.17	9.52 ± 0.27	10.93 ± 0.15	9.45 ± 0.82	9.96 ± 1.34	10.34 ± 1.11	9.71 ± 0.23
R27				32.74 ± 0.92	9.52 ± 0.12	13.23 ± 1.73	8.31 ± 0.55	9.34 ± 0.34	13.42 ± 0.66	9.23 ± 2.35
R4				32.93 ± 1.17	9.33 ± 2.15	10.41 ± 1.73	9.05 ± 0.62	9.12 ± 0.54	10.23 ± 0.61	9.16 ± 0.12
R19				33.29 ± 1.95	8.99 ± 0.92	12.23 ± 0.82	9.31 ± 0.45	8.86 ± 0.45	12.77 ± 0.36	9.36 ± 1.23
R24				33.66 ± 1.27	8.83 ± 0.64	10.43 ± 0.72	8.11 ± 0.76	8.71 ± 1.63	10.23 ± 0.36	9.62 ± 1.25
W3	Wastewater	Plant A	Selangor	32.45 ± 0.73	9.82 ± 0.43	12.43 ± 1.99	9.25 ± 0.62	9.43 ± 0.23	12.82 ± 0.58	9.23 ± 0.16
W22		Plant B	Selangor	32.59 ± 2.22	9.68 ± 0.72	11.46 ± 0.85	9.52 ± 0.45	9.23 ± 0.23	10.36 ± 0.15	9.34 ± 1.25
W17		Plant B	Selangor	32.63 ± 4.05	9.63 ± 0.58	12.43 ± 0.27	9.23 ± 0.12	9.35 ± 0.76	11.42 ± 0.99	9.12 ± 0.92
W7		Plant B	Selangor	32.66 ± 3.94	9.54 ± 0.91	11.74 ±0.82	8.54 ± 0.46	9.51 ± 0.78	11.93 ± 1.36	9.86 ± 1.52
W6		Plant A	Selangor	32.74 ± 4.13	9.45 ± 0.72	12.01 ± 0.53	8.52 ± 0.23	9.22 ± 0.12	12.43 ± 2.67	8.51 ± 0.61
W30		Plant B	Selangor	32.74 ± 0.78	9.37 ± 0.17	10.76 ± 1.86	9.32 ± 0.66	9.25 ± 0.51	10.54 ± 0.24	9.35 ± 0.62
W28		Plant A	Selangor	32.78 ± 0.14	9.31 ± 0.23	10.32 ± 0.97	8.31 ± 0.71	9.15 ± 0.12	12.23 ± 0.36	8.15 ± 2.15
W21		Plant B	Selangor	32.82 ± 0.61	9.23 ± 0.73	11.52 ± 0.47	8.21 ± 0.31	9.71 ± 1.34	11.23 ± 0.19	8.25 ± 0.23
W26		Plant B	Selangor	32.94 ± 0.68	9.31 ± 0.24	10.02 ± 1.22	9.23 ± 0.91	9.61 ± 1.78	10.43 ± 0.62	9.61 ± 0.61
W8		Plant A	Selangor	32.96 ± 0.47	9.30 ± 0.45	13.07 ± 0.37	8.52 ± 0.26	9.61 ± 0.76	13.34 ± 0.96	8.72 ± 0.21
W16		Plant A	Selangor	33.13 ± 0.38	9.11 ± 0.85	13.13 ± 0.88	9.23 ± 0.29	9.61 ± 1.37	13.32 ± 0.71	9.92 ± 0.83
W13		Plant B	Selangor	33.16 ± 2.58	9.23 ± 1.62	12.54 ± 0.18	9.14 ± 0.67	9.72 ± 0.96	12.93 ± 0.64	9.26 ± 1.34
W1		Plant A	Selangor	33.19 ± 0.15	9.21 ± 0.27	10.24 ± 1.36	9.23 ± 0.43	9.16 ± 0.93	12.22 ± 0.29	9.75 ± 1.23
W10		Plant B	Selangor	33.37 ± 0.26	8.94 ± 0.94	13.11 ± 0.26	9.62 ± 0.72	8.92 ± 0.95	13.71 ± 0.96	9.84 ± 0.62

Table 3.1. (contd.) Bacterial surface hydrophobicity, attachement and biofilm formation of 63 *Enterococcus faecalis* strains from various sources (Water sources, farm animal feces and UTI patients).

W25	Wastewater	Plant B	Selangor	33.65 ± 1.85	8.81 ± 0.61	11.84 ± 0.54	9.41 ± 0.45	8.26 ± 0.73	11.32 ± 0.86	9.73 ± 0.11
W20		Plant B	Selangor	33.71 ± 2.71	8.79 ± 0.12	13.01 ± 0.98	9.61 ± 0.46	8.84 ± 1.25	13.71 ± 0.12	9.81 ± 0.23
W19		Plant A	Selangor	34.1 ± 0.17	8.23 ± 1.52	13.92 ± 0.73	9.61 ± 0.72	8.75 ± 0.96	13.77 ± 0.26	9.57 ± 2.52
W29		Plant A	Selangor	34.24 ± 1.46	8.31 ± 0.36	10.12 ± 0.15	9.11 ± 0.21	8.36 ± 0.25	13.11 ± 0.84	9.71 ± 1.45
W11		Plant B	Selangor	34.26 ± 0.18	8.12 ± 0.82	10.29 ± 0.62	8.34 ± 0.23	8.26 ± 0.23	10.92 ± 0.16	8.11 ± 0.13
W9		Plant A	Selangor	35.29 ± 0.92	8.33 ± 0.91	13.34 ± 0.68	9.23 ± 0.23	8.84 ± 0.46	13.48 ± 0.61	9.56 ± 0.64
F25	Farm water	Farm D	Sabah	34.34 ± 0.46	8.32 ± 1.26	11.83 ± 0.26	9.12 ± 0.34	8.62 ± 0.78	11.35 ± 0.19	9.45 ± 0.62
F24		Farm D	Sabah	35.52 ± 0.13	8.24 ± 2.15	10.34 ± 1.26	8.23 ± 0.35	8.62 ± 0.42	10.94 ± 0.71	8.32 ± 0.23
Ck13	Chicken feces	Farm B	Selangor	30.32 ± 1.67	10.54 ± 0.62	14.99 ± 0.66	10.84 ± 0.81	10.51 ± 0.14	14.88 ± 0.11	10.72 ± 0.81
Ck27		Farm A	Selangor	31.68 ± 0.37	10.51 ± 0.23	13.51 ± 0.71	10.51 ± 0.68	10.54 ± 1.63	13.54 ± 0.52	10.54 ± 0.92
Ck20		Farm B	Selangor	32.28 ± 0.11	9.85 ± 0.51	14.85 ± 0.85	9.98 ± 0.24	9.62 ± 0.65	14.62 ± 0.13	9.99 ± 0.52
Ck28		Farm A	Selangor	32.54 ± 1.22	9.77 ± 0.42	13.56 ± 0.43	8.32 ± 0.21	9.61 ± 0.65	13.55 ± 0.31	8.32 ± 0.63
Ck40		Farm A	Selangor	32.58 ± 2.71	9.74 ± 0.21	10.52 ± 1.34	9.12 ± 0.54	9.26 ± 1.32	12.84 ± 0.01	9.96 ± 0.73
Ck25		Farm A	Selangor	32.85 ± 2.63	9.36 ± 0.69	11.23 ± 0.85	8.35 ± 0.12	9.71 ± 0.23	11.95 ± 2.16	9.61 ± 1.61
Ck35		Farm B	Selangor	33.18 ± 0.47	9.14 ± 0.18	13.24 ± 0.26	8.34 ± 0.23	9.57 ± 1.25	13.84 ± 0.57	9.84 ± 0.63
Ck3		Farm A	Selangor	33.26 ± 0.53	9.01 ± 1.92	12.43 ± 0.77	9.12 ± 0.26	9.91 ± 0.77	12.26 ± 0.16	9.26 ± 0.32
Ck26		Farm A	Selangor	33.59 ± 1.85	8.91 ± 0.22	10.23 ± 1.35	8.45 ± 0.56	8.71 ± 0.11	10.43 ± 0.35	8.62 ± 1.52
Ck37		Farm A	Selangor	33.63 ± 0.99	8.88 ± 1.26	11.81 ± 0.32	9.51 ± 0.34	8.73 ± 0.02	11.23 ± 0.21	8.91 ± 0.62
Ck6		Farm B	Selangor	33.97 ± 0.61	8.67 ± 0.85	12.06 ± 1.26	8.51 ± 0.23	8.62 ± 0.29	12.25 ± 0.53	9.84 ± 0.15
Ck50		Farm B	Selangor	35.22 ± 0.23	8.34 ± 0.63	13.19 ± 0.93	8.34 ± 0.62	8.11 ± 0.23	13.32 ± 0.34	9.45 ± 1.32
Cw47	Cattle feces	Farm F	Sabah	32.68 ± 2.11	9.53 ± 0.23	13.83 ± 1.26	9.32 ± 0.62	9.56 ± 0.67	13.24 ± 0.36	9.43 ± 1.54
Cw26		Farm E	Sabah	32.75 ± 0.72	9.33 ± 0.13	13.84 ± 0.37	8.23 ± 0.15	9.32 ± 0.91	13.92 ± 0.72	8.22 ± 1.25
Cw62		Farm E	Sabah	32.83 ± 1.72	9.12 ± 0.15	11.90 ± 0.62	9.23 ± 0.23	9.45 ± 0.85	11.43 ± 0.36	9.71 ± 2.61
Cw69		Farm F	Sabah	32.86 ± 2.16	9.31 ± 1.52	10.05 ± 0.32	9.21 ± 0.05	9.34 ± 1.32	10.42 ± 0.93	9.61 ± 0.62
Cw24		Farm D	Sabah	33.54 ± 1.83	8.89 ± 0.23	13.91 ± 1.63	9.72 ± 0.35	8.67 ± 0.25	13.34 ± 0.31	9.62 ± 0.15

Table 3.1. (contd.) Bacterial surface hydrophobicity, attachement and biofilm formation of 63 *Enterococcus faecalis* strains from various sources (Water sources, farm animal feces and UTI patients).

Cw55	Cattle feces	Farm C	Selangor	33.67 ± 0.26	8.83 ± 0.52	13.17 ± 0.26	9.12 ± 0.23	8.26 ± 2.22	13.23 ± 0.22	9.62 ± 1.66
Cw11		Farm E	Sabah	33.73 ± 1.27	8.71 ± 0.32	13.92 ± 0.26	8.21 ± 0.21	8.72 ± 0.34	13.53 ± 0.23	8.26 ± 0.43
Cw37		Farm D	Sabah	33.73 ± 0.94	8.69 ± 0.73	12.19 ± 1.77	9.42 ± 0.32	8.56 ± 0.91	12.25 ± 0.99	9.61 ± 0.72
Cw49		Farm F	Sabah	33.97 ± 1.73	8.56 ± 0.62	11.83 ± 0.62	8.21 ± 0.34	8.67 ± 0.48	11.33 ± 0.42	9.71 ± 0.11
Cw65		Farm E	Sabah	34.4 ± 1.52	8.34 ± 0.23	12.93 ± 0.32	9.81 ± 0.26	8.45 ± 0.19	12.26 ± 0.43	9.34 ± 0.54
Cw52		Farm C	Selangor	34.41 ± 0.54	8.13 ± 0.96	11.62 ± 0.77	9.18 ± 0.16	8.76 ± 0.56	11.16 ± 0.22	8.26 ± 1.23
Cw35		Farm C	Selangor	34.62 ± 0.46	8.34 ± 0.45	11.83 ± 0.62	8.23 ± 0.62	8.54 ± 1.22	11.93 ± 0.36	8.72 ± 0.63
Cw48		Farm F	Sabah	34.67 ± 2.35	8.23 ± 0.32	13.27 ± 2.16	9.13 ± 0.13	8.65 ± 0.62	13.23 ± 0.23	9.76 ± 0.32
Cw33		Farm C	Selangor	35.75 ± 0.55	8.13 ± 1.36	11.26 ± 0.33	9.12 ± 0.12	8.45 ± 0.45	11.23 ± 0.23	9.67 ± 0.43
P20	Patient	Ward A	Selangor	30.11 ± 1.27	10.79 ± 1.24	13.18 ± 1.25	10.67 ± 0.25	10.45 ± 0.21	13.23 ± 0.26	10.82 ± 0.06
P3		Ward C	Selangor	30.26 ± 3.63	10.57 ± 0.14	13.71 ± 0.24	9.03 ± 0.35	10.25 ± 1.35	13.35 ± 0.32	9.14 ± 0.28
P16		Ward E	Selangor	30.52 ± 0.07	10.43 ± 0.23	14.61 ± 0.13	10.49 ± 0.73	10.17 ± 0.64	14.34 ± 0.65	10.54 ± 0.84
P8		Ward D	Selangor	30.73 ± 1.12	10.38 ± 0.82	15.71 ± 0.43	9.16 ± 0.61	10.72 ± 0.32	15.15 ± 0.43	9.97 ± 0.77
P5		Ward F	Selangor	31.18 ± 1.35	10.35 ± 1.36	13.92 ± 0.19	10.48 ± 0.96	10.65 ± 1.35	13.32 ± 0.33	10.74 ± 1.72
P28		Ward H	Sabah	31.27 ± 1.84	10.32 ± 0.32	15.32 ± 0.37	10.46 ± 0.34	10.72 ± 0.42	15.72 ± 0.52	10.62 ± 0.74
P2		Ward B	Selangor	31.29 ± 3.15	10.03 ± 1.24	14.03 ± 0.11	10.26 ± 0.31	10.84 ± 0.97	14.84 ± 0.81	10.44 ± 0.31
P24		Ward G	Sabah	31.64 ± 0.86	9.99 ± 0.62	14.99 ± 0.17	9.99 ± 0.53	9.23 ± 0.63	14.23 ± 0.42	9.84 ± 0.56

3.4 Discussion

3.4.1 Effect of glucose, temperature and incubation duration on biofilm formation

This study reports a higher percentage of strong biofilm producing *E. faecalis* isolated from UTI patients and farm animal feces as compared to strains from water sources when supplemented with 1% glucose. Clearly therefore the presence of glucose supplementation significantly increases the biofilm forming ability of *E. faecalis* strains isolated from humans and animals but has a lower inpact on those from water sources. Our previous work (Daniel et al., 2017) revealed a higher presence of an enterococcal surface protein gene, *esp* in strains isolated from UTI patients (75%) and farm animal feces (65.38%) (Chapter 2, Section 2.3.3). In contrast, only 10.34% of the strains from water sources possessed this gene. The involvement of enterococcal surface protein in biofilm formation in the presence of a higher glucose concentration has been reported before (Tendolkar et al., 2004), in which *esp*-positive *E. faecalis* strains produced significantly more biovolume and thickness of biofilm than their *esp*-negative controls. Our results are consistent with the report of Tendolkar (2004). However, a statistically significant association with the presence of the *esp* gene could not be demonstrated for this samples ($p>0.05$), further analysis such as gene knock-out studies, is required to elucidate a more conclusive statement in regards to *esp*-positive *E. faecalis* strains and biofilm. The association between glucose in the medium and the capacity of biofilm formation has been reported for several other bacterial species as well (Sousa et al., 2008). The nutrient contents of the growth medium, such as glucose, influence biofilm production among various Gram-positive bacteria, including *E. faecalis* (Pillai et al., 2004). Studies have reported an increase in biofilm production of *E. faecalis* cells supplemented with as low as 0.2% of glucose as compared to cells grown in 0% glucose (Baldassarri et al., 2001, Kristich et al., 2004). This result show that *E. faecalis* strains isolated from humans and animals have a biofilm producing ability that is markedly glucose-dependant which may be of concern, particularly in diabetic patients (Tendolkar et al., 2004). The presence of glucose in the blood and urine may be one reason why these microbes are frequently found in urinary tract, wounds, bloodstream, and endocardium infections (Fisher & Philips, 2009).

The presence of glucose and that the presence of pathophysiological glucose concentrations, depending on other environmental variables, may reduce the time that the body has to respond to the pathogen before it is well established in biofilm. In light of this, postoperative glucose management may be critical in controlling the subsequent enterococcal infection rate.

In terms of biofilm formation at different incubation temperatures, this study reports no significant difference. A similar result was obtained by Marinho et al. (2013) and Peter et al. (2013) in which no significant difference was seen in biofilm formation at different incubation temperatures (28°C, 37°C and 45°C). This result substantiates the ability of *E. faecalis* to survive and form biofilms in both clinical and environmental reservoirs regardless of the ambient temperature. The ability of *E. faecalis* to produce biofilm in temperatures relevant to the reservoir of interest [humans (37°C), farm animals (~37 - 42°C) and surface water (~28°C)] has implications for both clinical treatments as well as for environmental measures to control the transmission of this opportunistic pathogen (Marinho et al., 2013).

Although there was a significant difference observed in the number of biofilm producing strains between 24 h and 48 h, there was no significant difference observed between 48 h and 72 h in this study. *E. faecalis* cells that attach irreversibly to the surfaces (i.e. those not removed by gentle rinsing) will begin cell division, form microcolonies, and produce extracellular polymers that define a biofilm within 24 h to 48 h, consistent with the increase in the number of biofilm-forming *E. faecalis* strains from 24 h to 48 h in our current study. However, the number of surface-adhered bacterial cells in this study remained relatively constant from 48 h to 72 h (Figure 3.1). This may suggest that only bacterial adhesion is observed during this period, and no formation of mature biofilm. Mature biofilm formation is noted to occur from 72 h to 144 h after initial adhesion and may take 240 h (De Silva et al., 2015, Meira et al., 2012). Maturity occurs mainly through population density increase as well as

by pronounced production and deposition of extracellular polymers, increasing biofilm thickness (Oliveira et al., 2010). Fully mature biofilms continuously shed planktonic bacteria, microcolonies and fragments of biofilm, which can disperse and attach to other parts of a wound bed or to other wounds, forming new biofilm colonies (De Silva et al., 2015). Matured biofilms also greatly enhance the tolerance of microorganisms embedded in the matrix to the immune system, antimicrobials and environmental stresses. This tolerance may approach complete resistance to factors that would easily kill these same microbes when growing in an unprotected, planktonic state. There is a need to develop methods or devices to quickly detect the presence of biofilm before and after selected treatments, this would help guide researchers and healthcare providers to develop effective biofilm management strategies and aid in the monitoring of treatment progress.

3.4.2 Attachment and biofilm formation on different abiotic surfaces

Stainless steel, polyurethane and silicone tubes are common materials used in various kinds of indwelling medical devices, such as artificial hip prostheses, intrauterine devices, prosthetic heart valves, central venous catheters, and urinary catheters (Donlan, 2002). Biofilm formation by *E. faecalis* on these abiotic surfaces may contribute to the persistence and survival of *E. faecalis* outside the host under environments which are detrimental to them. In the present study, all the *E. faecalis* strains tested attached to and formed biofilm on all the three abiotic surfaces used. This is consistent with previous findings which showed that *E. faecalis* has the ability to form biofilm on a variety of surfaces including stainless steel, polyurethane and silicone (Joyanes et al., 1999, Senechal et al., 2004, De Silva et al., 2015). Andrade et al. (1998) studied the adhesion properties of *Enterococcus faecium* to stainless steel surface and emphasized that in order for biofilm formation to occur, counts above 10^7 CFU/cm^2 are necessary. All strains in this study were observed to have bacterial counts above 10^8 CFU/cm^2 for attachment and biofilm formation on all surfaces tested. There was a positive

correlation in the bacterial count between initial bacterial attachment count and bacterial count after the incubation period (which has been taken as biofilm formation) on each of the three abiotic surfaces tested. Many hospital-acquired infections are associated with biofilm infections of implantable medical devices such as othapaedic prostheses and intravascular catheters. There is also a possibility of attachment and biofilm formation on hospital bedding and other items within a hospital ward. Although the antibiotic susceptibility testing in Chapter 2 (section 2.3.2) did not show a significant pattern in relation to strong biofilm production, the fact that there was a persistence of clinical *E. faecalis* strains in the hospitals (Chapter 2, section 2.3.5) stresses the need for evaluation of various control strategies remediating biofilm colonization of medical devices, and development of new methods for assessing the efficacy of these treatments.

Bacterial cell surface hydrophobicity has also been shown to play an essential role in mediating adherence of bacteria to a variety of surfaces and contributes to biofilm formation (Krasowska & Sigler, 2014). Water contact angles formed by bacterial lawns can be used as a qualitative indication of cell surface hydrophobicity, with lower values (≤50°) indicating a morehydrophilic surface (Krasowska & Sigler, 2014). The values obtained for all *E. faecalis* strains tested in this study ranged from contact angles of 30.11° to 35.75°, indicating that the strains are mostly hydrophilic in nature. Contact angle measurements for the abiotic surfaces tested in this study varied; 74.6° for silicone tube (hydrophobic), 72° for stainless steel (hydrophobic) and 50° for polyurethane (hydrophilic). In the present study, a significant positive correlation ($p<0.05$) was observed between cell surface hydrophilicity and attachment as well as biofilm forming ability of *E. faecalis* strains on all abiotic surfaces tested. In addition, strains from UTI patients have a higher degree of attachment and biofilm formation particularly on polyurethane (10.02 – 15.71 log CFU/cm^2 and 10.23 – 15.72 log CFU/cm^2, respectively), which is more hydrophilic compared to the other two abiotic surfaces. This supports a more accurate rephrasing of the second hypothesis of this chapter to state that *E. faecalis* isolated from clinical and environmental sources has a hydrophilic nature and a

high degree of attachment to abiotic surfaces that are hydrophilic in nature (i.e. polyurathene). Depending on the type of surface, the hydrophilicity of the bacterial cells can increase the propensity of microorganism adhesion. The more hydrophobic cells adhere more strongly to hydrophobic surfaces, while hydrophilic cells strongly adhere to hydrophilic surfaces (Kochkodan et al., 2008, Giaouris et al., 2009, Krasowska & Sigler, 2014). A study by Van Merode et al. (2006) reported the hydrophilic nature of clinical *E. faecalis* strains (23° - 32°) demonstrating a high degree of attachment ($\geq$8.0 log CFU/cm^2) to a hydrophilic surface, glass. The increasing use of medical devices in moderm medicine, from indwelling tubes or catheterization to surgical sutures, has been accompanied by the rise in device-relation infections such as enterococcal infections. As demonstrated by the results of this study, many such devices provide an ideal surface to which microorganisms can attach and form biofilms. Since biofilm-associated infections are extremely difficult to eradicate, this poses a serious concern, often associated with increased mortality and morbidity. Considering polyurathene is used often in medical device applications, and its use continues to grow, one of the solutions to this problem is using implats from anti-biofilm materials that can delay or completely avoid the adhesion of microorganisms. For example, the use of polymeric nanofibers on polysterene surface significantly delays bacterial biofilm formation (Krasowska & Sigler, 2014). Another strategy of preventing surfaces from bacterial colonization is the modification of surfaces by coating them with noble metals, i.e., silver nanoparticles, although the exact mechanism of antimicrobial action of silver is still not completely known (Menno et al., 2011). Cell surface hydrophobicity is an important feature of the adhesion process, hence considering the possibility of regulating this element of pathogenesis is worth the commitment.

3.5 Conclusion

E. faecalis are recognized as a major cause of nosocomial infections and form biofilms that are dependent on multiple genetic and environmental factors. To our knowledge this is the first report on the relationship between biofilm formation and cell surface hydrophobicity by clinical and

environmental *E. faecalis* strains isolated from Malaysia. The results support previous findings that indicate glucose supplementation had a significant effect on the biofilm formation of *E. faecalis* strains. In addition, our study also reports a hydrophilic nature of all strains and a high degree of attachment and biofilm formation particularly on hydrophilic material such as polyurethane. The capacity of strong biofilm-production of *E. faecalis* from various reservoirs is alarming since the biofilm formation contributes to survival, persistence and/or resistance genes in diverse environmental conditions. *E. faecalis* isolated from farm animals and hospitals appeared to show in general better biofilm producing abilities than those isolated from water bodies but these experiments should be repeated with a larger number of samples. There was a clear correlation between the hydrophobicity/hydrophilicity and the biofilm forming ability for the surfaces tested. While the number of studies involving biofilm production have increased in recent years, more research is needed to allow a better understanding of the regulation of biofilm production and its correlation with cell surface hydrophobicity. The key to success may hinge upon a more complete understanding on what makes the biofilm phenotype so different from the planktonic phenotype. Research on microbial biofilms is proceeding on many fronts, with particular emphasis on elucidation of the genes specificaly expressed by biofilm-associated organisms. A more complete understanding of the role of genetic and environmental factors in the development of biofilm may lead to improved strategies for biofilm control among enterococci.

Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Assembled and annotated the genome, performed analysis, wrote original manuscript and coordinated subsequent manuscript revisions	80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Sadequr Rahman	Participated in the development of ideas and commented on draft manuscripts.
Gary A. Dykes	Participated in the development of ideas and commented on draft manuscripts.
Lee Sui Mae	Participated in the development of ideas and commented on draft manuscripts.
Gan Han Ming	Participated in the development of ideas and commented on draft manuscripts.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature  **Date: 15/03/2018**

Main Supervisor's Signature  **Date: 15/03/2018**

CHAPTER 4

Draft Genome Sequencing and Comparative Analysis of *Enterococcus faecalis* Strains from Clinical and Environmental Origins

The work presented in this chapter represents the following publications:

Daniel, D.S., Lee, S.M., Gan, H.M., Dykes, G.A., and Rahman, S. (2017). Draft Genome Sequences of Six *Enterococcus faecalis* Strains Isolated from Malaysian Clinical and Environmental Origins. *Genome Announcement*.

Chapter 4 - Draft Genome Sequencing and Comparative Analysis of *Enterococcus faecalis* Strains from Clinical and Environmental Origins

4.1 Introduction

The Enterococcus, first distinguished as a separate genus by Shleifer and Kilpper-Balz (1984) is a common member of the normal intestinal flora in humans and animals. However, some species in the genus Enterococcus, such as *Enterococcus faecalis*, are leading causes of highly contagious hospital-acquired infections, including urinary tract, intra-abdominal, pelvic, and soft tissue infections, as well as bacteremia and endocarditis. Thus, members of the genus Enterococcus have been extensively studied. The first genome sequence of *E. faecalis* was published by Paulsen in 2003 (Paulsen et al., 2003). The virulence factors present in *E. faecalis* are well established, and include aggregation substances, surface adhesions, sex pheromones, toxin cytolysin, but novel virulence factors continue to be reported (Kayaoglu and Orstavik, 2004).

Comparative genomic analysis can be used to identify genes coding for virulence, antibiotic resistance and gene mobility as well as elucidate the evolutionary relationship among bacteria. The number of complete or draft genome sequences available for *E. faecalis* as of April 2017 is 503, comprising the bulk of enterococcal genome sequences available, as several comparative genomic studies of these species have been conducted (Palmer et al., 2012, Qin et al., 2012). However, there is a poor representation of genomic sequences for enterococci from Malaysia with only approximately 7 assemblies reported (Daniel et al., 2017). To the best of our knowledge, this is the first genomic comparison analysis on *E. faecalis* strains isolated from various sources in Malaysia.

The overall aim of this chapter is focused on the sequencing and subsequent analysis of these 6 *E. faecalis* genomes. The hypothesis is that there are genetic relationships between the isolates which have not been revealed by PFGE analysis (Chapter 2) but which will be revealed by whole genome sequencing. This hypothesis was tested by performing whole genome sequencing and comparative genomic analysis of 6 *E. faecalis* strains isolated from water sources, farm animal feces and hospitalized UTI patients. Comparison of genomic data from these strains with other genomes of *E. faecalis* may also improve our understanding of the virulence factors and pathogenesis present in Enterococcus. The second hypothesis for this chapter is that by comparing the sequences of abundant and poor biofilm producers in these samples, candidate genes involved in biofilm production can be identified. Furthermore, the present study intends to provide insights into the genomes of these strains that have been isolated in Malaysia, and this may lead to novel insights into *E. faecalis* genome organization and genetic history.

4.2 Materials and Methods

4.2.1 Bacterial strains

E. faecalis strains (n=6) recovered from water sources (n=2), farm animal feces (n=2) and UTI patients (n=2) in Selangor (Peninsular Malaysia) were examined in this study. The strains used in this study were selected based on different pulsed-field gel electrophoresis (PFGE) pulsotypes and attachment properties reported previously (Daniel et al., 2017). Two strains from each source (water, farm animal and UTI patients) were picked, in which one from each set had higher attachment and biofilm forming properties than the other. A detailed list of the 6 *E. faecalis* strains with the corresponding background information including attachment properties, biofilm forming properties and demographic distribution is provided in Table 4.1. All *E. faecalis* strains were phenotypically identified by growth and biochemical reactions as

described by Olutiola et al. (2000) and further characterized by 16S rDNA sequencing as a confirmation from phenotypic testing (Marchesi et al., 1998). For long term storage, strains were cryopreserved at -80°C in nutrient broth (Oxoid, UK) and 40% glycerol (R&M Chemical, Malaysia).

Table 4.1. Summary of background information on the 6 *Enterococcus faecalis* strains tested in this study

Bacterial Strains (Original Strain ID)	**Source**	**Bacterial Surface Hydrophobicity (°)**	**Attachment (log CFU/cm^2)**			**Biofilm Formation (log CFU/cm^2)**		
			Stainless steel	**Polyurethane**	**Silicone tube**	**Stainless steel**	**Polyurethane**	**Silicone tube**
S12 (R24)	River water (Selangor)	33.66 ± 1.27	8.83 ± 0.64	10.43 ± 0.72	8.11 ± 0.76	8.71 ± 1.63	10.23 ± 0.36	9.62 ± 1.25
S13 (Ck50)	Chicken feces (Farm B, Selangor)	35.22 ± 0.23	8.34 ± 0.63	13.19 ± 0.93	8.34 ± 0.62	8.11 ± 0.23	13.32 ± 0.34	9.45 ± 1.32
S14 (Ck27)	Chicken feces (Farm A, Selangor)	31.68 ± 0.37	10.51 ± 0.23	13.51 ± 0.71	10.51 ± 0.68	10.54 ± 1.63	13.34 ± 0.52	10.54 ± 0.92
S15 (R6)	River water (Selangor)	30.24 ± 0.35	10.63 ± 1.96	13.46 ± 0.07	10.43 ± 0.45	10.23 ± 0.81	13.23 ± 0.95	10.37 ± 0.72
S16 (P20)	UTI patient (Ward A, Selangor)	30.11 ± 1.27	10.79 ± 1.24	13.18 ± 1.25	10.67 ± 0.25	10.45 ± 0.21	13.23 ± 0.26	10.82 ± 0.06
S17 (P24)	UTI patient (Ward G, Sabah)	31.64 ± 0.86	9.99 ± 0.62	14.99 ± 0.17	9.99 ± 0.53	9.23 ± 0.63	14.23 ± 0.42	9.84± 0.56

4.2.2 Isolation of whole genome DNA

Genomic DNA was extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis, Malaysia). The final DNA concentration was estimated using a Nanodrop 8000 UV-VIS spectrophotometer (Thermo Scientific, Germany) following the instructions of the manufacturer. DNA integrity and the absence of RNA contamination were checked by electrophoresis on a 1% agarose gel.

4.2.3 Genomic sequencing and quality control

The extracted genomic DNA was tagmented with Nextera XT (Illumina, San Diego, CA) according to the manufacturer's instructions and sequenced on the MiSeq desktop sequencer located at the Monash University Malaysia Genomics Facility (2×250 bp run configuration). Genome sequencing of the strains generated paired-end data in FastQ format. Low-quality bases result in misassemblies by interfering in the assembly process. Hence, quality filter is the first and foremost requisite for all downstream computational analyses and results interpretation (Li et al., 2015), therefore additional quality control of raw data using FastQC was performed. The reads were pre-processed using CLC Genomics Workbench 7.0 (CLC bio, Denmark) to obtain clean paired-end data in a FastQ format which was also subjected to quality control using FastQC. The high quality, filtered reads were used for downstream analyses. All subsequent programs mentioned in this study were conducted on a Microsoft Windows platform.

De novo assembly was carried out using CLC Genomics Workbench 7.0. The de novo assembly pipeline was applied with automatic detection of best parameters. After the assembly process, all sequencing reads were remapped to all contigs to update the assembly. All contigs below a length threshold of 500 bp were removed.

4.2.4 Annotation and Subsystem Analysis

Genomes were annotated using the online platform RAST (Rapid Annotation using Subsystem Technology) version 2.0. We choose six RAST subsystems central to our analysis (cell wall and capsule, iron acquisition and metabolism, chemotaxis, phages and mobilome, regulation and cell signaling, virulence and disease) to build a presence/absence map which summarize all pairwise comparisons into a unique list, including genes assigned to the chosen subsystems.

4.2.5 Genome Comparison

We used several methods for whole-genome comparison to identify similarities and differences between each test strain's genome. First we retrieved the annotated genomes from RAST and aligned them against one reference genome (*E. faecalis* ATCC 19433) for each strain using MAUVE version 2.3.1 (Darling et al., 2004). Following this step we performed a multiple whole-genome alignment using the progressive alignment algorithm implemented in MAUVE. We used the output of this alignment to check for rearrangements in each genome. The relationship between the strains was also visualized using a dendrogram based on the gene content (presence or absence). UPGMA clustering was used to calculate the genome clustering through the OrthoANI algorithm (Lee et al., 2016).

4.2.6 Virulence Factor Predictor and Secondary Metabolite Biosynthetic Gene Clusters

Virulence genes were identified using VirulenceFinder (version 1.5), and antibiotic resistant genes using a combination of ResFinder (version 2.1) and the Comprehensive Antibiotic Resistance Database (CARDs). Secondary metabolite biosysntetis gene clusters 64

were identified using the Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH).

4.2.7 CRISPRs

To evaluate the presence of CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) we analyzed the assembled genomes of the six strains using the CRISPR Recognition Tool CRT (Bland et al., 2007).

4.3 Results

4.3.1 General properties of the *E. faecalis* genome

The general genome properties of *E. faecalis* strains S12, S13, S14, S15, S16, S17 and reference genome *E. faecalis* ATCC 19433 was automatically annotated by using the RAST server. The general features of the genomes are summarized in Table 4.2. The genomes of the 6 strains tested ranged in size from 2.84 – 3.06Mb, with *E. faecalis* S15 exhibiting the smallest and *E. faecalis* S14 the largest genome. The genomic GC content of the strains ranges between 37.3-37.6%. CRISPR PEGs (protein encoding genes) were detected in the genome of all strains tested except S15 and S16.

Table 4.2. Genome characteristics of *Enterococcus faecalis* strains isolated from various sources.

Strain	***E. faecalis* ATCC 19433 (reference genome)**	***E. faecalis* S12** (R24)	***E. faecalis* S13** (Ck50)	***E. faecalis* S14** (Ck27)	***E. faecalis* S15** (R6)	***E. faecalis* S16** (P20)	***E. faecalis* S17** (P24)
Number of contigs	11	140	133	90	87	109	230
GC content %	37.4	37.3	37.4	37.3	37.5	37.5	37.6
Accumulated length (bp)	2,881,400	3,002,129	2,934,970	3,065,309	2,846,800	2,855,348	2,860,937
Number of coding sequences[1]	2843	2921	2857	3033	2720	2699	2684
rRNA	12	4	3	3	4	5	7
tRNA[2]	68	59	42	55	37	51	49
Number of subsystems[1]	366	361	353	347	351	352	352
CRISPR locus[3]	1	1	1	1	0	0	1
N_{50}	578,583	38,596	57,066	152,272	81,546	87,184	31,473
Source	Human oral cavity	River water (Selangor)	Chicken Feces (Farm B, Selangor)	Chicken feces (Farm A, Seleangor)	River water (Selangor)	UTI patient (Ward A, Selangor)	UTI patient (Ward G, Sabah)

Information according to [1]RAST; [2]Sequin; [3]CRIPSR

MAUVE analysis showed an overall collinear relationship across the sequenced *E. faecalis* strains (Figure 4.1). Most of the locally co-linear blocks (LCBs) were highly homologous between the 6 assemblies, despite several gaps in between the blocks.

Major subsystems and metabolic pathways were conserved between *E. faecalis* strains, however the number of genes was increased in *E. faecalis* ATCC 19433 in several functional categories compared to the 6 strains tested (Figure 4.2); such as cell wall and capsule, and regulation and cell signaling subsystems. *E. faecalis* strain S17 (UTI patient) had the lowest number of genes under the Phages, prophages and transposable subsystem as compared to the other strains. Strain S12 (river water) was found to have higher number of genes (~85) in the virulence, disease and defense subsystems as compared to the other strains. At least 380 of annotated genes per strain (about 15% of the total number of annotated genes) in this study had a designated role for carbohydrate utilization.

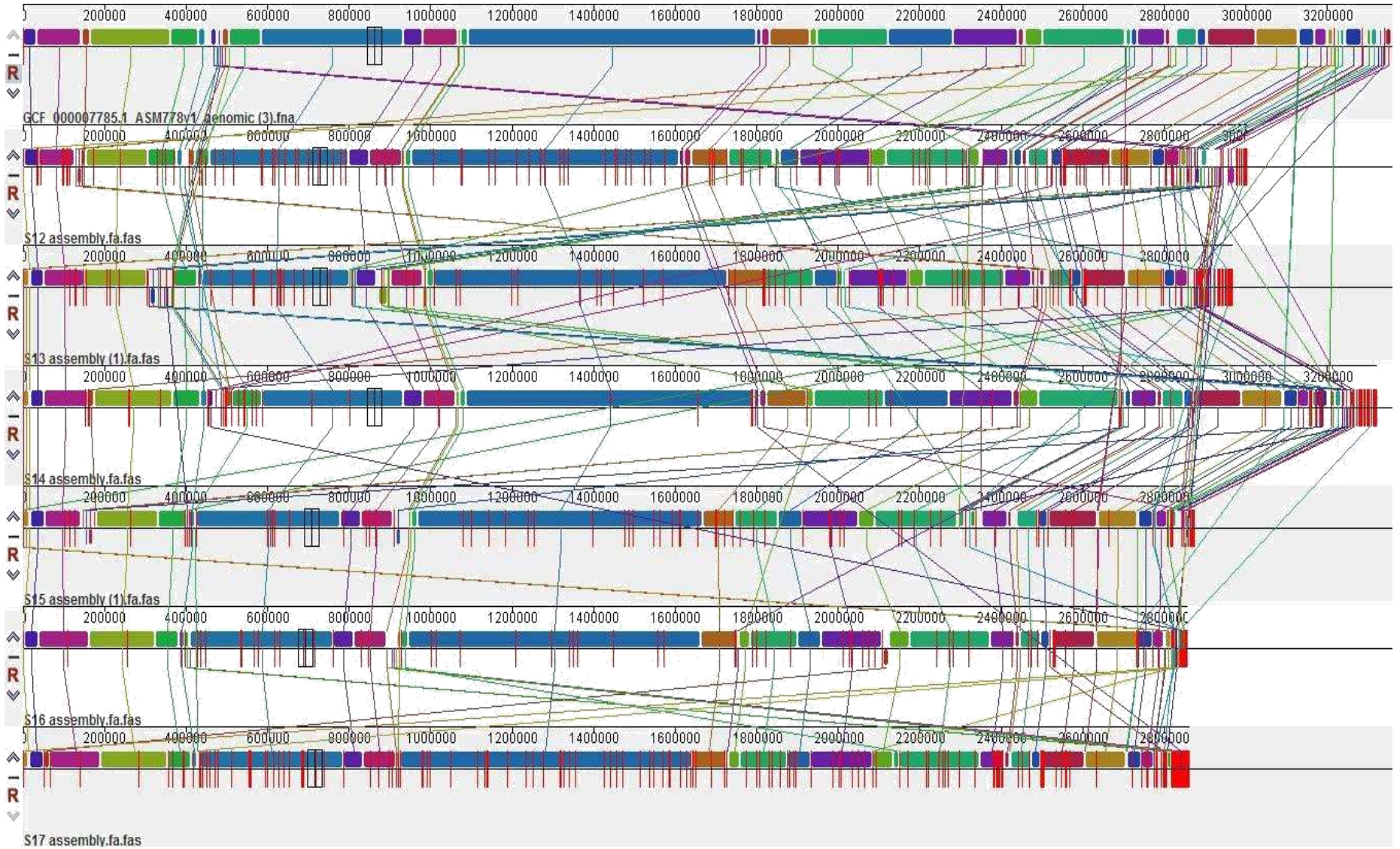


Figure 4.1. Genomic alignment of *Enterococcus faecalis*. MAUVE alignment of reference genome *E. faecalis* ATCC 19433 and the genome sequences of *E. faecalis* strains S12, S13, S14, S15, S16 and S17 (top to bottom). Same colour boxes represent homologous regions of sequence shared between *E. faecalis* genomes.

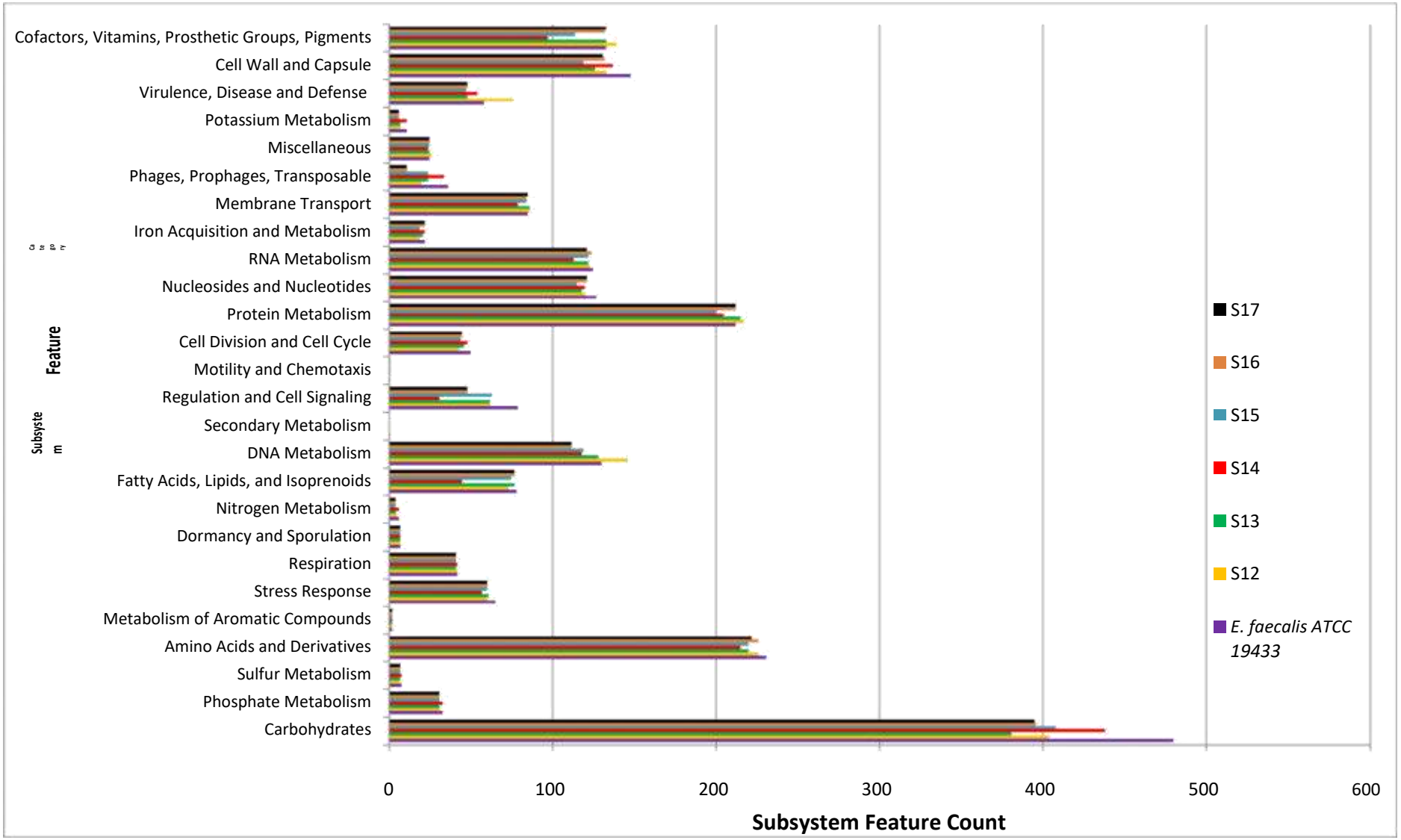


Figure 4.2. Comparison of subsystem features between *E. faecalis*. Genome sequences of strains S12, S13, S14, S15, S16, S17 and reference genome *E. faecalis* ATCC 19433 were uploaded to the SEED Viewer server independently. Functional roles of RAST annotated genes were assigned and grouped in subsystem feature categories as shown in the figure, and coloured bars indicate the number of genes assigned to each category.

4.3.2 Functional based comparison

The function based comparison tool compares two genomes to assess similarities and differences in the presence of functional roles that have been linked to subsystems. Table 4.3 shows the number of common functioning parts between two strains and what is singular in each one.

Table 4.3. Number of common and unique functioning parts of the genomes between A (reference genome) and B (comparison genome)

	Total number of functional parts	A + B Number of functional parts	%	A Number of functional parts	%	B Number of functional parts	%
S12 (A) and S13 (B)	1904	1820	95.59	56	2.94	28	1.47
S12 (A) and S14 (B)	1828	1677	91.73	93	5.08	58	3.17
S12 (A) and S15 (B)	1890	1806	95.56	51	2.69	33	1.74
S12 (A) and S16 (B)	1909	1800	94.29	67	3.50	42	2.20
S12 (A) and S17 (B)	1907	1799	94.33	68	3.57	40	2.09
S13 (A) and S14 (B)	1823	1655	90.78	85	4.66	83	4.55
S13 (A) and S15 (B)	1867	1807	96.78	25	1.33	35	1.87
S13 (A) and S16 (B)	1874	1826	97.43	22	1.17	26	1.38
S13 (A) and S17 (B)	1873	1824	97.38	24	1.28	25	1.33
S14 (A) and S15 (B)	1823	1642	90.07	85	4.66	96	5.26
S14 (A) and S16 (B)	1839	1671	90.86	80	4.35	88	4.78
S14 (A) and S17 (B)	1836	1671	91.01	80	4.35	85	4.62
S15 (A) and S16 (B)	1865	1792	96.08	40	2.14	33	1.76
S15 (A) and S17 (B)	1864	1790	96.03	42	2.25	32	1.71
S16 (A) and S17 (B)	1868	1863	99.73	4	0.21	1	0.05
ATCC (A) and S12 (B)	1916	1829	95.4	61	3.18	26	1.35
ATCC (A) and S13 (B)	1914	1806	94.35	87	4.54	21	1.09
ATCC (A) and S14 (B)	1851	1775	95.89	72	3.88	4	0.21
ATCC (A) and S15 (B)	1915	1805	94.25	83	4.33	27	1.41
ATCC (A) and S16 (B)	1930	1818	94.19	88	4.56	24	1.24
ATCC (A) and S17 (B)	1928	1817	94.24	89	4.61	22	1.14

Note: *Enterococcus faecalis* ATCC 19433 is referred to as ATCC in this table.

Overall, ≥90% of functioning parts were shared between all the strains tested, with S16 and S17 having the highest share (~99%). Figure 4.3 shows a 100% orthologous average nucleotide identity between S16 and S17. In comparison, the strain isolated from chicken feces (S13) has only 98% orthologous average nucleotide identity with S14.

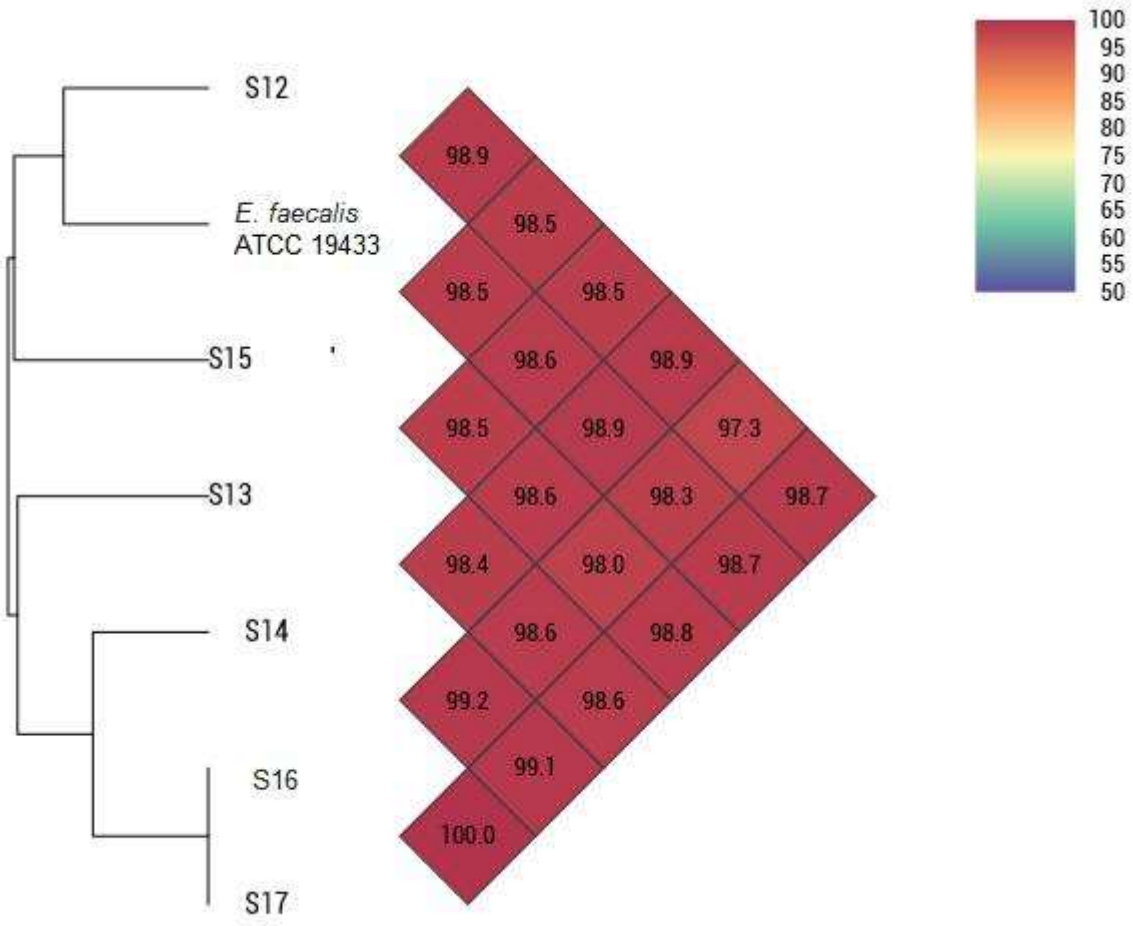


Figure 4.3. Dendrogram based on OrthoANI analysis and the presence of POGs in the genome of *E. faecalis* ATCC 19433 and the genomes of strains S12, S13, S14, S15, S16 and S17. An ANI phylogenetic tree was constructed using the orthologous average nucleotide identity tool based on OrthoANI values.

4.3.3 Sequence based comparison

The map below (Figure 4.4) illustrates a circular map of the sequence based comparison of the protein sequences encoded in the contigs/genes of the 6 *E. faecalis* strains against the reference strain *E. faecalis* ATCC 19433. Figure 4.4 confirms high homology between the 6 strains and *E. faecalis* ATCC 19433, with a high percentage of CDS similarity above 90%. Clearly, S14 appears to be most similar to the reference strains in the CDS although not on the basis of nucleotide sequence comparisons (Fig 4.3) and appears to be more distant to the others.

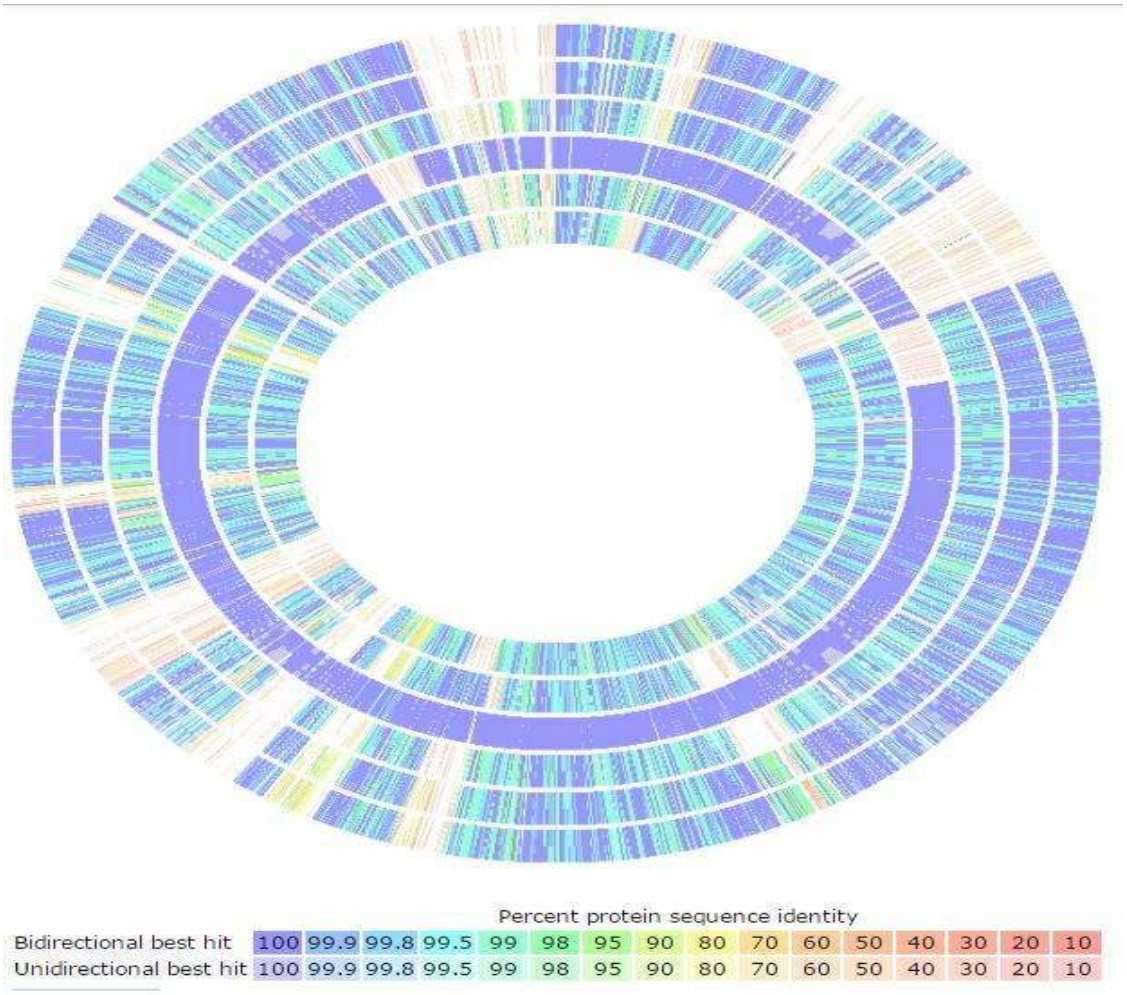


Figure 4.4. Circle plot showing the comparison between *E. faecalis* strains S12, S13, S14, S15, S16 and S17 genomes (inside to out) relative to *E. faecalis* ATCC 19433. In the legend the percent sequence identity is shown; the blue colour represents the highest sequence similarity and red represents the lowest.

4.3.4 Virulence factors and antibiotic resistance

In this study, a number of virulence genes were detected in the 6 *E. faecalis* strains tested. Table 4.4 shows the virulence and antibiotic resistant genes discovered through whole genome sequencing.

Table 4.4. Antibiotic resistant and virulence associated genes recovered from genome sequencing analysis of six *E. faecalis* strains

Strain	S12 (R24)	S13 (Ck50)	S14 (Ck27)	S15 (R6)	S16 (P20)	S17 (P24)
Isolation source	River water	chicken feces	Chicken Feces	River water	UTI patient	UTI patient
*vanBHRSXWY**	-	-	NBDW010 00006	-	-	-
tetM*	NBDR010 00040	-	-	-	-	-
*gelE**	NBDR010 00048	NBDS010 00053	NBDW010 00028	NBDV010 00016	NBDU010 00023	NBDT010 00088
*efaA**	NBDR010 00100	NBDS010 00066	NBDW010 00002	NBDV010 00033	NBDU010 00049	NBDT010 00053
*ace**	NBDR010 00038	NBDS010 00079	NBDW010 00009	NBDV010 00014	NBDU010 00054	NBDT010 00152
*ebpC**	NBDR010 00050	NBDS010 00024	NBDW010 00009	NBDV010 00060	-	NBDT010 00066
*ebpR**	NBDR010 00050	NBDS010 00024	NBDW010 00009	-	NBDU010 00043	NBDT010 00066
*eep**	NBDR010 00032	NBDS010 00004	NBDW010 00006	NBDV010 00036	NBDU010 00015	NBDT010 00113
*fsr**	-	-	NBDW010 00030	NBDV010 00018	NBDU010 00023	NBDT010 00090
*camE**	NBDR010 00070	NBDS010 00043	-	NBDV010 00025	NBDU010 00044	-
*cad**	NBDR010 00041	NBDS010 00051	NBDW010 00011	NBDV010 00028	NBDU010 00002	-
*salA**	NBDR010 00020	NBDS010 00058	NBDW010 00010	NBDV010 00003	NBDU010 00038	NBDT010 00008
*salB**	NBDR010 00005	NBDS010 00016	NBDW010 00017	NBDV010 00004	NBDU010 00026	NBDT010 00065

*The accession number of reference sequences for each gene is as follows: *vanBHRSXWY* (KC489787), *tetM* (U09422), *gelE* (DQ845100), *efaA* (JF512477), *ace* (HQ003827), *ebpC* (KJ710255), *ebpR* (EF646762) and *eep* (AF152237) and contigs containing the corresponding gene(s) exhibit ≥90% identity to their respective reference sequence.

Among the surface proteins that were present in all 6 strains were *SalA* and *SalB* (*SagA*-like), which resemble the *E. faecium* surface antigen *SagA* and codes for biofilm formation. The pili genes *eep* was found in all strains tested. Genes *ebpC* and *ebpR*, which are also biofilm-associated virulence factors, were identified in all strains except S16 and S15 respectively. Antibiotic resistant genes were also found in two of the sequenced isolates. E. *faecalis* S14 was found to have Vancomycin resistant genes (*vanB, vanH, vanR, vanS, vanX, vanW,* and *vanY*) whereas *E. faecalis* S12 was found to have Tetracycline resistant gene (*TetM*).

4.3.5 Genes related to biofilm forming ability

A comparison was made of the draft sequences of S12, S13 (low biofilm producing ability) in one group and S14, S15 and S16 (high biofilm producing ability) in another group in order to search for possible differences in the genetic constitution of the two types. S17 was not included as it behaved anomalously and had intermediate properties. Functional ortholog clustering was performed using the translated protein sequences as the input. BLASTALL was first performed among the protein sequences with the minimum score value and E-value in BLAST as 50 and 1e-8 respectively. The filtered BLAST results were clustered by MCL algorithm (Enright et al. 2002) and in order to group the same genes into the same cluster, the global match region would need to have at least 50% of the longest protein sequence and 50% sequence identity. The results show that 28 genes were putatively different between the two groups, being present in the low biofilm group but not in the other. No genes were found to be present in the high biofilm producing group that were not found in the low. Among them are genes coding for components in the Phosphotransferase system (PTS) and ATP binding cassette transporters and peptidoglycan acetylation genes.

4.4 Discussion

4.4.1 General properties of the *E. faecalis* genome

The genomes of the 6 strains tested ranged in size from 2.84 – 3.06Mb, with *E. faecalis* S15 (river water) exhibiting the smallest and *E. faecalis* S14 (chicken feces) the largest genome. The variations in genome sizes may suggest a difference in the size of the nucleoid genome between strains or the presence or absence of plasmids. In general, microorganisms with bigger genomes tend to have more genes, and more transposable elements than organism with smaller genomes.

Despite the difference in reservoirs, MAUVE analysis showed an overall collinear relationship across the six *E. faecalis* strains. Most of the locally co-linear blocks (LCBs) were highly homologous between the 6 assemblies, despite several gaps in between the blocks. The results from this study show that there are more conserved regions present among the genomes than unique regions. At least 380 of annotated genes per strain (about 15% of the total number of annotated genes) had a designated role for carbohydrate utilization in this study. *E. faecalis* strains, similar to other Enterococci, are characterized by their ability to use a wide range of carbon sources through their diverse carbohydrate metabolic pathways and transport systems (Kim et al., 2016).

Despite the similarities above, S14 appears to be the most different to the others. It has the largest genome, lowest percent similarity overall to the others in terms of shared functional parts and substantially higher similarity in protein coding sequences to the reference straincompared to others (Fig. 4.4). However, interstingly, its orthoANI and POG analysis showed it to be closely related to S16 and S17.

4.4.2 Functional and sequence based comparison

Overall, approximately ≥90% of functioning parts were shared between all the strains tested, with S16 and S17 having the highest share (~99%). Interestingly, S16 and S17 were isolated from different hospital wards in the same hospital. Their phylogenetic relationship based on the PFGE analysis was presented in our previous study (Daniel et al., 2017). The close relationship of the two strains deduced from sequencing demonstrates that the information about relatedness obtained through PFGE can be misleading. One study (Salipante et al., 2015) reports a similar result. Horizontal gene transfers among strains, mediated by plasmids and other mobile genetic elements, and recombination may significantly alter the genomic and phenotypic properties of a strain through single, discrete events (Salipante et al., 2015). It is speculated that, in the context of molecular strain typing, such events may result in shifts to PFGE patterns that are not proportional to the time or degree of strain divergence. In contrast, point mutations detectable by WGS accumulate at a far more predictable rate and consequently serve as a more reliable molecular clock for molecular epidemiology reconstructions (Salipante et al., 2015).

The genome analysis also provides other interesting insights. Figure 4.3 shows a 100% orthologous average nucleotide identity between S16 and S17. The analysis also revealed a 99% orthologous average nucleotide identity between S14 (farm chicken feces) and S16, as well as S17. However, strain S13 (farm chicken feces) has only 98% orthologous average nucleotide identity with S14 (farm chicken feces). Figure 4.4 also confirms high homology between the 6 strains and *E. faecalis* ATCC 19433, with a high percentage of CDS similarity above 90%. However, clearly S14 stands out in being much more closely related to the

reference strain than the others. Fig 4.4 is also interesting in that it shows a close relationship between the hospital strains and the river strain S12, suggesting at least the posibility of a shared reservoir.

4.4.3 Virulence factors and antibiotic resistance

Virulence genes contribute to the pathogenicity of an organism. In this study, a number of virulence genes were detected in the 6 *E. faecalis* strains tested including *efaA* (endocarditis antigen), *ace* (collagen adhesion), *gelE* (gelatinase production) and fsr (*E. faecalis* regulator). The ace genes are important for facilitating cell wall adhesion to host tissues. The *efaA* gene also plays a role in adherence to host tissues and is a virulence factor involved in endocarditis. The *gelE* gene encodes for gelatinase, which hydrolyses gelatin, collagen, casein and haemoglobin. Its expression is regulated by the two-component *fsr* system, with both *gelE* and *fsr* genes important in biofilm formation.

In addition to these virulence genes, a number of bacterial sex pheromone genes were also present in *E. faecalis* including *camE* and *cad*. Certain conjugative plasmids found in *E. faecalis* respond to the secretion of bacterial sex pheromone genes from plasmid-free enterococci inducing their transfer (Beukers et al., 2017). Sex pheromone response plasmids have rarely been described in other *Enterococcus spp*. Regarding the resistance to antibiotics, *E. faecalis* S14 (from chicken feces) was found to have Vancomycin resistant genes (*vanB, vanH, vanR, vanS, vanX, vanW,* and *vanY*) whereas *E. faecalis* S12 (from river water) was found to have Tetracycline resistant gene (*TetM*). These two strains, S14 and S12, also showed resistance to Vancomycin and Tetracyline respectively in Chapter 2 of this study. Samples S13

(from chicken feces) and S15 (from river water) was also found to be resistant to Tetracycline as shown in Chapter 2, however these two strains did not possess the *TetM* gene. Tetracylcine resistance is often due to the acquisition of new genes, which code for energy-dependant efflux of Tetracyclines or for a protein that protects bacterial ribosomes from the action of Tetracyclines. These genes, other than *TetM*, may be the contributing factor Tetracycline resistance characteristic as discovered in Chapter 2 of this study.

Other surface proteins that were present in all 6 strains include *SalA* and *SalB* (*SagA*-like), which resemble the *E. faecium* surface antigen *SagA* and codes for biofilm formation. Pili genes *eep* was found in all strains tested. Genes *ebpC* and *ebpR*, which are also biofilm-associated virulence factors, were identified in all strains except S16 and S15 respectively. The *ebp* pili genes assist in adherence and biofilm formation. Garsin and Willems (2010) suggests that pili are also key factors in *E. faecalis* pathogenesis, again affecting biofilm formation and virulence in model of ascending urinary tract infection. It is therefore hypothesized that pili are generally important for biofilm formation and pathogenesis in all pathogenic enterococcal strains. However, despite the presence of these adhesin and biofilm-associated genes, *E. faecalis* strain S12, S13, and S17 are not strong biofilm-forming strains based on our previous findings (Daniel et al., 2017).

Biofilms are considered to be environments where new or previously unrecognized, biological properties could be expressed. The search for unifying biofilm gene expression pattern has been rather unsuccessful. This is particularly the case when it comes to key regulatory pathways. Many of the new regulatory pathways that have been associated with biofilm production have not been demonstrated to be specific nor required in all biofilm situations (Beloin and Ghigo, 2005). This indicates that key proteins in putative biofilm

signalling pathways and/or their regulation is yet to be discovered.

Attempts to find biofilm related genes without any prior assumptions by carrying out comparisons between encoded proteins in two groups (see section 4.3.5) yielded 28 putative differences in the encoded proteins. This analysis would be clearly improved with more genome sequences in each of the two groups. Among the genes found in this analysis were those that encode for components of the phosphtransferase system and other surface proteins. Some of these genes may provide useful leads for future investigations in this area.

The data presented in this study provides further evidence that many of the virulence genes are not in themselves critical for biofilm formation. Clearly, far more detailed analysis of genomes and correlation with experimental data are required. A deep understanding of the mechanisms involved in biofilm formation will ultimatey shed light on the generation of alternative treatments for *E. faecalis* infections. There is no doubt that future studies will reveal additional biofilm matrix components and identify more elaborate regularoty circuits for biofilm formation.

4.5 Conclusion

In general, the genomes from *E. faecalis* S12, S13, S14, S15, S16 and S17 shared great similarity as well as with the reference genome *E. faecalis* ATCC 19433. Despite the phylogenetic distance of S16 and S17 as shown by PFGE, this study shows that the strains share almost 100% orthologous average nucleotide identity and more functional groups than the other pairs, suggesting a very close phylogenetic relationship of both strains. Furthermore, two strains isolated from chicken feces appear to be the most different from each other. To our knowledge this is the first genome comparison analysis report on clinical and environmental *E.*

faecalis strains isolated from Malaysia. This study also revealed multiple virulence and antibiotic resistance genes that might contribute to the survival and persistence in diverse environments. Despite the presence of multiple adhesin and biofilm-associated genes, *E. faecalis* strains S12, S13, and S17 did not exhibit high attachment and biofilm capabilities as compared to their counterparts S15, S14 and S16. This suggests that regulatory pathways that have been associated with biofilm production have not been demonstrated to be specific nor required in all biofilm situations. It is important to study the genome structure of *E. faecalis* as it enables discrimination of the pathogen strains at the single nucleotide level, essentially providing a genome-level typing tool that serves the same purpose as other typing tools (i.e. PFGE), but with a much higher resolution. Advances in this field suggests exciting directions for improving public health efforts for infectious disease treatment and prevention.

CHAPTER 5

General Discussion and Conclusion

Chapter 5 – General Discussion and Conclusion

5.1 Major Findings and Contributions of This Study

The primary aim of this study was to investigate the characteristics and genetic variation among *E. faecalis* strains from a variety of sources including water sources, farm animal feces and UTI patients. Genetic diversity, antibiotic resistance profiling, virulence factors as well as attachment and biofilm capabilities of this nosocomial opportunistic pathogen were addressed. This work was designed to serve as a framework for the development of new strategies to prevent and treat *E. faecalis* infections, as well as highlight the need of health care settings and industries in Malaysia to strictly regulate the use of antibiotics to curb the emerging threat of MDR enterococci. Alternative control strategies must also be devised for both testing the antibiotic susptibility of enterococci within a biofilm and implementing treatment strategies that disrupt or target specific components of the biofilm matrix. Based on the results obtained from this study, a number of conclusions can be drawn.

Data on phenotypic antibiotic resistance groupings revealed that 80% of the total isolated *E. faecalis* in this study were resistant to the 8 individual antibiotics tested, with resistance to Tetracycline being present in the highest proportions (62%), particularly isolates from farm animals. Antibiotic resistant enterococci have been detected previously in livestock in Malaysia (Getachew et al., 2012, Getachew et al., 2013), and this has led to suggestions of an epidemiological link between livestock and human infections (Getachew et al., 2012, Getachew et al., 2013). Distribution of 9 virulence markers tested in the study varied between sources, with the *gelE* gene, which is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and

other peptides, being present in the highest percentage (75.6%) in this study. With the exception of clinical *E. faecalis* isolates, these results support the hypothesis that there is a high prevalence of putative virulence genes and antibiotic resistance profile in *E. faecalis.* In addition, pulse-field gel electrophoresis-typing succeeded in providing insights into the genetic diversity of *E. faecalis* isolated from farm animal feces, water sources and UTI patients, as well as persistence of these strains after a period of 6 months (Chapter 2). High levels of genetic diversity were found between strains isolated from all three sources (Simpson's diversity index ≥ 0.901). The high diversity observed in each of the three sources is not particularly surprising as previous reports (Praveena et al., 2011, Getachew et al., 2013) recorded a high genetic diversity of *E. faecalis* strains from various sources. It is possible that the exposure to physical and chemical stresses may have resulted in evolution of wide diversity which is necessary for the adaptation of *E. faecalis* (Baureder et al., 2012). While an epidemiological link between livestock and human infections was stated by previous studies (Getachew et al., 2012, Getachew et al., 2013), no pulsotype was common to all the three sources in the current study. This is almost certainly due to the small numbers of pulsotypes examined, particularly from hospitals. Similar PFGE patterns were also recovered in all sources after at least 6 months of follow-up, particularly in each patient room which had its own unique PFGE pattern. Similar PFGE patterns were also recovered in all sources after at least 6 months of follow-up, particularly in each patient room which had its own unique PFGE pattern, refuting the third hypothesis of Chapter 2 which states that strains would not likely persist after the six-month period due to a high genetic diversity. This may suggest that the patients acquired *E. faecalis* from their individual rooms, i.e. hospital bedding or shared bathroom within the room. This highlights the need for better and more regular cleaning or sanitization of the hospital rooms including changing of bedding and disinfecting shared toilets within the rooms. An extensive

strengthening of infection control in Malaysian hospitals is needed, followed by a systematic evaluation of the effectiveness of this measures to ensure the policy has been followed thoroughly.

The findings of the study on attachment and biofilm forming capabilities of *E. faecalis* strains as well as the cell hydrophobicity properties of the strains were also notable (Chapter 3). Glucose supplementation had a significant effect on the biofilm formation of the *E. faecalis* strains. Our study also reports a higher percentage of strong biofilm producing *E. faecalis* isolated from UTI patients and farm animal feces as compared to strains from water sources when supplemented with 1% glucose. No difference was seen in biofilm formation with regard to incubation temperature. This result show that *E. faecalis* strains isolated from humans and animals have a glucose-dependant biofilm forming ability which may be of concern, particularly in diabetic patients (Tendolkar et al., 2004). The following section largely repeats above. The presence of glucose in the blood and urine may be one reason why these microbes are frequently found in urinary tract, wounds, bloodstream, and endocardium infections (Fisher and Philips, 2009). In addition, the current results report a hydrophilic nature of all strains and a high degree of attachment and biofilm formation particularly on hydrophilic material such as polyurethane. Several studies have reported that more hydrophobic cells adhere more strongly to hydrophobic surfaces, while hydrophilic cells strongly adhere to hydrophilic surfaces (Kochkodan et al., 2008, Giaouris et al., 2009, Krasowska & Sigler, 2014). There was, however, no direct correlation observed between biofilm formation and the virulence factors and antibiotic resistance profile analyzed in Chapter 2.

Previous studies have also reported 503 complete or draft genome sequences are available for *E. faecalis* as of April 2017 (https://www.ncbi.nlm.nih.gov/assembly/?term=Enterococcus+faecalis). Several comparative genomic studies of *E. faecalis* have also been conducted (Palmer et al., 2012, Qin et al., 2012). However, there is a poor representation of genomic sequences for enterococci from Malaysia with only 7 assemblies reported so far in the NCBI database (NCBI database, 2017). Whole genome sequencing was used to comparatively analyze 6 *E. faecalis* strains on the basis of their different sources, pulsotypes and attachment capabilities (Chapter 4). The whole genome sequencing analysis demonstrated that in the case of one pair at least (out of the six analysed) the close relationship contradicted the distant relationship as suggested by PFGE pulsotype comparisons in Chapter 2. A high homology between the 6 strains and *E. faecalis* ATCC 19433, with a high percentage of CDS similarity above 90% was observed. The results obtained in this chapter shows that strains share more functional groups that was not picked up by PFGE analysis, suggesting a closer phylogenetic relationship of the strains and possibly the occurance of some eventual cases of horizontal gene transfer. The whole genome analysis revealed virulence factors in all 6 strains that contribute to multiple adhesin and biofilm-associated genes, regardless of the attachment and biofilm capabilities demonstrated in Chapter 3. This suggests that regulatory pathways that have been associated with biofilm production have not been demonstrated to be specific nor required in all biofilm situations (Beloin & Ghigo, 2005). It is possible that key proteins in putative biofilm signaling pathways are yet to be discovered. Further studies are needed in order to confirm the role of these genes on biofilm formation by *E. faecalis*.

5.2 Future Directions

The collective findings of my work and that of recent studies could be useful for future studies to analyze the persistence of *E. faecalis* in the environment or develop more specific methods in examining the health significance of potentially virulent strains from environmental and clinical sources. Potential areas of future research that could be undertaken in order to gain more insight into characteristics and persistence of *E. faecalis* are listed below. In particular, the collective findings of our work and that of recent studies could provide useful information on the nature of *E. faecalis,* and this may help develop control strategies to curb the emerging threat of MDR enterococci not only in healthcare settings but also in agricultural and wastewater systems. This could be achieved by interrogation of genome sequences of strains with high and low biofilm capacity to identify critical genes. In addition, this research shows the widespread selection and dispersal of resistance genes to critical antibiotics. This issue needs to be combated by legislation limiting the use of such antibiotics in food systems and my work provides evidence to support such steps.

5.2.1 Whole genome comparison of *E. faecalis* using complete genome

Whole-genome comparison among *E. faecalis* strains isolated from water sources, farm animal feces and UTI patients, as described in Chapter 4, identified some potential genes that might be involved in biofilm formation or the pathogenesis of enterococcal infections. It would therefore be useful to obtain complete genomes of more *E. faecalis* strains which showed variation in virulence and antibiotic resistant genes and carry out a more comprehensive genomic comparison study, highlighting the divergence of gene family clusters and biological processes of unique proteins in each strain. Additional surveillance of virulence and antibiotic resistance genes among *E. faecalis* from different hosts and habitats should continue to better understand the occurrence and clinical relevance of these microbes in environmental and clinical settings.

5.2.2 Construction of single-gene knockout mutant

Gene candidates that are reported to be involved in biofilm formation and other virulence factors present in *E. faecalis* were identified through the whole genome comparison approach in this study (Chapter 4). The role of these genes in biofilm formation as well as pathogenesis could be further investigated through construction of single-gene knockout mutant followed by gene complementation studies. This will contribute to a better understanding of the molecular mechanism underlying pathogenesis by *E. faecalis*.

5.2.3 Investigation of the interactions between *E. faecalis* and other bacterial species in mixed-species biofilms under different conditions

Formation of mixed-species biofilm with other bacterial species has been shown to enhance survival and pathogenicity of *E. faecalis* (Pillai et al., 2004). The interactions between *E. faecalis* and other bacterial species could be further investigated in the future. It would also be useful to examine biofilm formation by *E. faecalis* in the presence of different types of pathogens commonly found in healthcare settings such as *Escherichia coli* and *Pseudomonas aeruginosa*, to mimic natural microbial biofilms under conditions relevant to the environment these bacteria are likely to face (atmospheric conditions, room temperature, etc.). Natural microbial biofilms often consist of multiple species. Other species of bacteria and even viruses will interact in a mature biofilm, and horizontal gene transfer between these different microbial species is greatly enhanced. This is of great significance in the evolution of organisms with altered characteristics in terms of antibiotic resistance and pathogenicity. It will also be useful to look into the attachment properties of *E. faecalis* on not only abiotic surfaces other than the ones tested in this study (Chapter 3), but also on biotic surfaces.

5.2.4 Investigation on the persistence of *E. faecalis* in various environments

The intrinsic robustness of *E. faecalis* may allow it to survive for extended periods of time, leading to its persistence and nosocomial spread (Waar, 2004). A follow-up sampling in this study (after 6 months) revealed persistence in *E. faecalis* strains, particularly in hospital wards (Chapter 2). A follow-up plan over a longer period of time may provide long-term trends or patterns in terms of an epidemiological link between clinical and environmental strains. This allows for proper public health planning, healthcare resources and workforce development in eradicating the impending issue of antibiotic resistant Enterococci. A particular limitation to this study was the low number of clinical strains obtained. A larger sample number of clinical strains from a wider range of hospitals (with detailed information on the patient) may also give insight into the epidemiological link between various sources. This epidemiology study may not only show a correlation between the different sources sampled from, but also provide evidence for the spread of enterococci in a hospital setting and nosocomial acquisition of enterococci, and further underline the need for better control measures in the healthcare setting.

References

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

Al-Gheethi A, Ismail N, Lalung J, Talib A, Efaq AN & Abd Kadir MO (2013) Susceptibility for antibiotics among fecal indicators and pathogenic bacteria in sewage treated effluents. *Water Prac Tech* **8**: 1-6.

Andrade FH, Reid MB, Allen DG & Westerblad H (1998) Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. *J Physiol* **509**: 565–575.

Araoka H, Kimura M & Yoneyama A (2011) A surveillance of high-level gentamicin-resistant enterococcal bacteremia. *JIC* **17**: 433-4.

Arias CA, Contreras GA & Murray BE (2010) Management of multidrug-resistant enterococcal infections. *Clin Microbial Infect* **16**: 1469-1691.

Arias CA & Murray BE (2012) The rise of the Enterococcus: beyond vancomycin resistance. Nature reviews. *Microbiology* **10**: 266-278.

Allison DG (2003) The biofilm matrix. *Biofouling* **19**: 139-50.

Bakshi U, Sarkar M, Paul S, Dutta C (2016) Assessment of virulence potential of uncharacterized Enterococcus faecalis strains using pan genomic approach – Identification of pathogen–specific and habitat-specific genes. *Scientific Reports* **6**: 1-11.

Baldassarri L, Cecchini R, Bertuccini L, Ammendolia MG, Iosi et al. (2001) *Enterococcus* spp. produces slime and survives in rat peritoneal macrophages. *Med Microbiol Immunol* **190**: 113-120.

Baldassarri L, Creti R, Recchia S, Pataracchia M, Alfarone G, Orefici G, Campoccia D, Montanaro L & Arciola CR (2006) Virulence factors in enterococcal infections of orthopedic devices. *Int J Artif Organs* **29**: 402–406.

Baureder M, Reimann R & Hederstedt L (2012) Contribution of catalase to hydrogen peroxide resistance in *Enterococcus faecalis*. *FEMS Microbiol Lett* **331**: 160–164.

Bazaka K, Crawford R J, Nazarenko EL & Ivanova EP (2011) Bacterial extracellular polysaccharides. *Adv Exp Med Biol* **715**: 213–226.

Beloin C & Ghigo J.M (2005) Finding gene-expression patterns in bacterial biofilms. *Trends Microbiol* **13**: 16–19.

Beukers AG, Zaheer R, Goji N, Amoako KK, Chaves AV, Ward MP & McAllister TA (2017) Comparative genomics of *Enterococcus* spp. Isolated from bovine feces. *BMC Microbiology* **17**: 52.

Bland C, Ramsey TL, Sabree F, Lowe M, Brown K, Kyrpides NC, et al. (2007) CRISPR recognition tool (CRT): a tool for automatic detection of clustered regularly interspaced palindromic repeats. *BMC Bioinformatics* **8**: 209.

Bos R, van der Mei H & Busscher H (1999) Physico-chemistry of initial microbial adhesive interactions - its mechanisms and methods for study. *FEMS Microbiol Rev* **23**: 179–230.

Butaye P, Devriese LA & Haesebrouck F (2001) Differences in antibiotic resistance patterns of *Enterococcus faecalis* and *Enterococcus faecium* strains isolated from farm and pet animals. *Antimicrob Agents Chemother* **45**: 1374-1378.

Chen AY & Zervos MJ (2009) Enterococcus: Antimicrobial resistance in Enterococci epidemiology, treatment, and control in antimicrobial drug resistance, clinical and epidemiological aspects, (Mayers DL Ed.). Humana press: New York, NY.

Chetinkaya Y, Falk P & Mayhall CG (2000) Vancomycin-resistance Enterococci. *Clin Microbiol Rev* **13**:686-707.

Chomel BB (1998) New emerging zoonoses: A challenge and an opportunity for the veterinary profession. *Comp Immunol Microbiol Infec Dis* **21**: 1–14.

Chow A, Win NN, Ng PY, Lee W & Win MK (2016) Vancomycin-resistant enterococci with reduced daptomycin susceptibility in Singapore: prevalence and associated factors. *Epidemiol Infect* **144**: 2540-2545.

Clinical and Laboratory Standards Institute (2012) Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement. CLSI document M100-S17. Clinical and Laboratory Standards Institute: Pennsylvania.

Cocconcelli PS, Porro D, Galandini S & Senini L (1995) Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci. *Lett Appl Microbiol* **21**: 376-379.

Coburn PS, Baghdayan AS, Dolan GT & Shankar N (2007) Horizontal transfer of virulence genes encoded on the *Enterococcus faecalis* pathogenicity island. *Mol Microbiol* **63**: 530–544.

Comerlato CB, Resende MC, Caierão J & d'Azevedo PA (2013) Presence of virulence factors in *Enterococcus faecalis* and *Enterococcus faecium* susceptible and resistant to vancomycin. *Mem Inst Oswaldo Cruz* **108**: 590–595.

Cook LC & Dunny GM (2014) The influence of biofilms in the biology of plasmids. *Microbiol Spectr* **2**:0012.

Coque TM, Patterson JE, Steckelberg JM & Murray BE (1995) Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and communitybased persons. *J Infect Dis* **171**: 1223-1229.

Creti R, Imperi M, Bertuccini L, Fabretti F, Orefici G, Di Rosa R & Baldassarri L (2004) Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. *J Med Microbiol* **53**: 13–20.

Critchley IA, Blosser-Middleton RS, Jones ME, et al. (2003) Baseline study to determine in vitro activities of daptomycin against gram-positive pathogens isolated in the United States in 2000-2001. *Antimicrob Agents Chemother* **47**: 1689-1693.

Dada AC, Ahmad A, Usup G & Heng LY (2012) Antibiotic resistance and virulence among Enterococci isolated from Teluk Kemang Beach, Malaysia. *Water Qual Expo Health* doi:10.1007/s12403-012-0070-2.

Dahlen G, Blomqvist S, Almstahl A & Carlen A (2012) Virulence factors and antibiotic susceptibility in enterococci isolated from oral mucosal and deep infections. *J Oral Microbiol* **4**: 10.3402/jom.v4i0.10855.

Daniel DS, Lee SM, Gan HM, Dykes GA & Rahman S. Genetic diversity of *Enterococcus faecalis* isolated from environmental, animal and clinical sources in Malaysia. *J Infec Public Health* doi.org/10.1016/j.jiph.2017.02.006.

Darling AC, Mau B, Blatter FR & Perna TN (2004) Mauve: Multiple alignment of conserved genomic sequence with rearrangements. *Genome Res* **14**: 1394–1403.

Davies J & Davies D (2010) Origins and evolution of antibiotic resistance. Microbiology and molecular biology reviews. *MMBR* **74**: 417-33.

De Silva D, Yu YT, Amunts A, Fontanesi F & Barrientos A (2015) Mitochondrial ribosome assembly in health and disease. *Cell cycle* **14**: 2226-2250.

Descheemaeker P, Lammens C, Pot B, Vandamme P & Goossens H (1997) Evaluation of arbitrarily primed PCR analysis and pulsed-field gel electrophoresis of large genomic DNA fragments for identification of enterococci important in human medicine. *Int J Syst Bacteriol* **47**: 555-561.

Diani M, Esiyok OG, Ariafar MN, Yuksel FN, Altuntas EG & Akcelik N (2014) The interactions between *esp*, *fsr*, *gelE* genes and biofilm formation and PFGE analysis of clinical *Enterococcus faecium* strains. *Afr J Microbiol Res* **8**: 129–137.

Dicuonzo G, Gherardi G, Lorino G, Angeletti S, Battistoni F, Bertuccini L, Creti R, Rosa R, Venditti M & Baldassarri L (2001) Antibiotic resistance and genotypic characterization by PFGE of clinical and environmental isolates of *Enterococci*. *FEMS Microbiology Letters* **201**: 205-211.

Di Rosa R, Creti R, Venditti M, D'Amelio R, Archiola CR, Montanaro L & Baldassarri L (2006) Relationship between biofilm formation, the enterococcal surface protein (Esp) and gelatinase in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. *FEMS Microbiol Lett* **256**: 145-50.

Domingues S, Harms K, Fricke WF, Johnsen PJ, da Silva GJ & Nielsen KM (2012) Natural transformation facilities transfer of transposons, integrons and gene cassettes between bacterial species. *PLOS Pathogens* **8**: e1002837.

Donabedian S, Thal LA, Bozigar P, Zervos T, Hershberger E & Zervos M (2003) Antimicrobial resistance in swine and chickens fed virginiamycin for growth promotion. *J Microbiol Methods* **55**: 739–743.

Donlan RM (2002) Biofilms: microbial life on surfaces. *Emerg Infect Dis* **8**: 881-90.

Donlan RM & Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15**: 167-193.

Dupont H (2007) The empiric treatment of nosocomial intra-abdominal infections. *Int J Infect Dis* **11**: 1-6.

Dutka-Malen S, Evers S & Courvalin P (1995) Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol* **33**: 24–27.

Dworniczek E, Wojciech L, Sobieszczanska B & Seniuk A (2005) Virulence of Enterococcus isolates collected in Lower Silesia (Poland). *Scand J Infect Dis* **37**: 630–636.

Ebert SC & Craig WA (1990) Pharmacodynamic properties of antibiotics: application to drug monitoring and dosage regimen design. *Infect Control Hosp Epidemiol* **11**: 319-326.

ECDC (2008) Annual Epidemiological Report on Communicable Diseases in Europe 2008, 2008, European Centre for Disease Prevention and Control: Stockholm.

Eisner A, Feierl G, Goriewicz G, Dieber F, Kessler H, Marth E & Kofer J (2005) High prevalence of VanA-type vancomycin-resistance *Enterococci* in Austrian poultry. *Appl Environ Microbiol* **71**: 6407–6409.

Eliopoulos GM & Moellering RC Jr (1996) Antimicrobial combinations, in Antibiotics in laboratory medicine, L. V., Editor Williams and Wilkins: Baltimore, Md. p. 330-396.

Enoch DA, Bygott JM, Daly ML, et al. (2007) Daptomycin. *J Infect* **55**: 205-213.

Enright AJ, et al. (2002) An efficient algorithm for large-scale detection of protein families. *Nucleic Acids Res.* **30**:1575–1584.

Fisher K & Philips C (2009) The mechanism of action of a citrus oil blend against *Enterococcus faecium* and *Enterococcus faecalis*. *J Appl Microbiol* **106**: 1343-1349.

Flemming HC & Wingender J (2010) The biofilm matrix. *Nat Rev Microbiol* **8**: 623-33.

Freitas AR, Coque TM, Novais C, et al. (2011) Human and swine hosts share vancomycin-resistant *Enterococcus faecium* CC17 and CC5 and *Enterococcus faecalis* CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. *J Clin Microbiol* **49**: 925-31.

Fukuda H, Lee J & Imanaka Y (2011) Costs of hospital-acquired infection and transferability of the estimates: a systematic review. *Infection* **39**: 185-99.

Garcia-Armisen T, Vercammen K, Passerat J, Triest D, Servais P & Cornelis P (2011) Antimicrobial resistance of heterotrophic bacteria in sewage-contaminated rivers. *Water Res* **45**: 788–796.

Garsin DA & Willems RJL (2010) Insights into the biofilm lifestyle of enterococci. *Virulence* **4**: 219-221.

Garsin DA, Frank KL, Silanpaa J, Ausubel FM, Hartke A, Shankar N & Murray BE (2014) Pathogenesis and models of enterococcal infection. In Gilmore MS, Clewell DB, Ike Y, Shankar N editors. Enterococci: From commensals to leading causes of drug resistant infections. Boston: Massachusetts Eye and Ear Infirmary.

Gelsomino R, Vancanneyt M, Condon S, et al. (2001) Enterococcal diversity in the environment of an Irish Cheddar-type cheesemaking factory. *Int J Food Microbiol* **71**: 177-88.

Getachew Y, Hassan L, Zakaria Z, Saleha AA, Kamaruddin MI, Che Zalina MZ, et al. (2012) Characterization and risk factors of vancomycin-resistant Enterococci (VRE) among animal-affiliated workers in Malaysia. *J Appl Microbiol* **113**: 1184-1195.

Getachew Y, Hassan L, Zakaria Z & Saleha AA (2013) Genetic variability of Vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* isolates from human, chickens, and pigs in Malaysia. *Appl Environ Microbiol* **79**: 4528.

Giaouris E, Chapot-Chartier M & Briandet R (2009) Surface physicochemical analysis of natural *Lactococcus lactis* strains reveals the existence of hydrophobic and low charged strains with altered adhesive properties. *Int J Food Microbiol* **131**: 2–9.

Gilmore MS (2002) The enterococci: pathogenesis, molecular biology, and antibiotic resistance. p. 150-250, Washington, DC: American Society for Microbiology.

Giridhara UPM, Umapathy BL & Ravikumar KL (2010) Comparative study for the presence of enterococcal virulence factors gelatinase, hemolysin and biofilm among clinical and commensal isolates of *Enterococcus faecalis*. *J Lab Physicians* **2**: 100-104.

Gordillo ME, Singh KV & Murray BE (1993) Comparison of ribotyping and pulsed-field gel electrophoresis for subspecies differentiation of strains of *Enterococcus faecalis. J Clin Microbiol* **31**: 1570-1574.

Goulter RM, Gentle IR & Dykes GA (2010) Characterisation of curli production, cell surface hydrophobicity, autoaggregation and attachment behaviour of *Escherichia coli* O157. *Curr Microbiol* **61**: 157-162.

Gregersen RH, Petersen A, Christensen H & Bisgaard M (2010) Multilocus sequence typing of *Enterococcus faecalis* isolates demonstrating different lesion types in broiler breeders. *Avian Pathology* **39**: 435-440.

Gulhan T, Aksakal A, Ekin IH, Savasan S & Boynukara B (2006) Virulence factors of *Enterococcus faecium* and *Enterococcus faecalis* strains isolated from humans and pets. *Turk J Vet Anim Sci* **30**: 477-482.

Gurevich A, et al. (2013) QUAST: quality assessment tool for genome assemblies. *Bioinformatics* **29**: 1072-1075.

Hall-Stoodley L, Costerton JW & Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2**: 95-108.

Hammerum AM, Lester CH & Heuer OE (2010) Antimicrobial-resistant *Enterococci* in animals and meat: A human health hazard. *Foodboren Pathog Dis* **7**: 1137-1146.

Hamzah A, Kipli SH & Rahil Ismail SII (2011) Microbiological study in coastal water of Port Dickson, Malaysia. *Sains Malaysiana* **40**: 93–99.

Harbarth S & Uckay I (2004) Are there patients with peritonitis who require empiric therapy for enterococcus? *Eur J Clin Microbiol Infec Dis* **23**: 73-77.

Heintz BH, Halilovic J & Christensen CL (2010) Vancomycin-resistant enterococcal urinary tract infections. *Pharmacother* **30**: 1136-1149.

Hidron AI, Edwards JR, Patel J, et al. (2008) NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. *Infect Control Hosp Epidemiol* **29**: 996-1011.

Hollenbeck BL & Rice LB (2012) Intrinsic and acquired resistance mechanisms in Enterococci. *Virulence* **15**:420-433.

Hufnagel M, Koch S, Creti R, Baldassarri L & Huebner J (2004) A putative sugar-binding transcriptional regulator in a novel gene locus in *Enterococcus faecalis* contributes to production of biofilm and prolonged bacteremia in mice. *J Infect Dis* **189**: 420-430.

Hunter PR & Gaston MA (1988) Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microb* **26**: 2465–2456.

Huycke MM, Sahm DF & Gilmore MS (1998) Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. *Emerg Infect Dis* **4**: 239-249.

Iversen A, Kuhn L, Rahman M, Franklin A, Burman L, Olsson-Liljquist B, Torell E & Mollyb R (2004) Evidence for transmission between humans and the environment of a nosocomial strain of *Enterococcus faecium*. *Environ Microbiol* **6**: 55–59.

Jana S & Deb JK (2006) Molecular understanding of aminoglycoside action and resistance. *Appl Microbiol Biotechnol* **70**: 140-50.

Jarvis WR & Martone WJ (1992) Predominant pathogens in hospital infections. *J Antimicrob Chemother* **29**: 19–24.

Joyanes P, Pascual A, Martinez-Martinez L, Hevia A & Perea EJ (1999) In vitro adherence of *Enterococcus faecalis* and *Enterococcus faecium* to plastic biomaterials. *Clin Microbiol Infect* **5**: 382–386.

Kanemitsu K, Nishino T, Kunishima H, Okamura N, Takemura H, Yamamoto H, et al. (2001) Quantitative determination of gelatinase activity among enterococci. *J Microbiol Methods* **47**: 11-16.

Kayaoglu G & Orstavik D (2004) Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease. *Crit Rev Oral Biol Med* **15**: 308–320.

Kim JY, Song HS, Kim YB, Kwon J, Choi J-S, Cho Y-J, Kim B-Y, Rhee J-K, Myoung J, Nam Y-D & Roh SW (2016) Genome sequenc eof commensal bacterium *Enterococcus faecalis* CBA7120, isolated from a Korean fecal sample. *Gut Pathog* **8**: 62.

Kochkodan V, Tsarenko S, Potapchenko N, Kosinova V & Goncharuk V (2007) Adhesion of microorganisms to polymer membranes: a photobactericidal effect of surface treatment with TiO_2. *Desalin* **220**: 380–385.

Kotra LP, Haddad J & Mobashery S (2000) Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. *Antimicrob Agents Chemother* **44**: 3249-3256.

Krasowska A & Sigler K (2014) How microorganisms use hydrophobicity and what does this mean for human needs. *Front Cell Infect Microbiol* **4**: 112.

Kristich CJ, Li YH, Cvitkovitch DG & Dunny GM (2004) *Esp*-independent biofilm formation by *Enterococcus faecalis*. *J Bacteriol* **186**: 154-163.

Kuehn BM (2007) Antibiotic-resistant ‘‘superbugs’’ may be transmitted from animals to humans. *J Am Med Assoc* **298**: 2125–2126.

Klare I, Konstabel C, Badstubner D, Werner G & Witte W (2003) Occurrence and spread of antibiotic resistances in *Enterococcus faecium*. *Int J Food Microbiol* **88**: 269-290.

Kuhn I, Burman LG, Haeggman S, Tullus K & Murray BE (1995) Biochemical fingerprinting compared with ribotyping and pulsed-field gel electrophoresis of DNA for epidemiological typing of enterococci. *J Clin Microbiol* **33**: 2812-2817.

Kuhn I, Iversen A, Burman LG, Olsson-Liljequist B, Franklin A, Finn M, et al. (2003) Comparison of enterococcal populations in animals, humans and the environment-a European study. *Int J Food Microbiol* **88**: 133-145.

Lam MM, Seemann T, Bulach DM, et al. (2012) Comparative Analysis of the First Complete *Enterococcus faecium* Genome. *J Bacteriol* **194**: 2334-2341.

Larsen J, Schonheyder HC, Lester CH, Olsen SS, Porsbo LJ, Garcia ML, et al. (2010) Porcine-origin gentamicin-resistant *Enterococcus faecalis* in humans, Denmark. *Emerg Infect Dis* **16**: 682–684.

Latasa C, Solano C, Penades JR & Lasa I (2006) Biofilm-associated proteins. *C R Biol* **329**: 849-857.

Leach KL, Brickner SJ, Noe MC, et al. (2011) Linezolid, the first oxazolidinone antibacterial agent. *Ann N Y Acad Sci* **1222**: 49-54.

Leavis H, Top J, Shankar N, et al. (2004) A novel putative enterococcal pathogenicity island linked to the esp virulence gene of *Enterococcus faecium* and associated with epidemicity. *J Bacteriol* **186**: 672-82.

Leclercq R, Derlot E, Duval J, et al. (1988) Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. *N Engl J Med* **319**: 157-161.

Lee I, Kim YO, Park SC, & Chun J (2016) OrthoANI: An improved algorithm and software for calculating average nucleotide identity. *Int J Syst Evol Microbiol* **66**: 1100-1103.

Lejeune P (2003) Contamination of abiotic surfaces: what a colonizing bacterium sees and how to blur it. *Trends Microbiol* **11**: 179-84.

Lester CH, Frimodt-Moller N, Sorensen TL, et al. (2006) In vivo transfer of the *vanA* resistance gene from an *Enterococcus faecium* isolate of animal origin to an *E. faecium* isolate of human origin in the intestines of human volunteers. *Antimicrob Agents Chemother* **50**: 596-9

Levering V, Cao C, Shivapooja P, Levinson H, Zhao X *et al*. (2016) Urinary catheter capable of repeated on-demand removal of infectious biofilms via active deformation. *Biomaterials* **77**: 77-86.

Li X, Nair A, Wang S & Wang L (2015) Quality control of RNA-seq experiments, in RNA Bioinformatics, Methods in Molecular Biology, Vol. 1269, (Picardi E, ed.) Springer: New York, NY.

Lindenstrauss AG, Pavlovic M, Bringmann A, et al. (2011) Comparison of genotypic and phenotypic cluster analyses of virulence determinants and possible role of CRISPR elements towards their incidence in *Enterococcus faecalis* and *Enterococcus faecium*. *System Appl Microbiol* **34**: 553-60.

Lopes SP, Ceri H, Azevedo NF, Pereira MO (2012) Antibiotic resistance of mixed biofilms in cystic fibrosis: impact of emerging microorganisms on treatment of infection. *Intl J Antimicrob Agents* **40**: 260–263.

Maccallum WG & Hastings TW (1899) A Case of Acute Endocarditis Caused by Micrococcus Zymogenes (Nov. Spec.), with a Description of the Microorganism. *J Exp Med* **4**: 521-34.

Magauran CE & Salgado CD (2010) Challenges and Advances in Infection Control of Hematopoietic Stem Cell Transplant Recipients. *Infect Disord Drug Targets* **11**: 18-26.

Malanovic N & Loher K (2016) Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. *BBA Biomembranes* **1858**: 936-946.

Malathum K, Singh KV, Weinstock GM & Murray BE (1998) Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. *J Clin Microbiol* **36**: 211-215.

Mallon D, Corkill J, Hazel S, Wilson JS, French N, Benette M & Hart CA (2002) Excretion of vancomycin resistant *Enterococci* by wild mammals. *Emerg Infect Dis* **8**: 636–638.

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Dymock D & Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16SrRNA. *Appl Environ Microb* **64**: 795-799.

Marinho VCC, Worthington HV, Walsh T & Clarkson JE (2013) Fluoride varnishes for preventing dental caries in children and adolescents. *Cochrane Database Syst Rev* **7**: CD002279.

Matar MJ, Tarrand J, Raad I, et al. (2006) Colonization and infection with vancomycin-resistant Enterococcus among patients with cancer. *Am J Infect Cont* **34**: 534-536.

Menno L, Knetsch W & Koole L (2011) New strategies in the development of antimicrobial coatings: the example of increasing usage of silver and silver nanoparticles. *Polymers* **3**, 340–366.

McBride SM, Fischetti VA, Leblanc DJ, et al. (2007) Genetic diversity among *Enterococcus faecalis*. *PloS one* **2**: e582.

Meira QGS, Barbosa IM, Athayde AJAA, Siqueira-Júnior JP, Souza EL (2012) Influence of temperature and surface kind on biofilm formation by *Staphylococcus aureus* from food-contact surfaces and sensitivity to sanitizers. *Food Cont* **25**: 469–475.

Moellering RC Jr (1992) Emergence of Enterococcus as a significant pathogen. *Infec Dis Soc Am* **14**: 1173-1176.

Molbak K (2004) Spread of resistant bacteria and resistance genes from animals to humans—the public health consequences. *J Vet Med Series B* **51**: 364–369.

Monds RD & O'Toole GA (2009) The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends Microbiol* **17**: 73-87.

Mohamed JA, Huang W, Nallapareddy SR, Teng F & Murray BE (2004) Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. *Infect Immun* **72**: 3658-63.

Mohamed JA & Huang DB (2007) Biofilm formation by enterococci. *J Med Microbiol* **56**: 1581-1588.

Montanato L, Poggi A, Visai L, Ravaioli S, Campoccia D, Speziale P & Archiola CR (2011) Extracellular DNA in biofilms. *Int J Artif Organs* 34: 824-831.

Moubareck C, Bourgeois N, Courvalin P, Doucet-Populaire F (2003) Multiple antibiotic resistance gene transfer from animal to human *Enterococci* in the digestive tract of gnotobiotic mice. *Antimicrob Agents Chemother* **47**: 2993–2996.

Munita JM, Mishra NN, Alvarez D, Tran TT, Diaz L, Panesso D, et al (2014) Failure of High-Dose Daptomycin for Bacteremia Caused by Daptomycin-Susceptible Enterococcus faecium Harboring LiaSR Substitutions. *Clin Infect Dis* **59**:1277-1280.

Murray BE (1990) The life and times of the Enterococcus. *Clin Microbiol Reviews* **3**: 46-65.

Murray BE, Singh KV, Heath JD, Sharma BR & Weinstock GM (1999) Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. *J Clin Microbiol* **28**: 2059-2063.

National Center for Biotechnology Information (NCBI) [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1988] – [cited 2017 May 2017]. Available from: https://www.ncbi.nlm.nih.gov/

Neela V, Ghaznavi-Rad E, Ghasemzadeh-Moghaddam H, Nor Shamsudin M, van Belkum A & Karunanidhi A (2013) Frequency of methicillin resistant *Staphylococcus aureus* in the noses of Malaysian chicken farmers and their chicken. *Iran J Vet Research* **14**: 226-231.

NORM/NORM-VET (2010) Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway. Tromsø / Oslo 2011. ISSN:1502-2307 (print) / 1890-9965 (electronic). 2010: Tromsø, Norway

O'Toole G, Kaplan HB & Kolter R (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* **54**: 49-79.

Oliveira MMM, Alves E & Piccoli RH (2010) Biofilm formation by *Listeria monocytogenes* on stainless steel surface and biotransfer potential. *Brazilian J Microbiol* **41**: 97-106.

Olutiola PO, Famurewa O & Sonntag HG (2000) An Introduction to General Microbiology: A Practical Approach. Bolabay Publications: Nigeria.

Palmer KL, Godfrey P, Griggs A, et al. (2012) Comparative genomics of enterococci: variation in *Enterococcus faecalis*, clade structure in *E. faecium*, and defining characteristics of *E. gallinarumand E. casseliflavus*. *mBio* **3**: e00318-11.

Palmer KL, van Schaik W, Willems RJ & Gilmore MS (2014) Enterococcal genomics. In: Enterococci: from commensals to leading causes of drug resistant infection. (Gilmore MS, Clewell DB, Ike Y, Shankar N, eds.) Massachusetts Eye and Ear Infirmary: Boston.

Papaparaskevas J, Vatopoulos A, Tassios PT, Avlami A, Legakis NJ & Kalapothaki V (2000) Diversity among high-level aminoglycoside-resistant enterococci. *J Antimicrob Chemother* **45**: 277283.

Parsek MR & Singh PK (2003) Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol* **57**: 677-701.

Paulsen IT, Banerjei L, Myers GS, Nelson KE, Seshadri R, Read TD, et al. (2003) Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* **299** :2071–2074.

Peter A, Mathew J & Zacharia SA (2013) Comparative study of virulence factors in Enterococci from clinical and nonclinical sources. *J Pharm Biomed Sci* **29**: 745-752.

Pillai SK, Sakoulas G, Eliopoulos GM, Moellering RC & Jr Murray BE et al. Effects of glucose on fsr-mediated biofilm formation in *Enterococcus faecalis. J Infect Dis* **190**: 967-970.

Pintado V, Cabellos C, Moreno S, et al. (2003) Enterococcal meningitis: a clinical study of 39 cases and review of the literature. *Medicine* **82**: 346-364.

Poulsen LL, Bisgaard M, Son NT, Trung NV, An HM & Dlasgaard A (2012) *Enterococcus faecium* clones in poultry and in humans with urinary tract infections, Vietnam. *Emerging Infec Dis* **18**: 1096-1100.

Prakash VP (2005) Clinical prevalence, identification and molecular characterization of enterococci. PhD thesis, Pondicherry University: India

Praveena SM, Siraj SS, Suleiman AK & Aris AZ (2011) A brush up on water quality studies of Port Dickson, Malaysia. *Res J Environ Science* **5**: 841–849.

Rao SC, Ahmed M & Hagan R (2006) One dose per day compared to multiple doses per day of gentamicin for treatment of suspected or proven sepsis in neonates. *Cochrane database of systematic reviews* **1**: CD005091.

Recht MI & Puglisi JD (2001) Aminoglycoside resistance with homogeneous and heterogeneous populations of antibiotic-resistant ribosomes. *Antimicrob Agents Chemother* 45: 2414-2419.

Reinthaler FF, Posch J, Feieri G, Wust G, Haas D, Ruckenbauer G, Mascher F & Marth E (2003) Antibiotic resistance of *E. coli* in sewage and sludge. *Water Res* **37**: 1685–1690.

Ribeiro T, Abrantes M, Lopes MdFS & Crespo MTB (2007) Vancomycin susceptible dairy and clinical enterococcal isolates carry vanA and vanB genes. *Intl Food Microbiol* **113**: 289-295.

Rich RL, Kreikemeyer B, Owens RT, LaBrenz S, Narayana SV, Weinstock GM, et al. (1999) Ace is a collagen-binding MSCRAMM from *Enterococcus faecalis*. *J Biol Chem* **274**: 26939–26945.

Roca I, Akova M, Baquero F, Carlet J, Cavaleri M, Coenen S, et al. (2015) The global threat of antimicrobial resistance: science for intervention. *New Microbes New Infect* **6**: 22–29.

Rougier F, Claude D, Maurin M, et al. (2004) Aminoglycoside nephrotoxicity. *Curr Drug Targets Infect Disord* **4**: 153-62.

Ruiz-Garbajosa P, Bonten MJ, Robinson DA, Top J, Nallapareddy SR, Torres C, et al. (2006) Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. *J Clin Microbiol* **44**: 2220-2228.

Ryu H, Henson M, Elk M, Toledo-Hernandez C, Griffith J, Blackwood D, et al. (2013) Development of quantitative PCR assays targeting the 16S rRNA genes of Enterococcus spp. and their application to the identification of enterococcus species in environmental samples. *Appl Environ Microbiol*. **79**:196–204.

Sarjit A, Tan SM & Dykes GA (2015) Surface modification of materials to encourage beneficial biofilm formation. *AIMS Bioeng* **2**: 404-422.

Savage VJ, Chopra I & O'Neill AJ (2013) *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. *Antimicrob. Agents Chemother*. **57**:1968–1970.

Salipante SJ, SenGupta DJ, Cummings LA, Land TA, et al (2015) Application of whole genome sequencing for bacterial strain typing in molecular epidemiology. *J Clin Microbiol* **53**: 1072-1079.

Schleifer KH & Kilpper-Balz R (1984) Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus Enterococcus nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *Int J System Evol Microbiol* **34** :31–34.

Semedo T, Santos MA, Lopes MFS, Figueiredo Marques JJ, Barreto Crespo MT & Tenreiro R (2003) Virulence factors in food, clinical and reference enterococci: a common trait in the genus? *Syst Appl Microbiol* **26**: 13–22.

Senechal A, Carrigan SD & Tabrizian M (2004) Probing surface adhesion forces of *Enterococcus faecalis* to medical-grade polymers using atomic force microscopy. *Langmuir* **20**: 4172-4177.

Seno Y, Kariyama R, Mitsuhata R, Monden K & Kumon H (2005) Clinical implications of biofilm formation by *Enterococcus faecalis* in the urinary tract. *Acta Med Okayama* **59**: 79–87.

Seputiene V, Bogdaite A, Ruzauskas M & Suziedeliene E (2012) Antibiotic resistance genes and virulence factors in *Enterococcus faecium* and *Enterococcus faecalis* from diseased farm animals: pigs, cattle and poultry. *Polish J Vet Science* **15**: 431-8.

Sghir A, Gramet G, Suau A, et al. (2000) Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. *Appl Environ Microbiol* **66**: 2263-2266.

Sharifi Y, Hasani A, Ghotaslou R, Varshochi M, Hasani A, Aghazadeh M & Milani M (2012) Survey of virulence determinants among vancomycin resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from clinical specimens of hospitalized patients of northwest of Iran. *Open Microbiol J* **6**: 34-39.

Sherman JM (1937) The Streptococci. *Bacteriological reviews* **1**: 3-97.

Shi X & Zhu X. Biofilm formation and food safety in food industries. *Trends Food Sci Tech* **20**: 407-413.

Sidhu JPS, Skelly E, Hodgers L, Ahmed W, Li Y & Toze S (2014) Prevalence of Enterococcus Species and their virulence genes in Fresh water prior to and after storm events. *Environ Sci Technol* **48**: 2979-2988.

Song JY, Cheong HJ, Jo YM, Choi WS, Noh JY, Heo JY, et al. (2009) Vancomycin-resistant Enterococcus colonization before admission to the intensive care unit: A clinicoepidemiologic analysis. *American J Infec Control* 37: 734-40.

Sorensen TL, Blom M, Monnet DL, Frimodt-Moller N, Poulsen RL & Espersen F (2001) Transient intestinal carriage after ingestion of antibiotic-resistant *Enterococcus faecium* from chicken and pork. *New England J Med* **345**: 1161–1166.

Sousa C, Henriques M, Azeredo J, Teixeira P & Oliveira R (2008) *Stapylococcus epidermidis* glucose uptake in biofilm versus planktonic cells. *World J Microbiol Biotechnol* **24**: 423-426.

Sutherland IW (2001) The biofilm matrix--an immobilized but dynamic microbial environment. *Trends Microbiol* **9**: 222-227.

Sydnor ER & Perl TM (2011) Hospital epidemiology and infection control in acute-care settings. *Clinical microbiology reviews* **24**: 141-73.

Tasina E, Haidich AB, Kokkali S, et al. (2011) Efficacy and safety of tigecycline for the treatment of infectious diseases: a meta-analysis. *The Lancet infectious diseases* **11**: 834-844.

Teale CJ & Moulin G (2012) Prudent use guidelines: a review of existing veterinary guidelines. *Rev Sci Tech* **31**:343–354.

Teh AHT, Wang Y & Dykes GA (2014) The influence of antibiotic resistance gene carriage on biofilm formation by two *Escherichia coli* strains associated with urinary tract infections. *Can J Microbiol* **60**: 105-111.

Teh AH, Lee SM & Dykes GA (2016) Draft genome sequences of three multiantibiotic-resistant *Campylobacter jejuni* strains (2865, 2868 and 2871) isolated from poultry at retail outlets in Malaysua. *Genome Announc* **5**: 4 pii: e00331-16.

Templer SP, Rohner P & Baumgartner A (2008) Relation of *Enterococcus faecalis* and *Enterococcus faecium* isolates from foods and clinical specimens. *J Food Prot* **71**: 2100–2104.

Tendolkar PM, Baghdayan AS, Gilmore MS & Shankar N (2004) Enterococcal surface protein Esp, enhances biofilm formation by *Enterococcus faecalis*. *Infect Immun* **72**: 6032–6039.

Texeira L, Carvalho M & Facklan R. (2007) *Enterococcus.* In: Manual of Clinical Microbiology, p. 430-442, (Murray P, ed.). ASM Publishing: Washington.

Texeira LM & Merquior VLC (2012) Enterococcus. In: Molecular Typing in Bacterial Infections, Infectious Disease, p. 17-23, (De Filippis I & McKee ML eds.). Springer Science: New York.

Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, Amonrena B, Leiva J, Penades JR & Lasa I (2001) The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. *Appl Environ Microbiol* 67: 4538-4545.

Top J, Willems R, Blok H, et al (2007) Ecological replacement of *Enterococcus faecalis* by multiresistant clonal complex 17 *Enterococcus faecium*. *Clin Microbiol Infect* **13**: 316-319.

Tremblay CL, Letellier A, Quessy S, Boulianne M, Daignault D & Archambault M (2011) Multiple-antibiotic resistance of *Enterococcus faecalis* and *Enterococcus faecium* from cecal contents in broiler chicken and turkey flocks slaughtered in Canada and plasmid colocalization of tetO and ermB genes. *Journal of Food Prot* **74**: 1639-48.

Qin X, Singh KV, Weinstock GM & Murray BE (2000) Effects of *Enterococcus faecalis* fsr genes on production of gelatinase and a serine protease and virulence. *Infect Immun* **68**: 2579–2586.

Qin X, Galloway-Pena JR, Sillanpaa J, Roh JH, Nallapareddy SR, Chowdhury S, et al. (2012) Complete genome sequence of *Enterococcus faecium* strain TX16 and comparative genomic analysis of *Enterococcus faecium* genomes. *BMC Microbiol* **12**: 135.

Ubeda C, Taur Y, Jenq RR, et al. (2010) Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. *J Clin Inves* **120**: 4332-41.

Uttley AH, Collins CH, Naidoo J, et al. (1988) Vancomycin-resistant enterococci. *Lancet* **1**: 57-58.

Vakulenko SB & Mobashery S (2003) Versatility of aminoglycosides and prospects for their future. *Clin Microbiol Rev* **16**: 430-450.

Van Merode AEJ, Van der Mei HC, Busscher HJ, Waar K & Krom BP (2006) *Enterococcus faecalis* strains show culture heterogeneity in cell surface charge. *Microbiol* **152**: 807–814.

Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D & Goossens H (2004) Evelopment of a multiplex PCR for the detection of *asa1*, *gelE*, *cylA*, *esp*, and *hyl* genes in *Enterococci* and survey for virulence eterminants among European hospital isolates of *Enterococcus faecium. J Clin Microbiol* **42**: 73-79.

Vidal L, Gafter-Gvili A, Borok S, et al. (2007) Efficacy and safety of aminoglycoside monotherapy: systematic review and meta-analysis of randomized controlled trials. *J Antimicrob Chemother* **60**: 247-257.

Waar K (2004) Pathogenesis of nosocomial infections with *Enterococcus faecalis*. Doctoral Thesis pp.152.

Wang JL & Hsueh PR (2009) Therapeutic options for infections due to vancomycin-resistant enterococci. Expert opinion on pharmacotherapy **10**: 785-96.

Weng PL, Ramli R, Shamsudin MN, Cheah YK & Hamat RA (2013) High genetic diversity of *Enterococcus faecium* and *Enterococcus faecalis* clinical isolates by pulsed-field gel electrophoresis and multilocus sequence typing from a hospital in Malaysia. *Biomed Res Int* **938937**: 1-6.

Werner G, Coque TM, Hammerum AM, et al. (2008) Emergence and spread of vancomycin resistance among enterococci in Europe. Euro surveillance : bulletin europeen sur les maladies transmissibles = European communicable disease bulletin 13(47).

Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS (2002) Extracellular DNA required for bacterial biofilm formation. *Science* **295**: 1487.

Yahav D, Lador A, Paul M, et al. (2011) Efficacy and safety of tigecycline: a systematic review and meta-analysis. *J Antimicrob Chemother* **66**: 1963-1971.

Yasufuku T, Shigemura K, Shirakawa T, Matsumoto M, Nakano Y, Tanaka K, et al. Mechanisms of and risk factors for fluoroquinolone resistance in clinical *Enterococcus faecalis* isolates from patients with urinary tract infections. *J Clin Microbiol* **49**: 3912–3916.

Yildirim M, Sencan I, Ozdemir D, Oksuz S, Yilmaz Z & Sahin I (2007) Vancomycin and high-level aminoglycoside resistant Enterococcus carriage and the risk factors related to resistance in hospitalized patients. *Mikrobiyol Bul* **41**: 271–277.

Yip T, Tse KC, Ng F, Hung I, Lam MF, Tang S, et al. (2011) Clinical course and outcomes of single-organism *Enterococcus peritonitis* in peritoneal dialysis patients. *Perit Dial Int* **3**: 522-528.

Appendix I

List of Antibiotics Used in Current Study

The following appendix contains the list of antibiotics used in the current study with interpretation breakpoints (Chapter 2).

Table A.1: The following antibiotics were tested with interpretation breakpoints in parentheses (MIC):

Antibiotics	**Minimum Inhibitory Concentration (MIC) (µg/ml)**
Vancomycin	≥32
Penicillin	≥16
Nitrofurantoin	≥128
Ciprofloxacin	≥4
High level Gentamicin	≥512
Tetracycline	≥16
Levofloxacin	≥8
Fosfomycin	≥256

Appendix II

Primers Used for the Detection of *Enterococcus*

faecalis Virulence Determinants

The following appendix contains the list PCR primers used for the detection of *Enterococcus faecalis* virulence determinants (Chapter 2).

Table A.2: PCR primers used for the detection of *Enterococcus faecalis* virulence determinants

Gene	Sequence (5′→3′)	Product size (bp)	GenBank accession no.	Position
esp	TTGCTAATGCTAGTCCACGACC	932	AF034779	1217
	GCGTCAACACTTGCATTGCCGA			2149
gelE	ACCCCGTATCATTGGTTT	405	M37185	762
	ACGCATTGCTTTTCCATC			1163
cylA	GACTCGGGGATTGATAGGC	688	AD1CLYL	6656
	GCTGCTAAAGCTGCGCTTAC			7344
asa1	CCAGCCAACTATGGCGGAATC	529	SFPASA1	3122
	CCTGTCGCAAGATCGACTGTA			3651
asa373	GGACGCACGTACACAAAGCTAC	619	AJ132039	3094
	CTGGGTGTGATTCCGCTGTTA			3713
ace	GGAATGACCGAGAACGATGGC	616	AF159247	160
	GCTTGATGTTGGCCTGCTTCCG			776
efaA	GCCAATTGGGACAGACCCTC	688	EFU03756	312
	CGCCTTCTGTTCCTTCTTTGGC			1000
EF0591	CGGAAGTATTGCGTTTGGTGGG	844	NC_004668	99
	CGTCTGCTTTAATAGACCCCAG			1003
EF3314	AGAGGGACGATCAGATGAAAAA	566	NC_004668	35
	ATTCCAATTGACGATTCACTTC			601

Appendix III

Minimum Inhibitory Concentration (MIC) Distribution of Antibiotics on *Enterococcus faecalis*

The following appendix contains the minimum inhibitory concentration (MIC) distribution of antibiotics used in this study on *Enterococcus faecalis* isolated from farm animals, water sources and hospitalized patients (Chapter 2).

Table A.3: Minimum Inhibitory Concentration (MIC) distribution of antibiotics for *Enterococcus faecalis* isolated from farm animals, water sources and hospitalized patients.

Rank[1]	Antibiotic and break points (µg/mL)[2]	Range of tested antibiotics and number of isolates farm animals/water/patients (120/100/30) inhibited by MIC (µg/mL)													
		0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**% of resistant isolates**
I	High-Level Gentamicin, (≥512)	0/0/5	0/0/7	7/3/2	10/3/1	7/5/4	10/5/4	9/2/3	8/12/3	11/16/0	9/10/0	10/11/0	15/18/0	24/16/0	20/16/0
	Vancomycin, (≥32)	19/6/0	21/7/0	17/7/3	16/10/3	9/16/5	11/17/7	12/9/6	7/12/6	5/7/0	3/7/0	0/2/0	0/0/0	0/0/0	7/16/0
	Penicillin, (≥16)	0/4/4	0/10/3	9/12/4	11/9/2	16/15/5	19/13/7	17/15/3	16/11/2	17/9/0	9/2/0	6/0/0	0/0/0	0/0/0	40/22/7
	Levofloxacin, (≥8)	16/19/6	15/12/5	10/9/9	19/15/5	16/17/3	16/16/0	13/10/1	11/2/1	4/0/0	0/0/0	0/0/0	0/0/0	0/0/0	23/12/7
	Ciprofloxacin, (≥4)	19/18/5	5/15/5	16/13/8	18/16/8	16/19/3	15/16/1	10/3/0	6/0/0	5/0/0	0/0/0	0/0/0	0/0/0	0/0/0	30/19/3
II	Tetracycline, (≥16)	0/0/0	0/8/0	8/5/4	7/8/8	11/12/9	12/7/5	8/11/3	15/16/1	19/18/0	11/8/0	17/7/0	12/0/0	0/0/0	62/49/3
III	Nitrofurantoin, (≥128)	11/7/0	14/9/0	13/5/0	11/6/4	12/8/4	10/10/5	14/12/9	9/8/4	10/13/3	9/8/0	7/12/1	0/2/0	0/0/0	6/14/0
	Fosfomycin, (≥256)	10/8/0	9/16/4	10/13/7	9/11/4	9/8/6	13/16/0	13/9/9	6/5/0	8/4/0	11/4/0	9/1/0	13/5/0	0/0/0	11/5/0

[1]Rank WHO categorization of critical antimicrobials in human health (Rank I - critically important, Rank II - highly important, Rank III - important) (Collignon et al. 2009)

[2]Microbroth dilution breakpoints, NARMS (http://www.ars.usda.gov) (additionally, the breakpoints are marked as vertical bold black lines)

Appendix IV

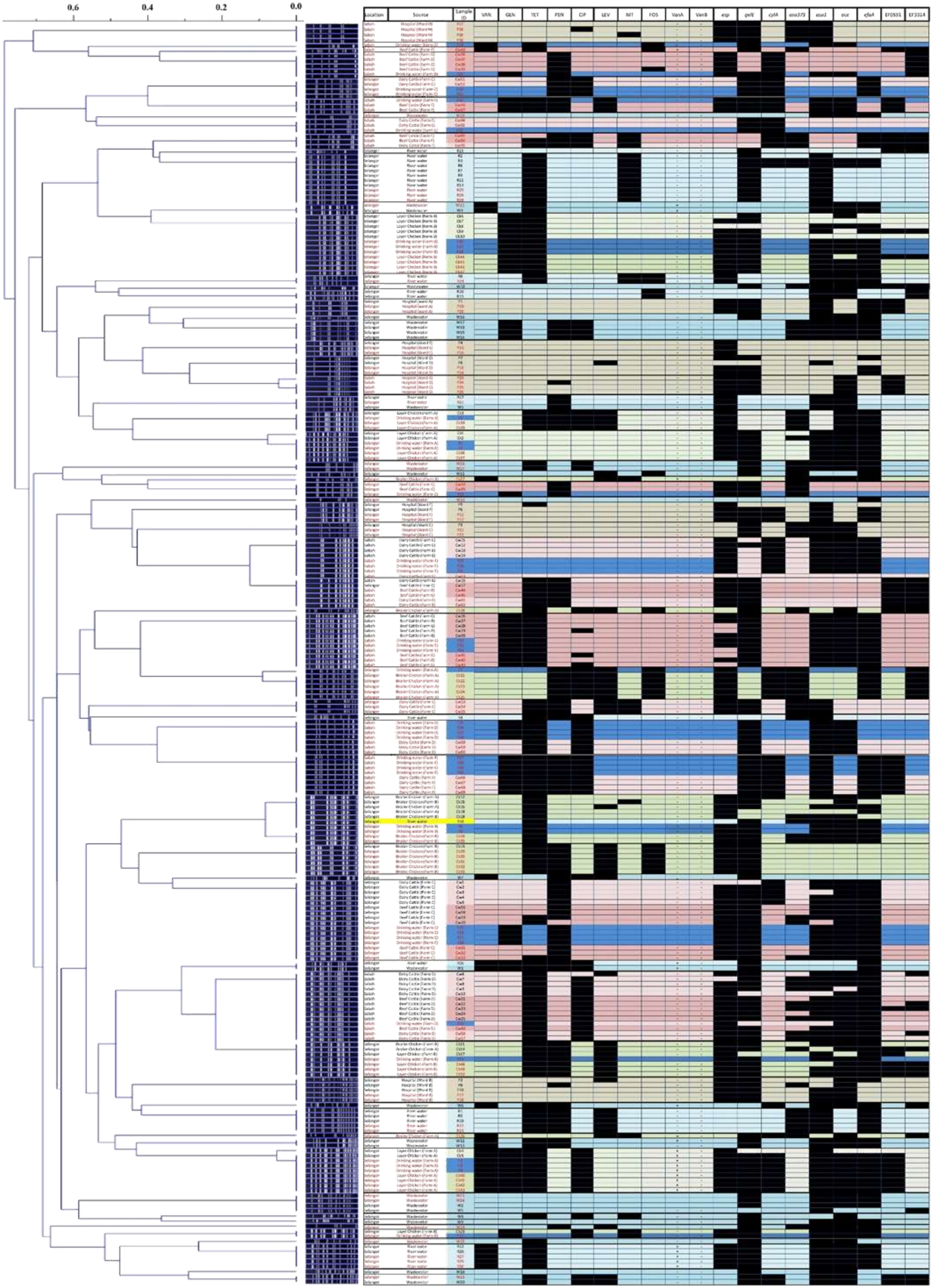
Cluster Analysis of *Enterococcus faecalis*

isolates Including Antibiotic Resistance and

Virulence Genes Profile

The following appendix contains the cluster analysis of all 250 *Enterococcus faecalis* isolates and an included set of antibiotic resistance and virulence genes using simple matching UPGMA clustering (Chapter 2).

Figure A.1: Cluster analysis of *Enterococcus faecalis* isolated and an included set of antibiotic resistance and virulence genes using siple matching UPGMA clustering. The dendrogram shows a simplified tree structure for 250 *E. faecalis* isolates from farm animal feces, water sources and hospital patients.



Note: Black text color indicates sampling in July 2014; Red text indicates sampling in December 2014. VAN = Vancomycin, GEN = Gentamicin, PEN = Penicillin, LEV = Levofloxacin, CIP = Ciprofloxacin, TET = Tetracycline, NIT = Nitrofurantoin, FOS = Fosfomycin

Hospital patients | Farm animal drinking water | Wastewater | River water | Dairy cattle | Beef cattle | Layer chicken | Broiler chicken

Appendix V

Demographic Distribution of Chosen

Enterococcus faecalis Strains with Antibiotic

Resistance and Virulence Gene Profile

The following appendix contains the demographic distribution of 63 *Enterococcus faecalis* strains (chosen on the bases of different pulsotypes) isolated from water sources, farm animal feces and hospitalized UTI patients, with the addition of the antibiotic resistance and virulence gene profile (Chapter 3).

Figure A.2. Demographic distribution of *Enterococcus faecalis* strains isolated from water sources, farm animals feces and hospitalized UTI patients; with the addition of the antibiotic resistance and virulence gene

			Antibiotic Resistance								Van resistance genes		Virulence genes							
Location	Source	Sample ID	VAN	GEN	TET	PEN	CIP	LEV	NIT	FOS	VanA	VanB	*esp*	*gelE*	*cylA*	*asa373*	*asa1*	*ace*	*efaA*	EF0591	EF3314
Selangor	River water	R27	■		■						+	-	■	■		■	■		■		
Selangor	River water	R19			■	■		■			-	-				■	■		■		
Selangor	River water	R6			■				■		-	-		■	■	■	■	■	■		
Selangor	River water	R13									-	-		■						■	■
Selangor	River water	R24			■				■	■	-	-	■				■	■	■		
Selangor	River water	R4			■	■	■				-	-				■	■				
Selangor	River water	R15								■	-	-					■	■			■
Selangor	Wastewater	W6	■		■	■					+	-			■		■	■	■		■
Selangor	Wastewater	W19									-	-		■			■	■			
Selangor	Wastewater	W20			■		■	■			-	-		■	■		■	■		■	■
Selangor	Wastewater	W11						■		■	-	-		■		■			■		
Selangor	Wastewater	W7	■			■	■	■			+	-		■							
Selangor	Wastewater	W9									-	-		■			■	■	■		
Selangor	Wastewater	W2												■			■	■	■		
Selangor	Wastewater	W10									-	-		■		■	■	■	■	■	■
Selangor	Wastewater	W8			■		■		■		-	-		■				■			
Selangor	Wastewater	W17		■		■	■				-	-				■	■	■	■		
Selangor	Wastewater	W16									-	-		■			■	■	■		
Selangor	Wastewater	W22	■		■						+	-					■	■	■		
Selangor	Wastewater	W13	■		■	■					+	-		■			■	■	■		
Selangor	Wastewater	W25			■						-	-		■			■	■			
Selangor	Wastewater	W26			■		■				-	-		■		■	■	■	■		
Selangor	Wastewater	W28				■		■			-	-		■		■	■	■	■		
Selangor	Wastewater	W29									-	-					■	■	■		
Selangor	Wastewater	W30									-	-							■		
Selangor	Wastewater	W1	■	■	■	■	■				+	-		■					■		
Selangor	Wastewater	W3				■					-	-	■				■	■			
Sabah	Farm water troughs	F24	■		■					■	+	-		■			■	■			
Sabah	Farm water troughs	F25		■	■		■				-	-		■			■	■			
Selangor	Chicken	Ck37									-	-	■	■		■	■	■	■		
Selangor	Chicken	Ck3			■	■	■	■			-	-	■			■		■	■		
Selangor	Chicken	Ck40	■	■		■	■				+	-	■	■	■			■	■	■	
Selangor	Chicken	Ck25				■		■		■	-	-	■		■	■			■		■
Selangor	Chicken	Ck26	■					■	■		+	-		■	■	■		■			■
Selangor	Chicken	Ck27	■					■	■		+	-				■		■	■		
Selangor	Chicken	Ck28			■		■			■	-	-		■		■		■		■	■
Selangor	Chicken	Ck6		■	■						-	-		■				■			
Selangor	Chicken	Ck50			■	■		■			-	-		■			■	■	■		
Selangor	Chicken	Ck20		■			■		■		-	-	■	■			■	■	■		
Selangor	Chicken	Ck35			■						-	-	■	■	■		■	■	■		
Selangor	Chicken	Ck13			■	■		■		■	-	-	■	■							
Selangor	Cattle	Cw33			■	■					-	-	■	■		■	■	■	■		
Selangor	Cattle	Cw35				■					-	-	■	■	■	■			■		
Selangor	Cattle	Cw52				■	■				-	-		■	■				■		
Selangor	Cattle	Cw55			■	■		■	■		-	-	■	■		■	■				
Sabah	Cattle	Cw11									-	-	■	■	■		■	■	■		
Sabah	Cattle	Cw62			■	■					-	-	■	■						■	■
Sabah	Cattle	Cw26		■	■						-	-	■	■			■	■	■		
Sabah	Cattle	Cw65									-	-		■	■						
Sabah	Cattle	Cw24			■		■				-	-	■	■		■		■	■		
Sabah	Cattle	Cw37				■					-	-	■		■						■
Sabah	Cattle	Cw47		■	■	■		■			-	-	■	■			■		■	■	
Sabah	Cattle	Cw49			■	■	■		■		-	-			■	■			■	■	■
Sabah	Cattle	Cw69		■	■	■	■				-	-		■	■		■		■	■	
Sabah	Cattle	Cw48	■		■					■	+	-		■		■				■	■
Selangor	Hospital	P5			■						-	-	■				■				
Selangor	Hospital	P8						■			-	-					■				
Selangor	Hospital	P16									-	-	■								
Selangor	Hospital	P2						■			-	-	■	■				■	■		
Selangor	Hospital	P20									-	-	■	■			■		■	■	
Selangor	Hospital	P3									-	-		■		■	■	■			
Sabah	Hospital	P24				■					-	-	■	■				■		■	
Sabah	Hospital	P28					■				-	-	■	■		■	■				

Note: VAN = Vancomycin, HL-GEN = High-Level Gentamicin, TET = Tetracycline, PEN = Penicillin, LEV = Levofloxacin, NIT = Nitrofuratoin, CIP = Ciprofloxacin, FOS = Fosfomycin.

River water | Wastewater | Water from troughs in farms | Farm chicken | Farm cattle | Hospitalized UTI patients

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Minireview

The public health risks of multiple-drug resistant (MDR) *Enterococcus* spp. in Southeast Asia

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spp.

Keywords: Antibiotics, *Enterococcus* spp, Public health, Southeast Asia, Transmission.

Abstract – 130 words

Enterococci rank as one of the leading causes of nosocomial infections, such as urinary tract infection, surgical wound infection and endocarditis in humans. These infections can be hard to treat due to the rising incidence of antibiotic resistance. Enterococci inhabiting nonhuman reservoirs appear to play a critical role in the acquisition and dissemination of antibiotic resistance determinants. The spread of antibiotic resistance has become a major concern in both human and veterinary medicine, especially in Southeast Asia where many developing countries have poor legislations and regulations to control the supply and excessive use of antimicrobials. This review addresses the occurrence of antibiotic resistant enterococci in ASEAN (Association of Southeast Asian Nations) countries and proposes infection control measures that should be applied to limit the spread of multiple drug resistant enterococci.

The enterococci are a complex and diverse group of bacteria. They are commonly found in the gastrointestinal tract, female genital tract, oral cavity and skin of humans and other animals. Enterococci are also found in soil, water, and foods. Different species of enterococci are able to grow between 10°C to 45°C and in environments with a broad range of pH values (1). These characteristics present a challenge to those who wish to control the spread of the pathogenic species of these organisms which can pose serious infections in humans and animals. In addition, enterococci have the capacity to acquire a wide variety of antimicrobial resistant factors through horizontal exchange of mobile genetic material which presents further problems in the management of patients with enterococcal infections (2). Enterococci can be transmitted to humans by various means including contaminated food and water sources (3). The presence of antibiotic resistant enterococci in the faecal material of animals has therefore become a major global concern in both human and veterinary medicine. Most of the studies concerning the transmission of microorganisms from food animals to humans have focused on pathogens that pose a direct threat to human health (4). Given the significant importance of *Enterococcus* spp. to public health and the farming industry, additional information on the genetics and transmission of multi-drug resistance in these species is essential.

Legislation and regulations to control the supply and excessive use of antimicrobials are

, 6, 7) and the prevalence of

56 antimicrobial resistance of major bacterial pathogens such as enterococci has been rapidly

57 increasing in Asia (8, 9, 10, 65). In particular the rise of multi drug resistant (MDR) enterococci

58 is of great concern.

This review briefly summarises the classification of enterococci and discusses the incidence and causes of MDR enterococci in non-human reservoirs, particularly farm animals and water supplies. The prevalence in hospitals is also reviewed and possible control measures are suggested with a particular focus on the Association of Southeast Asian Nations (ASEAN).

Human Reservoirs of *Enterococcus* spp.

Enterococcus spp. are normal flora of the human gastrointestinal tract (11). Enterococci are minority members of the bacterial community in humans, as molecular analysis has shown that these bacteria make up no more than 1% of the intestinal microflora of an adult (1, 12). However, the medical importance of these bacteria overshadows their relative numbers in the intestinal tract. This is due to *Enterococcus* spp. now ranking as among the leading causes of nosocomial infections in humans (13).

Enterococci are well adapted for living in biofilms where adhesion to extracellular matrix proteins of the human gut is the first step in colonization and infection (14, 15). The ability to form biofilms is a critical factor in causing endodontic and urinary tract infections as well as endocarditis. According to the National Institutes of Health, biofilms are involved in over 80% of microbial infections in the body (16). A mature biofilm can tolerate antibiotics at concentrations of 10 to 1000 times more than are required to kill planktonic bacteria (17). A recent study in

inical *Enterococcus faecalis* isolates in

77 their capacity to form biofilms when subjected to sub-minimum inhibitory concentration (MIC)

78 levels of antimicrobial compounds, Clindamycin and Tetracycline, found in endodontic

79 medicaments (18). A strong correlation between the presence of the virulence gene, *esp*, and the

80 ability of enterococci to form biofilms in vitro has also been reported (19, 20, 21). The

contribution of *esp* to biofilm formation was found to be most pronounced in the presence of 0.5% (wt/vol) or greater glucose (19). These results suggest that, whereas *esp* is important in biofilm formation, additional determinants in *E. faecalis* may also contribute to biofilm formation (19). Studies on antibiotic resistance and biofilm production of enterococci with relevance to Southeast Asia have not been focused on due to fragmented information.

Certain strains of enterococci have long been known as important causes of endocarditis and in the 1970's began to be recognized as common causes of hospital-acquired urinary tract and wound infections (13). While traditionally 90% of all enterococcal infections were caused by *E. faecalis* and only 10% by *E. faecium*, the proportion of *E. faecium* has gradually increased over the years to 40% (1). Other enterococcal species, including *E. avium, E. casseliflavus, E. cecorum, E. dispar, E. durans, E. gallinarum, E. hirae, E. malodoratus, E. mundtii, E. pseudoavium, E. raffinosus, E. saccharolyticus, E. seriolocida* and *E. solitarius* are primarily found in the gastrointestinal tract of various animals but occasionally isolated from human infections (1).

Non-human Reservoirs of *Enterococcus* spp.

Apart from humans, *Enterococcus* spp. are a natural part of the intestinal flora in most mammals

China and Papua New Guinea play a

major role globally in terms of meat production, contributing roughly 13 to 33% of global meat production from 1979 to 2004 (23). Southeast Asia also imports livestock from China, India, Australia and the US. The large importers of livestock, mainly cattle and pigs, are Singapore, Malaysia and Indonesia (23). Studies carried out in Malaysia, Thailand, Vietnam, Indonesia, and other Southeast Asian countries reported MDR enterococci isolated from livestock and animal

related products (22, 24). Many Southeast Asian nations such as Malaysia, Myanmar, Indonesia, Thailand and Vietnam have flourishing poultry and livestock industries, and are also major exporters around the Asian region (25). Countries that either export or import livestock or chickens could be inadvertently involved in the spread of MDR *E. faecalis* due to the widespread use of antimicrobials in these industries as discussed later (26).

Use of antimicrobials in Southeast Asia

In addition to the treatment of human infection, antimicrobial agents are used on food animals, on pets and for laboratory use. In modern food animal production, antimicrobial agents are used in four different ways: (i) therapy, the treatment of infections of animals; (ii) metaphylactics, the treatment of clinically healthy animals belonging to the same flock or pen as animals with clinical signs; (iii) prophylactics, the treatment of healthy animals in a period of stress to prevent disease, such as during early weaning; and (iv) growth promotion, the inclusion of antimicrobial agents continuously in animal feed to prevent infections and improve growth (27, 28).

It is challenging to obtain reliable data on quantities of antimicrobial agents used on food animals worldwide. In the US, the farm animal population (consisting of approximately 5.34 million lamb and sheep, 89.3 million cattle, 113.2 million pigs and 479 million poultry in 2012) used an estimated quantity of antimicrobial agents of 13542 tonnes, while the usage for humans

, 30). According to a 2013 report in

121 the UK from the Department for Environmental Food and Rural Affairs (DEFRA), it was

122 estimated that approximately 290 tonnes of antimicrobial agents were sold for food animals in

123 2011 (31). The UK farm animal population consisted of approximately 32 million lamb and

124 sheep, 9.7 million cattle, 4.8 million pigs and 162 million poultry in 2012 (32). Antimicrobial

consumption data are lacking in many developing countries including ASEAN countries (33). Table 1 shows the livestock population in ASEAN countries from the year 2010 as well as the estimated antimicrobial consumption in cattle, chicken and pigs (34). The estimates of antimicrobial consumption presented in Table 1 are based on antimicrobial consumption per population correction unit (PCU) as devised by Van Boeckal (35) for Organization for Economic Co-operation and Development (OECD) countries. The mean of the posterior for antimicrobial consumption in cattle was 45 mg/PCU, 148 mg/PCU for chickens and 172 mg/PCU for pigs (35). PCUs are used to compare population and production of different types of livestock across countries and correspond to 1kg of living or slaughtered animals (36) using an estimate of 2.5 kg per chicken (37), 100 kg per pig (38) and 600 kg per cattle (39). Assuming that antimicrobial consumption in chicken, cattle and pigs represent majority of antimicrobial consumption in food-producing animals, the total consumption of antimicrobials was calculated for each country by pooling the estimates collected by multiplying the per PCU figure by the total national livestock population for each type of livestock (35). Based on the estimated values of antimicrobial consumption in Table 1, Indonesia, Vietnam and Myanmar are the three leading users of antimicrobials for farm use on a total per country basis.

Although there is a large amount of data about the emergence of antimicrobial resistance

rmation is fragmented since it has

143 been published in different papers from different countries over several decades (40, 41, 42, 43).

144 However, several studies show the extent of unregulated and inappropriate use of antimicrobials

145 in food animals in developing Southeast Asian countries such as Vietnam and Malaysia (22, 24,

146 44). Usui et al. (24) obtained results that demonstrate the use of antimicrobials in chickens in

147 Southeast Asian countries, especially Vietnam, to be higher than developed countries (44). In

148 Vietnam, Colistin was reported as a commonly used antibiotic on poultry, representing 4 to 7%
149 of those used in quantitative terms compared with 1.6% reported from nine European countries
150 (45). The use of antimicrobials in Vietnamese aquaculture has also been reported to be high with
151 700g per tonne of production compared to 1 to 200g per tonne in three European countries,
152 Canada and Chile (46). In Malaysia, there are currently 97 antimicrobials registered for use
153 according to the National Pharmaceutical Control Bureau (NPCB) of the Ministry of Health,
154 Malaysia. Most of these registered drugs are used in poultry and pig farms. Unfortunately, more
155 than half of the antibiotics registered with the Ministry of Health for food animals are not
156 recommended for veterinary use by the World Health Organization (WHO). These antibiotics
157 include Ampicillin, Amoxycillin, Cefadroxil, Chlortetracycline, Oxytetracycline, Doxycycline,
158 Sulfadiazine, Sulfadimethoxine, Erythromycin, Spiramycin, Neomycin, Gentamicin and
159 Flumequine (47). Macrolides, Trimethoprim, Sulfonamides, Fluroquinolones and Tetracyclines
160 are classes of antibiotics that are commonly used in animal husbandry and human medicine in
161 the Southeast Asian region (6, 7, 48).

162 To summarise, in comparison with western countries, geographic variations in the use of
163 antimicrobials for poultry and livestock are notable in Southeast Asia due to different standards
164 and fragmented policies for antimicrobial usage between countries (40). Countries such as

e control of residues of veterinary

drugs, however, issues relating to facilities, human resources and law enforcement need to be controlled (49). The department of livestock and fisheries in Laos lack consistent methods in evaluating and addressing antimicrobial resistant issues (50). Myanmar also has a major existing problem of inappropriate usage of antimicrobials and most farmers use antimicrobials without any consultation by veterinarians (51). Much work is needed in elucidating the level of

171 antimicrobial resistance in these countries entailing cost, man power resources, and policy
172 reviews (6, 7).

173 Monitoring systems in developed countries, such as The Danish Integrated Antimicrobial
174 Resistance Monitoring and Research Programme in Denmark established in 1995, are used to
175 assess antimicrobial resistance in bacteria, including enterococci, from healthy food producing
176 animals (41). Control measures set by the World Organization for Animal Health (OIE) and the
177 Food and Agricultural Organization (FAO) in 2010 includes published guidelines for national
178 antimicrobial surveillance programs in animals and the responsible use of antimicrobials in them
179 (42). The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
180 reported a decrease in MDR *E. faecalis* from 40% in 2011 to 34% in 2012 from pigs. Prevalence
181 of MDR *E. faecalis* in broilers has also decreased from 13% in 2009 to 5% in 2013 (41).

182 In December 1998, the European Commission decided to ban the use of Bacitracin,
183 Spiramycin, Tylosin, and Virginiamycin for growth promotion beginning July 1 1999 (52).
184 These initiatives follow the recommendations by the WHO and have had significant effects on
185 the types and amounts of antimicrobial agents used. In comparison to the legislation and policies
186 in most ASEAN nations, the European Union has a stronger control over regulating non-
187 therapeutic uses of antibiotics in animals. The European Union leads the world in reducing

zerland were the first countries to

189 unilaterally ban all non-therapeutic antibiotic growth promoters in animal feed (47). A more

190 organized monitoring system of antimicrobial resistance in both agricultural and clinical settings

191 and restricting their use is essential for preserving the therapeutic value of antibiotics in

192 Southeast Asia.

Enterococcus spp. in the Environment and Water

Environmental and water samples often contain enterococci (53). Large amounts of human and animal wastes are distributed into the environment through sewage or non-sewage systems. For almost a century, enterococci have been used as indicators of faecal contamination of water and food for human consumption (1). Pathogenic bacteria in environmental surface waters originate mainly from the final effluent discharge from sewage wastewater treatment plants. Treated sludge, a by-product from treated sewage waste water containing the faecal contents of animals and humans, can be used as fertilizers on agricultural land which could potentially pass on MDR strains to food supply (54). Challenges for effective wastewater management differ in South East Asian countries as well. These include poor sanitation levels, especially in rural areas, inadequate sewerage network coverage, and lack of sewage treatment facilities (55). Many countries in Southeast Asia still depend on septic tanks and other low cost onsite sanitation facilities. However, most of these countries do not have specific policies, legal and institutional framework for appropriate septage management. Unfortunately septic tanks are poorly designed and not accurately constructed, operated and maintained in many cases. In Vietnam (56) a low treatment performance efficacy of only 20 to 30% BOD removal was observed. According to the AECOM and SANDEC 2010, the amount of generated septage that has been treated varies among

a, 5% in Metro Manila of Philippines,

211 less than 4% in Vietnam and 30% in Thailand (57). In environmental water such as agricultural

212 wells on animal farms, coastal waters, rivers and canals, the species considered as faecal

213 contaminants are mainly *E. faecalis* and *E. faecium*, but other species can also be recovered (1).

214 The water cycle has been suggested as a transmission route for resistance to antibiotics (54) and

215 this may be particularly true if incentives for monitoring the water quality are lacking and the

possibility of direct discharge of poorly treated sewage into seawater and rivers is present. Two studies (58, 59) have isolated MDR enterococci in coastal bathing waters and storm waters which lead to recreational beaches around Malaysia. The findings suggest that these recreational beaches may contribute to the dissemination of MDR enterococci and virulence characteristics. Another study carried out in Thailand found a high prevalence of MDR enterococci, out of which 10.3% were VRE isolates, from environmental water including agricultural wells on animal farms, rivers and canals (54). This again suggests a potential transfer route of MDR enterococci and resistance genes into the human-food chain and environment which could potentially pose a threat to public health. Table 2 summarizes studies carried out in Southeast Asian countries investigating incidences on antibiotic resistant *Enterococcus* species in the environment, namely water sources and farm animals.

Transfer of Resistance between Nonhuman and Human Reservoirs

Infections with enterococci in animals are rarely specifically targeted with antimicrobial agents. However, as normal inhabitants of the intestinal tract, enterococci are exposed to antimicrobial selection every time animals are subjected to antimicrobial therapy or are given antimicrobial agents for growth promotion (60).

Enterococci are one of the traditional bacterial markers for faecal contamination of food

or several decades that enterococci

234 from nonhuman sources could contaminate food intended for human consumption (54). Clearly
235 enterococci with resistance genes may reach humans in several ways, including direct contact
236 with farm personnel (22, 61), via waste and surface water (54, 58, 59), or by contact with or
237 consumption of food animals and food of animal origin (22, 24). Although the hygienic

standards of meat production are high in most developed countries, faecal contamination of meat products cannot be completely eliminated (62). Figure 1 shows the complex epidemiology of enterococci and its ecological relationship between different reservoirs (63). The interaction between the different reservoirs contributes to the widespread of MDR enterococci.

Transmission of resistance can take place through food animals or directly through contact between animals and humans. Studies have suggested the potential for zoonotic transmission of enterococci. Research in Vietnam documented the isolation of the same clone of *E. faecalis* in a patient's urine and poultry from the same households in which patients had close contact with the poultry. In 23% of urinary tract infection cases, identical or closely related pulsed-field gel electrophoresis patterns to that found in poultry were detected (64). In another study carried out in Malaysia, one vancomycin-resistant *E. faecium* strain isolated from a chicken was found to be clonal to that of humans (22). Treated sewage sludge, a by-product from treated sewage waste water containing the faecal contents of animals and humans, can be used as fertilizers which potentially pass on MDR strains to food supply. A study conducted in Vietnam found similar relative occurrences of *E. faecium*, *E. faecalis* and other *Enterococcus* spp. in the water–sediment of ponds and manure samples of pigs, suggesting that *Enterococcus* spp. isolated in the ponds originated mainly from the pig manure (65). Insufficient data on the interaction between

cocci in Southeast Asian countries.

256 **Use of Antimicrobials in Hospitals and Antimicrobial Resistance**

257 Generally, the antibiotic of choice for the treatment of enterococcal infections in humans is
258 Ampicillin, and Vancomycin is an alternative agent (66). Prudent antibiotic use is an essential
259 component for control of the spread of vancomycin-resistant enterococci (VRE). The Healthcare

Infection Control Practices Advisory Committee (HICPAC) guidelines insist on curtailing the use of antibiotics for routine surgical prophylaxis and empiric therapy (67).

Although the full extent of MDR *Enterococcus* spp. in Southeast Asia remains undiscovered, data is available from some countries. A linezolid-resistant *E. faecalis* strain was isolated in July 2010 from a diabetic patient in Thailand who received Linezolid for at least 3 months prior to the isolation of the resistant strain (68). From 1999 to 2009, 1.9% of VRE isolates were recovered from patients in the Rajavithi Hospital, Thailand. Out of this 1.9%, there was a significantly higher prevalence of VRE isolates from the inpatient department compared to the outpatient department (10). In Indonesia, antibiotics can easily be obtained without a prescription from medical retailers despite existing regulations (69). According to the National Surveillance of Antimicrobial Resistance in Malaysia, antibiotic susceptibility testing was carried out on bacterial isolates from hospitalized patients whereby analysis was based on one isolate per patient (70). This analysis revealed that roughly 1.2% of the *E. faecalis* isolates was vancomycin-resistant in 2012 and 1.4 % in 2013, a longer time-frame is required to determine if the rate is increasing over time. There was also an increase in Ciprofloxacin resistant *E. faecalis* from 248 patients (20.6%) in 2012 to patients 437 (21.1%) in 2013 and Penicillin resistant *E. faecium* from 309 patients (84.4%) in 2012 to 415 patients (89.6%) in 2013 (70). A study in

278 resistant Enterococci strains from hospitalised patients (66). Another case study in 2008
279 discovered Vancomycin, Teicoplanin, Ampicillin and Gentamicin resistant *E. faecium* strains
280 from two patients with chronic diabetes mellitus and urinary tract infection under a 3 to 12 days
281 course treatment of Cloxacilin, Ceftriaxone, Erythromycin and Vancomycin (71). The first VRE
282 isolated in Singapore was observed in 1994 from a patient at the Singapore National Burns

Centre (72). Two consecutive outbreaks followed later on in 2004 (73) and 2005 (74). According to the Network for Antimicrobial Resistance Surveillance in Singapore (NARSS) in 2006, VRE constitutes 0.8% of all enterococci isolates in Singapore public hospitals (75). An epidemiology study in Singapore documenting VRE in public hospitals from 2006 to 2010 reported 24.4% clinical VRE isolates (9). While the prevalence of VRE clinical isolates remain low in Singapore public hospitals the need for continued vigilance is necessary to prevent any further increase in VRE prevalence. Documented cases of antibiotic resistant *Enterococcus* species were reported in Myanmar from hospitalized patients during 2009 to 2013 in which 30.8% were found to be resistant to Ampicillin and 68.8% were resistant to Erythromycin (76). In 2012, a case study in Vietnam reported vancomycin-resistant *E. faecium* in a patient with liver cirrhosis undergoing antimicrobial therapy consisting Imipenem and Vancomycin for one week (77). Thus not only regulation of antibiotic, but also diligent prescribing of other broad-spectrum antimicrobials should be carried out in hospitals around the region in an attempt to decrease colonization with MDR *E. faecalis*.

Source Control for Infections

In past years, the source of infection for most patients was thought to be their own endogenous enterococci (1). However, with the increase of sophisticated molecular typing techniques and the

erococci in the 1980s and 1990s, studies

301 have clearly demonstrated transmission of enterococci among patients in acute care hospital

302 settings (2). A recent study in Malaysia discovered clinical strains of MDR *E. faecium* with the

303 presumed mode of spread from patient to patient via the hands of health care workers (22).

304 Transient carriage of *E. faecalis* on the hands of health care workers has also been documented

in other studies (78). Transmission of enterococci from transiently colonised health care worker's hand to a patient may involve direct contact with hands, environmental surfaces or medical equipment, but it is more likely that transmission results in colonization of the patient's gut (78). The acquired antibiotic resistant strain is able to survive in the gastrointestinal tract of humans with the aid of selective pressure of broad-spectrum antibiotics which is used frequently in hospitalized patients (78). Infections consequently arise from these newly acquired enterococcal strains.

Various guidelines have been set up by countries in Southeast Asia to provide infection control information for hospitals, healthcare facilities, and livestock/animal health to prevent the spread of MDR enterococci. Indonesia aims to strengthen the implementation of regulations for the production, distribution, sale and prescription of antibiotics as well as establish the Antimicrobial Resistance Control Programme as a national programme. This programme will aid in developing regulations for antibiotic use in veterinary practices as well as guidelines for community acquired infection and public access to it (47). Myanmar is currently establishing a national multisectoral steering committee for antimicrobial resistance and is in the process of constituting a national policy for antibiotic use in humans and animals. Data collection is ongoing in Thailand to understand trends in antimicrobial resistance and develop antibiotic

ant *Enterococcus* spp. especially

323 those due to VRE are limited. Therefore measures to minimize the spread of these resistant

324 organisms within a facility are essential. Each facility should establish a comprehensive infection

325 control program aimed at decreasing transmission of VRE among patients (79). Specific policies

326 should be based on the rates of resistance within the facility and should be appropriate for the

327 specific health care setting. In 1995, the Centers for Disease Control and Prevention Hospital

Infection Control Practices Advisory Committee (HICPAC) published recommendations aimed at controlling the nosocomial transmission of VRE (67). These recommendations provide a base on which specific policies can be developed for individual facilities. The major recommendations of HICPAC focus on (i) prudent use of Vancomycin to decrease the selective pressure for emergence of VRE; (ii) education of health care personnel about the importance of VRE and its mode of transmission; (iii) use of the microbiology lab to quickly identify patients with VRE; and (iv) infection control measures that minimize transmission to other patients. The emergence and severity of VRE has also been reported in other regions of Southeast Asia (5, 40). These findings suggest that early detection of VRE is necessary in preventing further spread in healthcare settings.

Conclusion

Enterococci inhabiting nonhuman reservoirs appear to play a critical role in the acquisition and distribution of antibiotic resistance determinants (60, 80). The introduction of antimicrobial agents in clinical medicine and animal husbandry has been one of the most important medical achievements, however surveillance and enforcement of the use of antibiotics in hospital settings and farms is often lax in most Southeast Asian countries. In addition, the Southeast Asian region lacks in systemic studies to understand the epidemiology of MDR enterococci. The most

ance, and thereby extend the usefulness of

346 antimicrobials, is through their restricted use (47). As a consequence, it has been recommended
347 that antimicrobial agents that select for resistance to antibiotics used for human therapy should
348 not be used for growth promotion in animal husbandry. Growth promoters should be limited to
349 agents that are of no value for therapeutic use (47). To limit the emergence of antimicrobial

resistance and the consequences for human and animal health, it is necessary to collect data on factors affecting the occurrence, emergence, and spread of resistance. At the present, knowledge of antimicrobial resistance among food animals in Southeast Asia is fragmentary. This review highlights the need of health care settings, industries and governments in Southeast Asian countries to strictly regulate the use of antibiotics to curb the emerging threat of MDR enterococci.

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References

1. **Gilmore MS.** 2002. The enterococci: Pathogenesis, molecular biology, and antibiotic resistance. ASM Press, Washington, DC.
2. **Handwerger S, Raucher B, Altarac D, Monka J, Marchione S, Sing KV, Murray BE, Wolff J, Walters B.** 1993. Nosocomial outbreak due to *Enterococcus faecium* highly resistant to vancomycin, penicillin and gentamicin. Clin. Infect. Dis. **16:**750-755. http://cid.oxfordjournals.org/content/16/6/750.short
3. **Leclercq R, Leclercq R.** 2009. Epidemiological and resistance issues in multidrug-resistant staphylococci and enterococci. Clin. Microbiol. Infect. **15:**224-231.

ls: impacts on human

health. Clin. Microbiol. Rev. **24:**718-733. http://cmr.asm.org/content/24/4/718.short

5. **Jean SS, Hsueh PR.** 2011. High burden of antimicrobial resistance in Asia. Int. J. Antimicro. Agents. **37:**291-295.

6. **Food and Agriculture Organization of the United Nations.** 2012. Proceedings of the international workshop on the use of antimicrobials in livestock production and antimicrobial resistance in the Asia-pacific region 2012. Regional office for Asia and the Pacific, Sri Lanka.

7. **World Health Organization.** 2012. Report of regional workshop on antimicrobial resistance 2012. Regional Office for Southeast Asia, Thailand.

8. **Kang CI, Song JH.** 2013. Antimicrobial resistance in Asia: current epidemiology and clinical implications. Infect. Chermother. **45:**22-31. http://synapse.koreamed.org/DOIx.php?id=10.3947/ic.2013.45.1.22

9. **Cai Y, Chan JP, Fisher DA, Hsu LY, Koh TH, Krishnan P, Kwa AL, Tan TY, Tee NW.** 2012. Vancomycin-resistant Enterococci in Singaporean hospitals: 5 year results of a multi-centre surveillance programme. Ann. Acad. Med. Singapore **41**:77-81. http://www.ncbi.nlm.nih.gov/pubmed/22498854

10. **Thongkoom P, Kanjanahareutai S, Chantrakooptungool S, Rahule S.** 2012. Vancomycin resistant enterococci (VRE) isolates isolated in Rajavithi Hospital between 1999 and 2009. J. Med. Assoc. Thai **95**:S7-S15. http://www.ncbi.nlm.nih.gov/pubmed/22619881

aecalis strains isolated from

388 male patients with complicated urinary tract infection. Korean J. Urol. **54:**388-393.

389 http://synapse.koreamed.org/DOIx.php?id=10.4111/kju.2013.54.6.388

390 12. **Sghir A, Gramet G, Suau A, Rochet V, Pochart P, Dore J.** 2000. Quantification of

391 bacterial groups within the human faecal flora by oligonucleotide probe hybridization.

392 Appl. Environ. Microbiol. **66:**2263-2266.

13. **Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK.** 2008. NHSN annual update: Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infect. Control Hosp. Epidemiol. **29:**996-1011. http://www.jstor.org.ezproxy.lib.monash.edu.au/stable/10.1086/591861

14. **Mohamed JA, Huang DB.** 2007. Biofilm formation by enterococci. J. Med. Microbiol. **56**:1581–1588. http://jmm.sgmjournals.org/content/56/12/1581.long

15. **Mika KB, Imamura G, Chang C, Conway V, Fernandez G, Griffith JF, Kampalath RA, Lee CM, Lin CC, Moreno R, Thompson S, Whitman RL, Jay JA.** 2009. Pilot- and bench-scale testing of faecal indicator bacteria survival in marine beach sand near point sources. J. Appl. Microbiol. **107**:72–84. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3938294/

16. **Romling U, Balsalobre C.** 2012. Biofilm infections, their resilience to therapy and innovative treatment strategies. J. Int. Med. **272**:541-561. http://onlinelibrary.wiley.com/doi/10.1111/joim.12004/abstract;jsessionid=9FF87142714 1B5FEE85EA52403FD555D.f01t01

b. Agents Chemother. **45**:999–

1007.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC90417/pdf/ac000999.pdf

18. **Wilson CE, Cathro PC, Rogers AH, Briggs N, Zilm PS.** 2015. Clonal diversity in biofilm formation by *Enterococcus faecalis* in response to environmental stress associated with endodontic irrigants and medicaments. Int. Endod. J. **48**:210-219.

http://onlinelibrary.wiley.com/doi/10.1111/iej.12301/abstract;jsessionid=FAD003FAD16
1C8901306ED3EDE6546DE.f04t03

19. **Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, CucarellaC, Lamata M, Amorena B, Leiva J, Penades JR, Lasa I.** 2001. The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. Appl. Environ. Microbiol. **67**:4538–4545.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC93200/pdf/am1001004538.pdf

20. **Tondolkor PM, Baghdayan AS, Gilmore MS, Shankar N.** 2004. Enterococcal Surface Protein, Esp, Enhances Biofilm Formation by *Enterococcus faecalis*. Infec. Immun. **72**:6032-6039.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC517584/pdf/0802-04.pdf

21. **Upadhyaya GPM, Lingadevaru UB, Lingegowda RK.** 2011. Comparative study among clinical and commensal isolates of *Enterococcus faecalis* for presence of *esp* gene and biofilm production. J. Infect. Ctries. **5**:365-369.
http://www.jidc.org/index.php/journal/article/view/21628813

22. **Getachew Y, Hassan L, Zakaria Z, Saleha AA.** 2013. Genetic variability of Vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* isolates from

biol. **79:**4528.

http://aem.asm.org/content/79/15/4528.short

23. **Del Rosario BP, Aquino AP, Tidon AG, Gerpacio RV.** 2008. Livestock sector training needs assessment report for Southeast Asia, China and Papua New Guinea. ILRI Publication Unit, Ethiopia.

438 24. **Usui M, Ozawa S, Onozato H, Kuge R, Obata Y, Uemae T, Ngoc PT, Heriyanto A,**
439 **Chalemchaikit T, Makita K, Muramatsu Y, Tamura Y.** 2014. Antimicrobial
440 susceptibility of indicator bacteria isolated from chickens in Southeast Asian countries
441 (Vietnam, Indonesia and Thailand). J. Vet. Med. Sci. **76:** 685-692.
442 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4073337/
443 25. **Food and Agriculture Organization of the United Nations.** 2007. Subregional report
444 on animal genetic resources: Southeast Asia 2007. Regional office for Asia and the
445 Pacific, Rome.
446 26. **Hammerum AM.** 2012. Enterococci of animal origin and their significance for public
447 health. Clin. Microbiol. Infec. **18**:619-625.
448 http://onlinelibrary.wiley.com/doi/10.1111/j.1469-0691.2012.03829.x/epdf
449 27. **Donskey CJ, Chowdhry TK, Hecker MT, Hoyen CK, Hanrahan JA, Hujer AM,**
450 **Hutton-Thomas RA, Whalen CC, Bonomo RA, Rice LB.** 2000. Effect of antibiotic
451 therapy on the density of vancomycin resistant enterococci in the stool of colonized
452 patients. N. Engl. J. Med. **343:**1925-1932.
453 28. **Aarestrup SM.** 2002. Food safety assurance and veterinary public health – Volume 1 –
454 Food safety assurance in the pre-harvest phase, p 153-170. *In* Veterinary drug use in farm

ageningen Academic

Publishers, Netherlands.

29. **Centre for Science and Environment.** 2014. Centre for science in the public interest: Antibiotic resistance in food pathogens. The Organization, New Delhi, India.

459 30. **United States Department of Agriculture.** 2012. Overview of U.S. Livestock, Poultry,
460 and Aquaculture Production in 2012. National Animal Health Monitoring System, U.S.A.
461 http://www.aphis.usda.gov/animal_health/nahms/downloads/Demographics2012.pdf
462 31. **Veterinary Medicines Directorate.** 2013. United Kingdom Veterinary Antibiotic
463 Resistance and Sales Surveillance 2013. Government Department for the Environment,
464 Food and Rural Affairs, U.K.
465 https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/404554/V
466 ARSS.pdf
467 32. **Agriculture in the United Kingdom.** 2012. Department of Environment, Food and
468 Rural Affairs. Department of Agriculture and Rural Development (Northern Island). The
469 Scotish Government, Rural and Environment Research and Analysis Directorate. Welsh
470 Assembly Government, The Department for Rural Affairs and Heritage.
471 https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/208436/au
472 k-2012-25jun13.pdf
473 33. **Huttner A, Harbath S, Carlet J, Cosgrove S, Goossens H, Holmes A, Jarlier V, Voss**
474 **A, Pittet D, World Healthcare-Associated Infections Forum participants.** 2013.
475 Antimicrobial resistance: a global view from the 2013 World Healthcare-Associated

http://www.aricjournal.com/content/pdf/2047-2994-2-31.pdf

34. **Food and Agricultural Organisation.** 2010. FAOSTAT on livestock population in ASEAN countries. http://faostat3.fao.org/browse/E/EK/E

480 35. **Van Boeckel TP, Browner C, Gilbert M, Grenfell BT, Levin SA, Robinson TP,**
481 **Teillant A, Laxminarayan R.** 2015. Global trends in antimicrobial use in food animals.
482 PNAS **112**:5649-5654.
483 36. **European Medicines Agency.** 2013. Sales of Veterinary Antimicrobial Agents in 25
484 EU/EEA Countries in 2011: Third ESVAC Report (Eur Med Agency, London), London.
485 www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/document_listing/document_
486 37. **CIWF – Compassion in World Farming.** 2013. The life of: Broiler chickens. Farm
487 Animal Welfare Compendium, US.
488 https://www.ciwf.org.uk/media/5235306/The-life-of-Broiler-chickens.pdf
489 38. **Lawlor P.** 2010. What is the optimum slaughter weight for pigs? Pig Development Unit,
490 Animal and Grassland Research Centre, Teagasc.
491 http://www.teagasc.ie/pigs/articles/farming_independent/2010/Optimum_slaughter_weig
492 hts_May2010.pdf
493 39. **Explore Beef.** 2009. Cattlemen's Beef Board and National Cattlemen's Beef
494 Association.
495 http://www.explorebeef.org/CMDocs/ExploreBeef/FactSheet_ModernBeefProduction.pd
496 f
497 40. **Lestari ES, Severin JA, Verbrugh HA.** 2012. Antimicrobial resistance among

. Trop. Med. Public. Health.

43:385-422.

41. **The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP).** 2013. Use of antimicrobial agents and occurrence of

antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Rosendahls-Schultz Grafisk, Denmark.

42. **World Organization for Animal Health.** 2010. FAO-OIE-WHO: A Tripartite Alliance for 'One Health' 2010. http://www.90.oie.int/en/Dates-to-remember/TIMELINE/April/FAO-OIE-WHO-A-Tripartite-alliance-for-One-Health

43. **Teale CJ, Moulin G.** 2012. Prudent use guidelines: A review of existing veterinary guidelines. Rev. Sci. Tech. **31:**343-354. http://europepmc.org/abstract/med/22849288

44. **Duong VN, Paulsen P, Suriyasathaporn W, Smulders FJ, Kyule MN, Baumann MP, Zessin KH, Pham HN.** 2006. Preliminary analysis of tetracycline residues in marketed pork in Hanoi, Vietnam. Ann. N. Y. Acad. Sci. **1081:**534–542. http://www.ncbi.nlm.nih.gov/pubmed/17135565

45. **European Medicines Agency.** 2011. Trends in the sales of veterinary antimicrobial agents in nine European countries (2005–2009). http://www.ema.europa.eu/docs/en_GB/document_library/Report/2011/09/WC500112309.pdf

46. **Smith P.** 2008. Antimicrobial resistance in aquaculture. Rev. Sci. Tech. **27:**243–264.

d Health Organization. 2011. WHO Advisory Group on Integrated Surveillance of
520 Antimicrobial Resistance (AGISAR), critically important antimicrobials for human
521 medicine, 3rd Revision 2011. The Organization, Norway.
522 48. **Managaki S, Murata A, Takada H, Tuyen BC, Chiem NH.** 2007. Distribution of
523 macrolides, sulfonamides and trimethoprim in tropical waters: Ubiquitous occurrence of

524 veterinary antibiotics in the Mekong delta. Environ. Sci. Technol. **41:**8004–801010.
525 http://pubs.acs.org/doi/abs/10.1021/es0709021
526 49. **Suandy I, Suparno.** 2012. Country report: Indonesia. The 36th APHCA-Animal
527 Production and Health Commission for Asia and the Pacific Session. Antimicrobial Use
528 and resistance in livestock in the Asia-Pacific region, Negombo.
529 50. **Stoesser N, Crook DW, Moore CE, Phetsouvanh R, Chansamouth V, Newton PN,**
530 **Jones N.** 2012. Characteristics of CTX-M ESBL-producing *Escherichia coli* isolates
531 from the Lao People's Democratic Republic, 2004–09. J Antimicrob. Chemother.
532 **67**:240–2. http://dx.doi.org/10.1093/jac/dkr434 PMID:21987239
533 51. **Sone P, Aung YH.** 2012. Country Report: Myanmar. The 36th APHCA-Animal
534 Production and Health Commission for Asia and the Pacific Session. Antimicrobial Use
535 and resistance in livestock in the Asia-Pacific region, Negombo.
536 http://cdn.aphca.org/dmdocuments/Events/36th_APHCA_Session/Papers/Country%20Re
537 port_Myanmar%20F.pdf
538 52. **Casewell M, Friis C, Marco E, McMullin P, Phillips I.** 2003. The European ban on
539 growth-promoting antibiotics and emerging consequences for human and animal health.
540 J. Antimicrob. Chemother. **52:**159-161.

542 53. **Paul JH, Rose JB, Jiang S, Kellogg C, Shinn EA.** 1995. Occurrence of fecal indicator
543 bacteria in surface waters and the subsurface aquifer in Key Largo, Florida. Appl.
544 Environ. Microbiol. **61:**2235-2241.
545 54. **Tansuphasiri U, Khaminthakul D,Pandii W.** 2006. Antibiotic resistance of
546 enterococci isolated from frozen foods and environmental water. Southeast Asian J. Trop.

547 Med. Public Health. **37:**162-170.

548 http://search.proquest.com.ezproxy.lib.monash.edu.au/docview/201463317?accountid=12

549 528

550 55. **McIntosh AC.** 2014. Urban water supply and sanitation in Southeast Asia: A guide to

551 good practice. Asian Development Bank, Mandaluyong.

552 http://www.adb.org/sites/default/files/publication/42583/urban-water-supply-sanitation-

553 southeast-asia.pdf

554 56. **Anh N, Ha TD, Nhue TH, Heinss U, Morel A, Moura M, Schertenleib R.** 2002.

555 Decentralised wastewater treatment - new concept and technologies for Vietnamese

556 conditions. Proceedings of the 5th Specialised Conference on Small Water and

557 Wastewater Treatment Systems, Istanbul, Turkey.

558 http://www2.gtz.de/Dokumente/oe44/ecosan/en-decentralised-wastewater-treatment-

559 vietnam-2002.pdf

560 57. **AECOM International Development, Inc. and the Department of Water and**

561 **Sanitation in Developing Countries (Sandec) at the Swiss Federal Institute of**

562 **Aquatic Science and Technology (Eawag).** 2010. A Rapid Assessment of Septage

563 Management in Asia: Policies and Practices in India, Indonesia, Malaysia, the

NDEC/EAWAG,

565 Bangkok, Thailand. http://pdf.usaid.gov/pdf_docs/PNADS118.pdf

566 58. **Al-Gheethi A, Ismail N, Lalung J, Talib A, Efaq AN, Abd Kadir MO.** 2013.

567 Susceptibility for antibiotics among fecal indicators and pathogenic bacteria in sewage

568 treated effluents. Water Prac. Tech. **8:**1-6.

569 http://www.iwaponline.com/wpt/008/wpt0080001.htm

59. **Dada AC, Ahmad A, Usup G, Heng LY.** 2013. Speciation and antimicrobial resistant of Enterococci isolated from recreational beaches in Malaysia. Environ. Monit. Assess. **185:**1583-1599. http://link.springer.com/article/10.1007/s10661-012-2653-6

60. **Hammerum AM, Lester CH, Heuer OE.** 2010. Antimicrobial-resistant enterococci in animals and meat: a human health hazard? Foodborne. Pathog. Dis. **10:**1137.

61. **Getachew Y, Hassan L, Zakaria Z, Zaid CZM, Yardi A, Shukor RA, Marawin LT.** 2012. Characterization and risk factors of vancomycin-resistant Enterococci (VRE) among animal-affiliated workers in Malaysia. J. Appl. Microbiol. **113:**1184-1195

62. **Hayes RJ, English LL, Carter PJ, Proescholdt T, Lee KY, Wagner DD, White DG.** 2003. Prevalence and antimicrobial resistance of *Enterococcus* species isolated from retail Meats. Appl. Environ. Microbiol. **69:**7153-7160.

63. **Aerestrup FM, Butaye P, Witte W.** 2002. Nonhuman reservoirs of enterococci, p. 79. *In* Gilmore MS, ASM Press, Washington, DC.

64. **Poulsen LL, Bisgaard M, Son NT, Trung NV, An HM, Dlasgaard A.** (2012) *Enterococcus faecium* clones in poultry and in humans with urinary tract infections, Vietnam. Emerging infectious diseases 18: 1096-1100.

65. **Dang ST, Petersen A, Van Truong D, Chu HT, Dalsgaard A.** 2011. Impact of

nce in bacteria at integrated

588 pig-fish farms in Vietnam. Appl. Environ. Microbiol. **77:** 4494–4498

589 66. **Weng PL, Ramli R, Shamsudin MN, Cheah YK, Hamat RA.** 2013. High genetic

590 diversity of *Enterococcus faecium* and *Enterococcus faecalis* clinical isolates by pulsed-

591 field gel electrophoresis and multilocus sequence typing from a hospital in

592 Malaysia. Biomed. Res. Int. 938937. http://dx.doi.org/10.1155/2013/938937

593 67. **The Healthcare Infection Control Practices Advisory Committee.** 1995.
594 Recommendations for preventing the spread of vancomycin resistant recommendations of
595 the hospital infection control practices advisory committee. Morbidity and mortality
596 weekly report. **44:**1-13. http://www.cdc.gov/mmwr/PDF/RR/RR4412.PDF
597 68. **Diaz L, Kiratisin P, Mendes RE, Panesso D, Singh KV, Arias CA.** 2012. Transferable
598 plasmid-mediated resistance to linezolid due to cfr in a human clinical isolate of
599 *Enterococcus faecalis*. Antimicrob. Agents Chemother. **56:**3917–22.
600 http://aac.asm.org/content/early/2012/04/02/AAC.00419-12.full.pdf
601 69. **Hadi U, Van den Broek P, Kolopaking EP, Zairina N, Gardjito W, Gyssens IC, the**
602 **study group 'Antimicrobial resistance in Indonesia: Prevalence and Prevention**
603 **(AMRIN).** 2010. Cross sectional study of availability and pharmaceutical quality of
604 antibiotics requested with or without prescription (over the counter) in Surabaya,
605 Indonesia. Infec. Dis. **10**:203. http://www.biomedcentral.com/content/pdf/1471-2334-10-
606 203.pdf
607 70. **National Surveillance of Antibiotic Resistance 2013.** 2013. National Surveillance of
608 Antibiotic Resistance Report. Ministry of Health Malaysia, Malaysia.

/Summary_of_NSAR_2013

_report.pdf

71. **Weng PL, Hamat RA, Kqueen YK, Zainol N, Aziz MN, Shamsudin MN.** 2012. Vancomycin-resistant *Enterococcus faecium* of multi-locus sequence type 18 in Malaysia. Med. J. Malaysia. **67:**639–640. http://www.e-mjm.org/2012/v67n6/vancomycin-resistant-enterococcus-faecium.pdf

615 72. **Ang SW, Seah CS, Lee ST.** 1996. Vancomycin-resistant enterococcus in the Singapore
616 National Burns Centre: a case report. Ann. Acad. Med. Singapore **25**:270-2.
617 http://www.ncbi.nlm.nih.gov/pubmed/8799020
618 73. **Chlebicki MP, Kurup A.** 2008. Vancomycin resistant Enterococcus – A review from a
619 Singapore perspective. Ann. Acad. Med. Singapore **37**:861-869.
620 http://www.annals.edu.sg/pdf/37VolNo10Oct2008/V37N10p861.pdf
621 74. **Yang KS, Fong YT, Lee HY, Kurup A, Koh TH, Koh D, Lim MK.** 2007. Predictors
622 of vancomycin-resistant enterococcus (VRE) carriage in the first major VRE outbreak in
623 Singapore. Ann. Acad. Med. Singapore **36**:379-83.
624 http://www.annals.edu.sg/pdf/36VolNo6Jun2007/V36N6p379.pdf
625 75. **Hsu LY, Tan TY, Jureen R, Koh TH, Krishnan P, Lin RTP, Tee NWS, Tambyah**
626 **PA.** 2007. Antimicrobial drug resistance in Singapore hospitals. Emerg. Infect. Dis.
627 **13**:1944-7. http://wwwnc.cdc.gov/eid/article/13/12/07-0299_article
628 76. **Myat TO, Prasad N, Thinn KK, Win KK, Htike WW, Zin KN, Murdoch DR,**
629 **Crump JA.** 2014. Bloodstream indefections at a tertiary referral hospital in Yangon,
630 Myanmar. Trans. R. Soc. Trop. Med. Hyd. **108:**692-698.
631 http://trstmh.oxfordjournals.org/content/early/2014/09/23/trstmh.tru151.short

vanA and vanB

633 *Enterococcus faecium* of clonal complex 17 in a patient with bacteremia due to vanA *E.*

634 *faecium*. Diagn. Microbiol. Infect. Dis. **79:**141-143.

635 http://www.sciencedirect.com/science/article/pii/S0732889314001035

636 78. **Ang SW, Seah CS, Lee ST.** 1996. Vancomycin-resistant Enterococcus in the Singapore
637 National Burns Centre: a case report. Ann. Acad. Med. Singapore. **25:**270-272.
638 http://europepmc.org/abstract/med/8799020
639 79. **Ostrowsky BE, Trick WE, Sohn AH, Quick SB, Holt S, Carson LA, Hill BC,**
640 **Arduino MJ, Kuehnert MJ, Jarvis WR.** 2001. Control of vancomycin resistant
641 Enterococcus in health care facilities in a region. N. Engl. J. Med. **344:**1427-1433.
642 80. **Fisher K, Phillips C.** 2009. The ecology, epidemiology and virulence of Enterococcus.
643 Microbiol. **155:**1749.
644 81. **Getachew YM, Hassan L, Zakaria Z, Saleha AA, Kamaruddin MI, Che Zalina MZ.**
645 2009. Characterization of vancomycin-resistant Enterococcu isolates from broilers in
646 Selangor, Malaysia. Trop. Biomed. **26:**280-288.

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648 Table 1. Livestock population and total antimicrobial consumption in chicken, cattle and pigs
649 from ASEAN countries

650 Table 2. Summary of key studies investigating incidences of antibiotic resistant *Enterococcus*
651 species in the environment.

652 Figure 1. Ecological relationships between different reservoirs (63)

Table 1. Livestock population and total antimicrobial consumption in chicken, cattle and pigs from ASEAN countries

Country	Livestock population (in thousands)			PCU (in thousands)			Total amount of antimicrobial consumption in chicken, cattle and pigs in mg/PCU (in millions)
	Chicken	Cattle	Pig	Chicken	Cattle	Pig	
Brunei	16,000	1	1.3	40,000	600	130	5.9
Cambodia	17,448	3,484	2,057	43,620	2,090,400	205,700	135.9
Indonesia	1,622,750	1,363	7,212	4,056,875	817,800	721,200	761.2
Lao PDR	23,000	1,400	3,400	57,500	840,000	340,000	104.7
Malaysia	225,790	909	1,711	564,475	545,400	171,100	137.5
Myanmar	125,000	13,000	7,900	312,500	7,800,000	790,000	533.1
Philippines	158,984	2,570	13,398	397,460	1,542,000	1,339,800	358.6
Singapore	3,300	0.2	270	8,250	120	27,000	5.8
Thailand	231,918	6,498	7,623	579,795	3,898,800	762,300	392.3
Vietnam	218,201	5,916	27,373	545502.5	3,549,600	2,737,300	711.2

FAOSTAT – FAO Statistics Division 2010

10 Table 2. Summary of key studies investigating incidences of antibiotic resistant *Enterococcus* species in the environment.

Study	Country	Location	Resistance rate (%)	Reference number
Tansuphasiri et al. 2006	Thailand	Environmental water (Agricultural wells on animal farm, rivers and canals)	48.4% resistant to Ciprofloxacin, 46.8% resistant to Tetracycline	54
Getachew et al. 2009	Malaysia	Feces from live broiler chickens	VRE [*Enterococcus faecalis* (48%), *Enterococcus faecium* (25.7%), *Enterococcus gallinarum* (12.1%), *Enterococcus casseliflavus* (1.4%) and other *Enterococcus species* (12.8%)]	81
Dada et al. 2013	Malaysia	Coastal bathing waters	76.63% resistant to Kanamycin, 10.87% resistant to novobiocin, 8.38% resistant to chloramphenicol	59
Al-Geethi et al.	Malaysia	Sewage treated effluent	71.4% resistant to Ampicillin, 4.7% resistant to Ciprofloxacin,	58

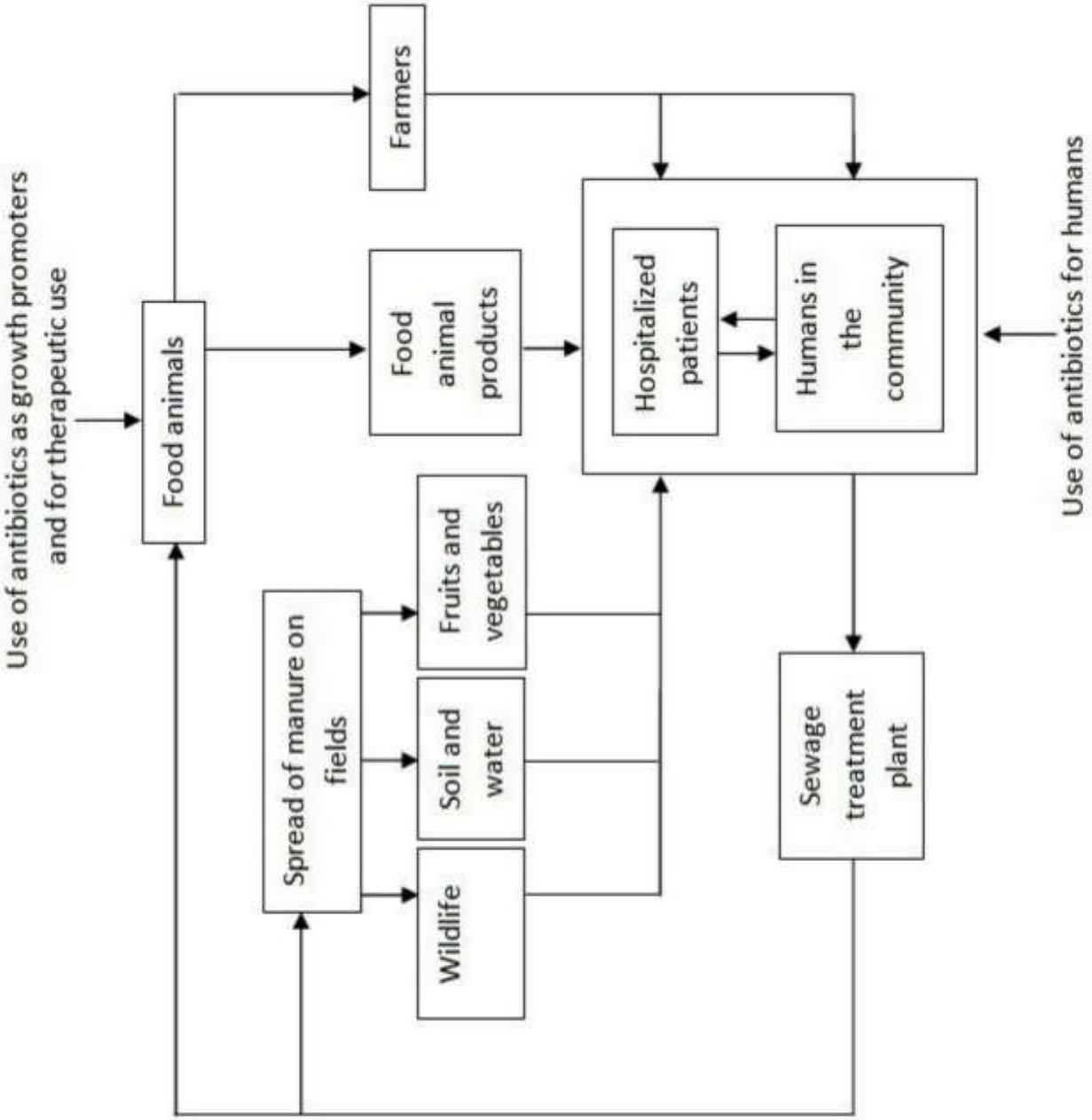
nt

to lincomycin, 86.5% resistant to enrofloxacin)

2

11 Table 2 (contd.) Summary of key studies investigating incidences of antibiotic resistant *Enterococcus* species in the environment.

			E. faecalis (79.3% resistant to Lincomycin, 77.6% resistant to erythromycin, 65.5% resistant to oxytetracycline)	
	Indonesia	Feces from live chicken	*E. faecium* (81% resistant to oxytetracycline, 69% resistant to enrofloxacin, lincomycin and kanamycin)	
Usui et al. 2014				24
			E. faecalis (56.8% resistant to oxytetracycline, 54% resistant to lincomycin, 48.5% resistant to erythromycin)	
	Thailand	Feces from live chicken	*E. faecium* (92.2% resistant to oxytetracylcine, 83.9% resistant to lincomycin, 82.8% resistant to enrofloxacin)	



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Genetic diversity of Enterococcus faecalis isolated from environmental, animal and clinical sources in Malaysia

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Enterococcus faecalis ranks as one of the leading causes of nosocomial infections. A strong epidemiological link has been reported between E. faecalis inhabiting animals and environmental sources. This study investigates the genetic diversity, antibiotic resistance and virulence determinants in E. faecalis from three sources in Malaysia. A total of 250 E. faecalis isolates were obtained consisting of 120 isolates from farm animals, 100 isolates from water sources and 30 isolates from hospitalized patients. Pulse-field gel electrophoresis-typing yielded 63 pulsotypes, with high diversity observed in all sources (D = ≥0.901). No pulsotype was common to all the three sources. Each patient room had its own unique PFGE pattern which persisted after six months. Minimum inhibitory concentrations of Vancomycin, Gentamicin, Penicillin, Tetracycline, Nitrofurantoin, Levofloxacin, Ciprofloxacin and Fosfomycin were evaluated. Resistance to Tetracycline was most prevalent in isolates from farm animals (62%) and water sources (49%). Water isolates (86%) had a higher prevalence of the asa1 gene, which encodes for aggregation substance, whereas clinical (78%) and farm animal isolates (87%) had a higher prevalence of the esp gene, encoding a surface exposed protein. This study generates knowledge on the genetic diversity of E. faecalis with antibiotic resistance and virulence characteristics from various sources in Malaysia.

Introduction

Enterococcus faecalis is found in a variety of environments, such as soil, water, plants, and animals [1]. In humans, as well as in other mammals, these microbes are mainly found in the gastroin-testinal tract as commensals. However, E. faecalis may become an opportunistic pathogen in individuals whose immune systems are compromised [2]. The virulence associated genes in human pathogenic E. faecalis encode among others a collagen-binding pro-tein (ace) [3], an aggregation substance (asa1) [4], a haemolysin activator (cylA) [4], an endocarditis antigen (efaA) [5], a surface protein (esp) [4], gelatinase (gelE) [6] and two recently identified putative surface antigens, EF0591 and EF3314 [7]. E. faecalis has also been shown to acquire resistance to a wide range of antibi-otics [8]. As a result, enterococci have emerged as one of the leading therapeutic challenges associated with enterococcal infec-tions including urinary tract infections (UTI) [2]. Around the world E. faecalis remains one of the most frequently recovered species from enterococcal infections in humans [9].

Due to the prevalence of E. faecalis in nosocomial infections, studies have suggested hospital settings as a source for antibiotic-resistant strains [10]. Additional studies suggest environmental sources including animals and water can serve as important sources for antibiotic resistant E. faecalis strains [1] as human populations, animal populations, and the environment are all interconnected [1]. Selection and persistence of antibiotic resistance might be attributed to a variety of factors including horizontal transfer of resistance genes among bacteria, the misuse or overuse of antibi-otics in humans and animals, and environmental contamination through livestock slurry and plant wastewater. The rate of devel-opment of resistance appears to have accelerated in the past decade and today multiple antibiotic resistant bacteria constitute a global problem [11].

It is important to investigate the genetic relationships between microbes, such as E. faecalis, that are found in both the environ-ment and hospitals, as a possible relationship between the different sources may be established. Although a number of studies have investigated the prevalence and characteristics of antibiotic resis-tance among enterococci in clinical and environmental settings in

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Malaysia [12–17], such studies are typically limited to vancomycin-resistant enterococci and/or studies of a limited geographical area. In this study, E. faecalis from the feces of farm animals, water sources and hospital patients in Malaysia were characterized. The genetic relationships, virulence determinants and antibiotic susceptibili-ties shared between human and environmental E. faecalis isolates from different sources were assessed. In addition, these same char-acteristics were assessed from the same location after a period of six months to assess the persistence of each type of isolate in each source.

Materials and methods

Study site and sample collection

Sampling was carried out in two states representing different geographical regions in Malaysia; Selangor (West Malaysia) and Sabah (East Malaysia). Study sites comprised of chicken and cattle farms, wastewater treatment plants, rivers and hospitals. All farms and water sources were located within a 15 km radius of the hos-pitals in Selangor and Sabah respectively. The sampling areas in Sabah comprised of small to medium residential communities sur-rounded by rural agricultural regions as opposed to Selangor which included sampling areas around semi-urban development consti-tuting smallholder farms. Sampling was conducted at two different sampling times, June and December 2014. Details of the sampling procedure and the distribution of samples obtained in this study can be found in Supplementary material 1.

Isolation and identification of E. faecalis

Suspected E. faecalis appearing as typical black to brown colonies on BAA agar, indicating esculin hydrolysis, were transferred on Slanetz and Bartley (SlaBa) agar (Oxoid, UK) and identified by growth and biochemical reactions as described by Olutiola et al. [18].

Confirmation of E. faecalis identity by sequencing of 16S ribosomal DNA

All presumptive E. faecalis isolates, including the clinical E. faecalis isolates obtained from hospital patients, were further characterized by 16S rDNA sequencing as a confirmation from phe-notypic testing as proposed by Marchesi et al. [19]. Total DNA was extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis, Malaysia). Primers were obtained from First BASE Laboratories, Malaysia. Species identification was determined from the best-scoring reference sequence of the BLAST output and whether the best-scoring reference sequence in the database had a sequence identity of 98% with e-values 10^{-5} and at least 96% query coverage.

PFGE analysis

Pulse Field Gel Electrophoresis (PFGE) was performed (3 repli-cates per isolate) subsequent to DNA digestion with SmaI (Promega, USA) as described by Weng et al. [17]. The PFGE marker (Promega, USA) containing lambda concatemers and lambda-digested HindIII fragments was used as a size standard. Comparison of the PFGE fin-gerprints was analyzed with Cliqs 1D Pro software (Cliqs 1D Pro, USA).

Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) was determined for all E. faecalis isolates against a range of antibiotics using the

broth microdilution technique according to standard recommen-dations [20]. The list of antibiotics tested in this study is provided in Supplementary material 2. These antibiotics were chosen because they are either used in both human medicine and animal husbandry or because previous studies have reported E. faecalis resistance to them [21]. All antibiotics were purchased from Oxoid (UK) and Nacalai Tesque (Japan). The results were interpreted according to the cut-off levels proposed by CLSI guidelines [20].

Screening for vanA and vanB genes

All isolates were subjected to PCR for vanA and vanB genes according to Dutka-Malen et al. [22]. Primers were obtained from First BASE Laboratories, Malaysia.

Putative virulence markers

All primers for testing the presence of putative virulence mark-ers were selected according to Creti et al. [7]. Primers for all virulence markers tested in this study are listed in Supplementary material 3. Primers were obtained from First BASE Laboratories, Malaysia.

Statistical analysis

The prevalence of antibiotic resistance phenotype to each antibi-otic among E. faecalis isolates from all sources was compared using the chi-squared test. A P-value of <0.05 was considered to be statistically significant. Simpson's index of diversity (D) was cal-culated [23] to assess the differentiation of E. faecalis pulsotypes by PFGE. PFGE analysis was based on Dice similarity coefficient and unweighted pair group method using arithmetic averages (UPGMA) clustering with position tolerance and optimization coefficient of 1.5%.

Results

Sample collection

In this study, one isolate per sample was haphazardly picked for analysis. A total of 250 E. faecalis isolates were obtained throughout this study; 120 from farm animal feces, 100 from water sources and 30 from hospital patients (Supplementary material 1).

Diversity of Enterococcus faecalis isolates by PFGE

The analysis based on the dendrogram generated from the PFGE profiles grouped the E. faecalis isolates into 63 pulsotypes (with ≥90% similarity) with 44 clonal populations and 19 isolates that were treated as unique. The PFGE patterns of samples from Selangor and Sabah showed distinct differences. The complete dendrogram is shown in Fig. 1.

A total of 27 pulsotypes for isolates from farm animal feces, 47 for isolates from water sources and 8 for clinical isolates were obtained. Isolates from the same farm clustered together, with the exception of four isolates in pulsotypes XLII and XLVIII which displayed identical PFGE patterns between Farm A and Farm B, as shown in Fig. 1. There was no overlapping of PFGE patterns between isolates from chicken and cattle feces. All isolates from animal drinking water showed similar PFGE patterns to those from farm animals with respect to the farms sampled. Isolates from river water and wastewater showed large genetic variability. E. faecalis from wastewater did not cluster according to the two wastewater treatment plants that were sampled, although farm samples did cluster according to the source farm. In addition, this study found identical PFGE patterns between two pulsotypes consisting both

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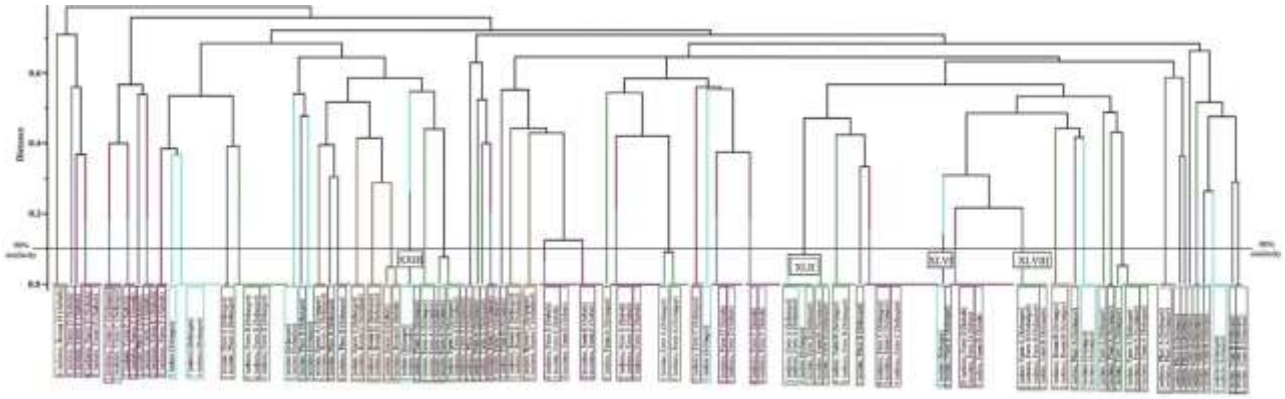

Fig. 1. Dendrogram of similarity among the observed PFGE macrorestriction patterns of SmaI-digested DNA from 250 Enterococcus faecalis isolates from farm animal feces, water sources and hospital patients at two different sampling times (July and December 2014). Dendrogram was generated using Dice similarity coefficient and UPGMA clustering with position tolerance and optimization coefficient of 1.5%. A total of 63 pulsotypes were identified at 90% similarity.

Note: Black text denotes samples collected in July 2014; Red text denotes samples collected in December 2014. Cattle, chicken, water from troughs, wastewater, river water, hospital patients.

wastewater and river water isolates as shown in clusters XXIII and XLVI in Fig. 1. Clinical strains isolated from patients occupying the same room had the same PFGE pattern, which differed from one room to another (Fig. 1). There was no overlapping of PFGE pat-terns between the three sources. The PFGE patterns obtained were highly variable for pooled isolates from each of the three sources (Simpson's diversity index; river and sewage wastewater D = 0.975, farm animals D = 0.951 and hospital patients D = 0.901).

Persistence of Enterococcus faecalis pulsotypes

All the pulsotypes obtained for the clinical strains from each of the rooms in Selangor persisted after six months (Fig. 1). Similarly, previously observed PFGE patterns were recovered in all farms after a follow-up period of at least six months (Fig. 1); in addi-tion some variant pulsotypes were observed after the six month sampling period. In contrast, pulsotypes for samples from river water and wastewater after a period of six months showed con-siderable genetic transience and diversity among E. faecalis isolates. The complete dendrogram and correlation between the pulsotypes, antibiogram and virulence genes are presented in Supplementary material 4.

Antibiotic susceptibility test

Antibiotic resistance patterns of all E. faecalis isolates are pre-sented in Figs. 2 and 3. Additional data on the antibiotic resistance profile of E. faecalis from all sources tested is available in Supple-mentary material 5. Of the total isolated E. faecalis in this study, 80% were resistant to at least one of the antibiotics tested. Compar-ison of the prevalence of antibiotic resistance of E. faecalis between Sabah and Selangor revealed variable differences in the proportion of antibiotic resistant E. faecalis, depending on the antibiotic tested (Table 1).

Isolates from farm animal feces and water sources were most commonly resistant to Tetracycline (Fig. 2). In contrast, 7 out of 30 clinical E. faecalis isolates were found to be resistant to Peni-cillin (2 isolates), Levofloxacin (2 isolates), Ciprofloxacin (1 isolate), Tetracycline (1 isolate) and Nitrofurantoin (1 isolate). The highest frequency of resistance in this study (except to Vancomycin and Nitrofurantoin), was found among isolates from farm animal feces. Multi-resistance (≥2 antibiotics) was common among isolates from water sources (74%) and farm animal feces (73%) (Fig. 3). River water held a higher percentage (83%) of multi-resistant E. faecalis

isolates compared to wastewater (60%). None of the clinical isolates in this study demonstrated multi-resistance.

Twenty four out of 250 isolates (9.6%) in the present study that possessed vanA were resistant to high levels of Vancomycin (MIC 32 g/ml to 128 g/ml) with the exception of one isolate from river water that possessed the vanA gene but did not express Vancomycin resistance. There was no specific correlation observed between antibiogram patterns and the groupings obtained by PFGE (Supplementary material 4).

Prevalence of virulence markers

Distribution of nine virulence markers tested in the study varied between sources. All isolates carried at least one of the virulence genes tested, except for one isolate from cattle feces. Virulence gene gelE was found to be the most common factor (75.6%) in E. faecalis isolates in this study (Table 2). Water isolates had a statistically ($P < 0.05$) higher prevalence of the asa1 gene than the other two sources as shown in Table 2. A high proportion of isolates from river water were found to have the asa1gene (93%), whereas isolates from wastewater had an equally high prevalence of both asa1 (83%) and ace (83%) genes. Clinical isolates revealed high prevalence of the esp (87%) and gelE (83%) genes. However the EF3314 gene was not present in any of the clinical isolates tested. Isolates with the same PFGE pattern showed different virulence profiles in a few cases in this study (Supplementary material 4).

Discussion

Genetic variability of E. faecalis

The genetic relationship between E. faecalis isolates from the dif-ferent sources mentioned was analyzed by genotyping using PFGE which has previously been used to identify clonal relationships among isolates [24].

The clustering of PFGE patterns according to Selangor and Sabah suggests geographical localization. The high diversity observed in each of the 3 sources ($D = \geq 0.901$) is not particularly surprising. It is possible that the exposure to physical and chemical stresses may have resulted in evolution of wide diversity which is neces-sary for the adaptation of E. faecalis. The evolutionary process such as mutation, selection and recombination might have played a role in the evolution of environmental stress tolerance and resulted in observed high diversity. [25]. E. faecalis is also a ubiquitous colo-nizer in the gut of mammals and sauropods [1].

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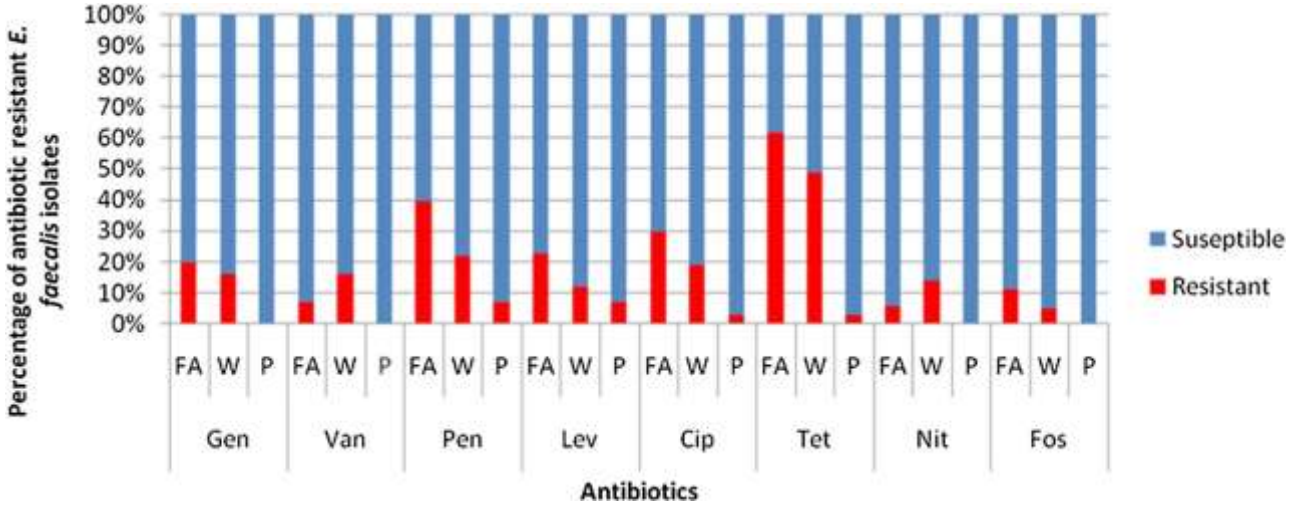

Fig. 2. Distribution of antibiotics for Enterococcus faecalis isolated from farm animal feces, water sources and hospital patients.

Note: GEN = Gentamicin, VAN = Vancomycin, PEN = Penicillin, LEV = Levofloxacin, CIP = Ciprofloxacin, TET = Tetracycline, NIT = Nitrofuratoin, FOS = Fosfomycin, FA: farm animals, W: water, P: patients.

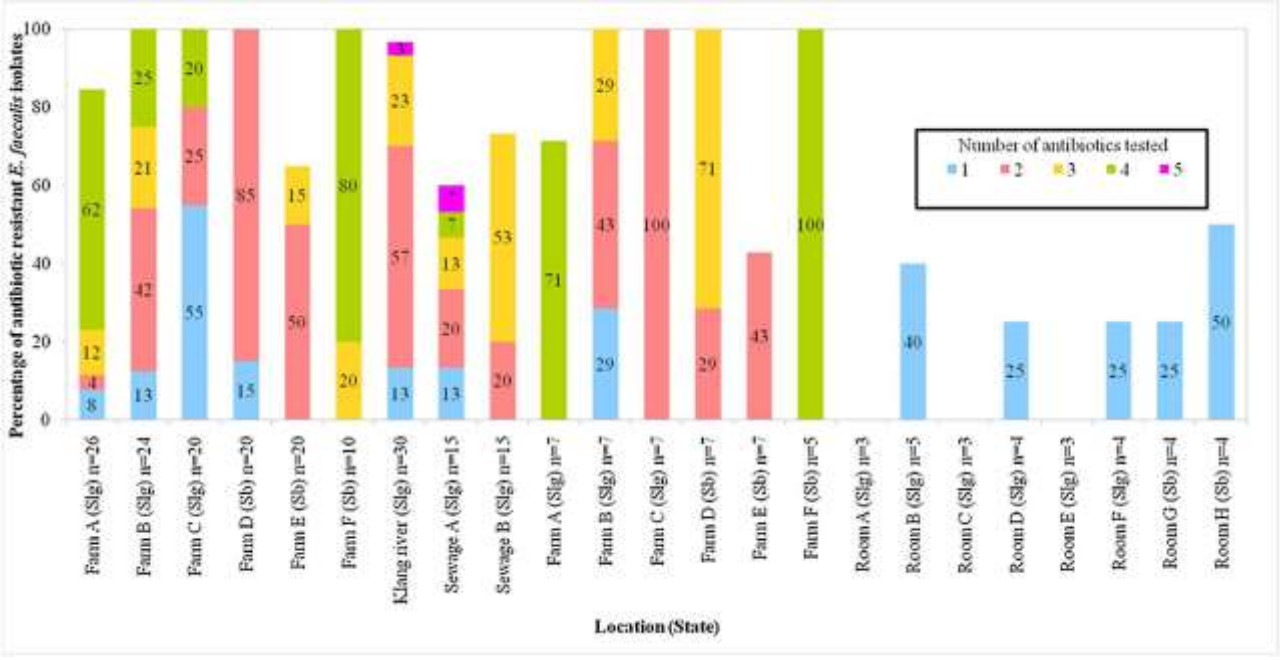

Fig. 3. Prevalence of multi resistant antibiotic (≥2 antibiotics) Enterococcus faecalis isolates from farm animal feces, water sources and hospital patients.

Note: Slg: Selangor, Sb: Sabah.

Table 1

Prevalence of antibiotic resistant Enterococcus faecalis isolates in Sabah (n = 77) and Selangor (113) in percentage (number of isolates).

	VAN	HL-GEN	TET	PEN	CIP	LEV	NIT	FOS
Sabah	2.59% (2)	35.06% (27)	67.53% (52)	32.46% (25)	45.45% (35)	5.19% (4)	5.19% (4)	3.89% (3)
Selangor	9.73% (11)	24.78% (28)	49.56% (56)	44.25% (50)	23% (26)	28.31% (32)	7.08% (8)	11.50% (13)

Note: VAN = Vancomycin, HL-GEN = high-level Gentamicin, TET = Tetracycline, PEN = Penicillin, CIP = Ciprofloxacin, LEV = Levofloxacin, NIT = Nitrofuratoin, FOS = Fosfomycin.

This study reports overlapping pulsotypes between Farm A and Farm B (Fig. 1) which are both chicken farms. The two farms are approximately 5 km distance from each other. Farms traditionally do not operate in isolation and farm staff within a locality may well visit other farms with some regularity, as well as using shared resources such as delivery trucks [26].

All isolates from animal drinking water in this study showed identical PFGE patterns to those from farm animal feces with respect to the farms sampled from. These results may indicate that E. faecalis is disseminated or maintained within a herd by contam-inated water. This study also reports identical PFGE patterns of isolates from wastewater and river water that were approximately 8 km from each other (Fig. 1). Waste from hospitals and farms in the areas investigated are discharged into the sewer system. The treated sewage effluent from both treatment plants is discharged into the Klang river [16].

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Table 2

Prevalence of virulence genes among Enterococcus faecalis isolates from all sources sampled.

Source	Location (State)	Number of isolates with virulence gene present								
		esp	gelE	cylA	asa373	asa1	ace	efaA	EF0591	EF3314
Chicken (n = 50)	Farm A (Selangor) (n = 26)	23	15	12	13	14	19	23	7	8
	Farm B (Selangor) (n = 24)	22	21	0	3	13	13	13	6	5
Cattle (n = 70)	Farm C (Selangor) (n = 20)	17	19	7	8	13	12	14	1	1
	Farm D (Sabah) (n = 20)	16	15	4	8	15	15	13	1	8
	Farm E (Sabah) (n = 20)	13	16	6	0	12	12	12	5	5
	Farm F (Sabah) (n = 10)	2	7	9	4	6	2	7	10	4
River (n = 30)	Klang river (Selangor) (n = 30)	9	18	2	9	28	14	16	1	3
Treated sewage wastewater (n = 30)	A (Selangor) (n = 15)	1	10	1	2	12	12	10	1	3
	B (Selangor) (n = 15)	0	9	2	7	13	13	12	2	2
Animal drinking water (n = 40)	Farm A (Selangor) (n = 7)	7	5	4	2	5	6	7	3	1
	Farm B (Selangor) (n = 7)	7	7	0	0	7	7	7	0	0
	Farm C (Selangor) (n = 7)	5	7	2	1	4	4	6	0	0
	Farm D (Sabah) (n = 7)	2	6	1	0	6	7	1	0	1
	Farm E (Sabah) (n = 7)	5	4	4	0	6	6	6	0	0
	Farm F (Sabah) (n = 5)	1	5	5	0	5	1	4	5	0
Hospital Serdang (n = 22)	Room A (Selangor) (n = 3)	3	3	0	0	3	0	3	3	0
	Room B (Selangor) (n = 5)	5	5	0	1	3	5	1	0	0
	Room C (Selangor) (n = 3)	0	3	0	3	3	3	0	0	0
	Room D (Selangor) (n = 4)	3	3	0	0	1	0	3	0	0
	Room E (Selangor) (n = 3)	3	0	3	0	0	0	0	0	0
	Room F (Selangor) (n = 4)	4	3	0	0	4	0	3	0	0
Hospital LahadDatu (n = 8)	Room G (Sabah) (n = 4)	4	4	0	0	0	4	0	4	0
	Room H (Sabah) (n = 4)	4	4	0	4	4	0	0	0	0
Total		156	189	62	65	177	155	161	49	41

For each patient room, clinical strains had the same PFGE pat-tern, which was different from other rooms. This suggests probable hospital to patient transfer, possibly via contact with fixed materi-als within the specific patient room.

Genomic persistence of E. faecalis

Mostly identical PFGE pattern was recovered in all farms after at least six months of follow-up although some variant pulsotypes were recovered as well (Fig. 1). Consequently, the farm animals examined appeared to be sources of E. faecalis, whose persistence over time may be a function of survival and proliferation of some resident population. In contrast, a great diversity was observed among E. faecalis isolated from water sources after a six month interval. Isolates from river and wastewater appear to be transient populations that fluctuate [26]. All clinical strains from Selangor persisted after 6 months in each of the rooms tested. This may sug-gest the patients picked up E. faecalis from the individual patient rooms, i.e. hospital bedding, shared bathroom within the room, as a result of infection; this confirms the nosocomial nature of E. faecalis. Similar results were seen by Papaparaskevas et al. [27] which found persisting clusters of E. faecalis PFGE patterns within a specific ward over a period of seven months.

of concern for both clinical treatments as well as for the ecological implications for the transmission of this opportunistic pathogen.

Antibiotic susceptibility patterns of E. faecalis

A number of studies in Malaysia have reported antibiotic resis-tant E. faecalis from farm animals [12,13], water sources [14–16] and clinical sources [17]. So far in Malaysia, little emphasis has been given to the prevalence and diversity of MAR (multiple antibiotic resistant) E. faecalis and it was of interest to assess this. Most of the antibiotics used in this study were categorized by the World Health Organization as Rank I, i.e. critically important to human health (Supplementary material 5). Therefore, the high percentage of resistant isolates (80%) observed among E. faecalis isolates are

Antibiotic resistant enterococci have been detected previously in livestock in Malaysia [12,13], and has led to suggestions of an epidemiological link between livestock and human infections [12,13]. A high level of Tetracycline resistance in E. faecalis iso-lated from farm animals (62%) in this study pose similar results to Butaye et al. [28] in Belgium, which reported Tetracycline resistant E. faecalis in almost all isolates (79%) from broilers. As intestinal inhabitants, enterococci are under selective pressure due to the routine supplement of antibiotics in livestock feed. In Malaysia, there are currently 97 antimicrobials registered for use according to the National Pharmaceutical Control Bureau (NPCB) of the Ministry of Health, Malaysia, unfortunately more than half of the antibiotics registered with the Ministry of Health for food animals in Malaysia are not recommended for veterinary use by the World Health Orga-nization (WHO) [29]. A high percentage of multi-antibiotic resistant E. faecalis isolates was obtained from both chicken (82%) and cat-tle (70%). Tetracycline is one of the classes of antibiotics that are commonly and currently used in animal husbandry and human medicine in the Southeast Asian region [29].

In previous reports, core issues affecting the bacteriological quality of rivers available in Malaysia have been highlighted [14–16]. The current study, found comparable rates of antibiotic resistant E. faecalis isolates from river water (83%) as compared to sewage wastewater (60%). It is clear that a more integrated water management and monitoring system is vital for the com-munity. While only 23% of clinical E. faecalis isolates were observed as antibiotic resistant in this study, reports in Turkey and Japan demonstrated that underlying urinary tract diseases predispose patients to repeated UTIs and exposure to antibiotics such as Fluro-quinolones, leading to the selection of resistant E. faecalis isolates and the development of UTIs which may be caused by Quinolone resistant E. faecalis [30,31]. Although clinical strains of patients in the same room had the same PFGE pattern, the antibiotic resistant profiles were not identical in all the strains from the same patient

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room (Supplementary material 4). No correlation was observed between antibiotic treatment and resistance of isolates for specific patients with antibiotic resistant E. faecalis. This suggests diversity and an exchange of antibiotic determinants among the population in a particular clinical setting.

vanA and vanB resistance have been linked with outbreaks of VRE and may be transferred to other organisms [32]. Studies have suggested that the occurrence of vanA in feces of animals may be of risk to humans through direct contact or ingestion of contami-nated products [32]. The risk factors for VRE infection in humans are hospitalization and antibiotic treatment [33]. The vanA pheno-type is related to a high level of inducible resistance to Vancomycin and cross-resistance to Teicoplanin, whereas and the vanB pheno-type has variable levels of inducible resistance only to Vancomycin [34]. The absence of resistant behavior even when the vanA gene is present, displayed by one of the isolates in this study, was also observed by Ribeiro et al. [35].

Prevalence of virulence markers

A number of genes suggested to play a role in the virulence prop-erties of E. faecalis were assessed in this study. The gelE gene, which is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other peptides, was found to be the most common marker (75.6%) in E. faecalis isolates in this study. Similar results were seen by other researches in a number of countries [36,37].

A high frequency of the esp gene was found in both farm animals (87%) and clinical (78%) E. faecalis isolates in this study. The esp gene encodes a surface exposed protein and is important for the initial adherence during biofilm formation and urinary tract colonization.

The asa1 gene, which encodes for aggregation substance, was found to be more common in river water (93%) and wastewa-ter (83%) isolates as compared to the other sources. The ace gene (83%) was also a common virulence marker found in isolates from wastewater. A study by Sidhu et al. [38] of reported high prevalence of ace (74%) and asa gene was found in 47% of E. faecalis isolates in that study.

The EF3314 gene was not present in any clinical isolates. No cor-relation was apparent between PFGE pulostypes and the virulence profiles of the strains. This observation is in agreement with the findings of Comerlato et al. [8] who observed no clonal relation-ship among E. faecalis isolates that influenced the distribution of virulence determinants.

To the best of our knowledge, this report remains to be the first to describe phenotypic and genotypic characteristics of E. fae-calis isolates from farm animals, water and patients in East and West Malaysia. Although the study design of this experiment is insufficient to fully address the transmission of E. faecalis from farms and environmental sources to hospitals, due to low num-ber of samples and great diversity of E. faecalis strains, the present investigation gives insight into the genetic diversity of E. faecalis isolates recovered from different sources in Sabah and Selangor, Malaysia. The high antibiotic resistance level with MAR pattern among the strains should be of concern for public health. A bet-ter knowledge of genotypic traits of E. faecalis might help in the design of strategies for the prevention and treatment of E. faecalis infections.

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Competing interests

None declared.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jiph.2017.02.006.

References

[1] Mallon D, Corkill J, Hazel SM, Wilson JS, French NP, Bennett M, et al. Excre-tion of vancomycin resistant enterococci by wild mammals. Emerg Infect Dis 2002;8:636–8.

[2] Texeira LM, Merquior VLC. Enterococcus. In: De Filippis I, McKee ML, editors. Molecular typing in bacterial infections, infectious disease. New York: Springer Science; 2012. p. 17–23.

[3] Rich RL, Kreikemeyer B, Owens RT, LaBrenz S, Narayana SV, Weinstock GM, et al. Ace is a collagen-binding MSCRAMM from Enterococcus faecalis. J Biol Chem 1999;274:26939–45.

[4] Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, et al. Evelopment of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among Euro-pean hospital isolates of Enterococcus faecium. J Clin Microbiol 2004;42:73–9.

[5] Templer SP, Rohner P, Baumgartner A. Relation of Enterococcus faecalis and Enterococcus faecium isolates from foods and clinical specimens. J Food Prot 2008;71:2100–4.

[6] Qin X, Singh KV, Weinstock GM, Murray BE. Effects of Enterococcus faecalis fsr genes on production of gelatinase and a serine protease and virulence. Infect Immun 2000;68:2579–86.

[7] Creti R, Imperi M, Bertuccini L, Fabretti F, Orefici G, Di Rosa R, et al. Survey for virulence determinants among Enterococcus faecalis isolated from different sources. J Med Microbiol 2004;53:13–20.

[8] Comerlato CB, Resende MC, Caierão J, d'Azevedo PA. Presence of virulence fac-tors in Enterococcus faecalis and Enterococcus faecium susceptible and resistant to vancomycin. Mem Inst Oswaldo Cruz 2013;108:590–5.

[9] Giridhara UPM, Umapathy BL, Ravikumar KL. Comparative study for the pres-ence of enterococcal virulence factors gelatinase, hemolysin and biofilm among clinical and commensal isolates of Enterococcus faecalis. J Lab Physicians 2010;2:100–4.

[10] Ruiz-Garbajosa P, Bonten MJ, Robinson DA, Top J, Nallapareddy SR, Torres C, et al. Multilocus sequence typing scheme for Enterococcus faecalis reveals hospital-adapted genetic complexes in a background of high rates of recombi-nation. J Clin Microbiol 2006;44:2220–8.

[11] Roca I, Akova M, Baquero F, Carlet J, Cavaleri M, Coenen S, et al. The global threat of antimicrobial resistance: science for intervention. New Microbes New Infect 2015;6:22–9.

[12] Getachew Y, Hassan L, Zakaria Z, Saleha AA, Kamaruddin MI, Che Zalina MZ, et al. Characterization and risk factors of vancomycin-resistant entero-cocci (VRE) among animal-affiliated workers in Malaysia. J Appl Microbiol 2012;113:1184–95.

[13] Getachew Y, Hassan L, Zakaria Z, Saleha AA. Genetic variability of vancomycin-resistant Enterococcus faecium and Enterococcus faecalis isolates from human, chickens, and pigs in Malaysia. Appl Environ Microbiol 2013;79:4528.

[14] Hamzah A, Kipli SH, Rahil Ismail SII. Microbiological study in coastal water of Port Dickson, Malaysia. Sains Malays 2011;40:93–9.

[15] Praveena SM, Siraj SS, Suleiman AK, Aris AZ. A brush up on water quality studies of Port Dickson, Malaysia. Res J Environ Sci 2011;5:841–9.

[16] Dada AC, Ahmad A, Usup G, Heng LY. Antibiotic resistance and virulence among enterococci isolated from Teluk Kemang Beach, Malaysia. Water Qual Expo Health 2012;4:113–22.

[17] Weng PL, Ramli R, Shamsudin MN, Cheah YK, Hamat RA. High genetic diversity of Enterococcus faecium and Enterococcus faecalis clinical isolates by pulsed-field gel electrophoresis and multilocus sequence typing from a hospital in Malaysia. Biomed Res Int 2013;2013. ID 938937:1–6.

[18] Olutiola PO, Famurewa O, Sonntag HG. An introduction to general microbiol-ogy: a practical approach. Nigeria: Bolabay Publications; 2000.

[19] Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, et al. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16SrRNA. Appl Environ Microb 1998;64:795–9.

[20] Clinical and Laboratory Standards Institute. Performance standards for antimi-crobial susceptibility testing; seventeenth informational supplement. CLSI

Please cite this article in press as: Daniel DS, et al. Genetic diversity of Enterococcus faecalis isolated from environmental, animal and clinical sources in Malaysia. J Infect Public Health (2017), http://dx.doi.org/10.1016/j.jiph.2017.02.006

document M100-S17. Pennsylvania: Clinical and Laboratory Standards Insti-tute; 2012.

[21] Arias CA, Contreras GA, Murray BE. Management of multidrug-resistant ente-rococcal infections. Clin Microbial Infect 2010;16:1469–691.

[22] Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance geno-types and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol 1995;33:24–7.

[23] Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typ-ing systems: an application of Simpson's index of diversity. J Clin Microb 1988;26:2465–6.

[24] Teixeira L, Carvalho M, Facklan R. Enterococcus. In: Murray P, editor. Manual of clinical microbiology. Washington: ASM Publishing; 2007. p. 430–42.

[25] Baureder M, Reimann R, Hederstedt L. Contribution of catalase to hydrogen per-oxide resistance in Enterococcus faecalis. FEMS Microbiol Lett 2012;331:160–4.

[26] Kuhn I, Iversen A, Burman LG, Olsson-Liljequist B, Franklin A, Finn M, et al. Comparison of enterococcal populations in animals, humans and the environment—a European study. Int J Food Microbiol 2003;88:133–45.

[27] Papaparaskevas J, Vatopoulos A, Tassios PT, Avlami A, Legakis NJ, Kalapothaki V. Diversity among high-level aminoglycoside-resistant enterococci. J Antimicrob Chemother 2000;45:277283.

[28] Butaye P, Devriese LA, Haesebrouck F. Differences in antibiotic resistance pat-terns of Enterococcus faecalis and Enterococcus faecium strains isolated from farm and pet animals. Antimicrob Agents Chemother 2001;45:1374–8.

[29] Neela V, Ghaznavi-Rad E, Ghasemzadeh-Moghaddam H, Nor Shamsudin M, van Belkum A, Karunanidhi A. Frequency of methicillin resistant Staphylococcus aureus in the noses of Malaysian chicken farmers and their chicken. Iran J Vet Res 2013;14:226–31.

[30] Yildirim M, Sencan I, Ozdemir D, Oksuz S, Yilmaz Z, Sahin I. Vancomycin and high-level aminoglycoside resistant Enterococcus carriage and the risk factors related to resistance in hospitalized patients. Mikrobiyol Bul 2007;41:271–7.

[31] Yasufuku T, Shigemura K, Shirakawa T, Matsumoto M, Nakano Y, Tanaka K, et al. Mechanisms of and risk factors for fluoroquinolone resistance in clinical Enterococcus faecalis isolates from patients with urinary tract infections. J Clin Microbiol 2011;49:3912–6.

[32] Donabedian S, Thal LA, Bozigar P, Zervos T, Hershberger E, Zervos M. Antimicro-bial resistance in swine and chickens fed virginiamycin for growth promotion. J Microbiol Methods 2003;55:739–43.

[33] Song JY, Cheong HJ, Jo YM, Choi WS, Noh JY, Heo JY, et al. Vancomycin-resistant Enterococcus colonization before admission to the intensive care unit: a clini-coepidemiologic analysis. Am J Infect Control 2009;37:734–40.

[34] Yip T, Tse KC, Ng F, Hung I, Lam MF, Tang S, et al. Clinical course and outcomes of single-organism Enterococcus peritonitis in peritoneal dialysis patients. Perit Dial Int 2011;3:522–8.

[35] Ribeiro T, Abrantes M, Lopes MdFS, Crespo MTB. Vancomycin-susceptible dairy and clinical enterococcal isolates carry vanA and vanB genes. Int J Food Micro-biol 2007;113:289–95.

[36] Gulhan T, Aksakal A, Ekin IH, Savasan S, Boynukara B. Virulence factors of Ente-rococcus faecium and Enterococcus faecalis strains isolated from humans and pets. Turk J Vet Anim Sci 2006;30:477–82.

[37] Kanemitsu K, Nishino T, Kunishima H, Okamura N, Takemura H, Yamamoto H, et al. Quantitative determination of gelatinase activity among enterococci. J Microbiol Methods 2001;47:11–6.

[38] Sidhu JPS, Skelly E, Hodgers L, Ahmed W, Li Y, Toze S. Prevalence of Enterococcus species and their virulence genes in fresh water prior to and after storm events. Environ Sci Technol 2014;48:2979–88.

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