ANTIMICROBIAL RESISTANCE IN DIRECT-FED MICROBIAL PREPARATIONS USED IN CATTLE

by

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B.S., Wichita State University, 2012

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

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Abstract

The use of antimicrobials in animal feed has come under increasing scrutiny from the public and regulatory agencies. Direct-fed microbials (DFM) are considered valuable alternatives to antimicrobials in food animal nutrition. DFM are products containing live (viable microorganisms). Studies in Europe have reported antimicrobial resistance (AMR) in organisms used in DFM. This is of serious concern because of the potential for transferring resistance to pathogenic bacteria in the gut. The aim of the present study is to characterize phenotypic and genotypic AMR profiles for 20 different antimicrobials in bacterial strains isolated from 10 commercially available DFM used in. Two antimicrobial susceptibility testing methods, disc diffusion and broth micro-dilution based assay were performed. *Enterococcus faecium* isolates showed resistance towards metronidazole (n=9/9) with a MIC of > 32 μg/mL, erythromycin (n=5/9) with a MIC of ≥ 8 μg/mL, ciprofloxacin (n=2/9) with a MIC ≥ 4 μg/mL, ceftriaxone (n=6/9) with a MIC ≥ 0.25 μg/mL, rifampin (n=8/9) with a MIC of > 4 μg/mL, trimethoprim/sulfamethoxazole (n=4/9) with a MIC ≥ 1 μg/mL and clindamycin (n=5/9) with a MIC of > 0.5 μg/mL. A *Propionibacterium freudenreichii* isolate showed resistance towards kanamycin with a MIC of > 64 μg/mL. The same strain also had a MIC of 16 μg/mL for levofloxacin. Two *Lactobacillus acidophilus* were resistant to vancomycin (n=2/6) with a MIC ≥ 32 μg/mL. All the *Lactobacillus* species including *L. acidophilus* (n=6), *L. casei* (n=4) and *L. plantarum* (n=2) were resistant to metronidazole, MIC > 32 μg/mL. Two strains of *Bacillus subtilis* showed resistance to clindamycin, with an MIC of 4 μg/mL and erythromycin with an MIC of > 8 μg/mL, and one strain had no zone of inhibition for metronidazole (MIC > 32 μg/mL). Microarray analysis revealed resistance genes in *E. faecium* strains of 3 different DFM, including aminoglycoside resistance genes, *ant(4')-Ia*, erythromycin resistance genes, *ere(*A2*)*

and *erm*B, tetracycline resistance genes, *tet39, tet*31, *tet*K and *tet*C, and beta-lactam resistance gene, *pbp*5. Conjugation with filter mating showed erythromycin resistance gene transfer, *msr*C gene, from donor strains to a recipient strain (*E. faecium* 45-24). These studies show that AMR is prevalent among bacterial strains used as DFM in the cattle industry in the U.S., justifying further characterization, detection and observation of transferable antibiotic resistance between the same genus.

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Acknowledgements

I would like to take this opportunity to thank my parents who have been very supportive, financially and emotionally. I would also like to extend my sincerest gratitude to my major professor, Dr. Sanjeev Narayanan, and my graduate committee members, Dr. T.G. Nagaraja and Dr. Michael Apley. I would also like to thank my colleague and mentor, Dr. Deepti Pillai, my friends from other laboratories, including Leigh Ann Feurbacher, for their continuous guidance, support and patience with me.

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Dedication

I dedicate this to my parents, whose sacrifices I can only try to repay.

Preface

Reason is the natural order of truth; but imagination is the organ of meaning.
- C. S. Lewis

Chapter 1 - Introduction

Antibiotics and Antimicrobial Resistance

Antimicrobial agents, which include antibiotics, have long been used in veterinary and human medicine, including treating infectious disease in patients, working effectively to decrease illness and death since the 1940s (4, 26). According to the Centers for Disease Control and Prevention (CDC) (5), the most important factor that contributes to antibiotics resistance globally is the unnecessary use, overuse, and misuse of antibiotics, including providing incorrect doses. Overuse of antibiotics has also caused selective pressure, which inhibits bacteria that are susceptible to these antibiotics, thereby making additional resources available for resistant bacterial populations (2, 26). With antibiotics being used in such manner, antimicrobial resistance (AMR) is becoming a serious issue, continuing to negatively impact globally and in U.S. healthcare. Centers for Disease Control and Prevention (CDC) (5) define antimicrobial resistance as the "ability of microbes to resist the effects of drugs, where the germs are not killed and their growth is not stopped". In the U.S., at least 23,000 people die annually from infections caused by AMR bacteria and approximately 2.3 million people are infected with microbes that are resistant to antibiotics (4). Use of antibiotics is influenced by knowledge, prescription choice, patient behavior, economics and health system (25).

Antibiotics have long been used in food animals for treatment, prevention and control of infectious diseases, and growth promotion for the purpose of improving feed production and utilization (19). However, usage of antibiotics and the accompanying rise of resistant bacteria in food-producing animals have become a serious concern since these animals act as reservoirs for pathogens with the potential to transfer resistance to other pathogenic and opportunistic bacteria

(4, 5). Microbes allow mutations in their genes or exchange resistant traits between each other contribute to the natural phenomenon of the evolution of resistant strains (31).

The development of resistant bacteria in food products and in production animals have been connected to resistant strains causing human food-borne infections, resulting from antibiotics used as antimicrobial growth promoters (AGP) (25). Suspicious usage of AGP has caused some countries within the European Union to act on this new research, such as Sweden in 1986, Denmark in 1998-1999 and Norway in 1995 (25). However, while there have been documentations on foodborne pathogens with multiple resistances, the vast majority of most bacteria on meat are susceptible to most antibiotics. Antibiotics used in human medicine have been recommended to be banned for use as growth promotants in animals (19, 20). These recommendations took effect in European countries and Canada, with the use of growth promoters being banned throughout Europe in 2006 (19, 20, 32). This resulted in more than 50% reduction in antibiotics usage, specifically macrolides) in pigs from 1992 to 2008, without any decrease in productivity (32). The United States still continued the usage of these growth promoters. Use of antibiotics for growth enhancement in livestock has been said to be the most controversial (19, 20). Other cases include more than 57% and 59% of dairy operations in the United States treating respiratory and diarrheal disorders with antibiotics in dairy cattle. More than 85% of operations use antibiotics like aminoglycosides, tetracycline and macrolides to treat mammary infections in mature cows (20).

In addition to the decline of development and research of newer antibiotics to curb this imbalance, there is a lack of regulation of antibiotic use in agriculture especially in food animals, a lack of surveillance and control, and inadequate coordination efforts (4). AMR calls for immediate attention due to the fact that resistant microbes do not respond to the typical

treatment. Failure of standardized medical treatments will result in persistent illness, higher risk of death along with increasing health care cost (31). Other negative effects were observed in poultry, including colonization of chickens with tetracycline-resistant as well as other drug-resistant *Escherichia coli* strains. *Enterococcus* species, on the other hand, were found to harbor various tetracycline resistance genes in chicken regardless of being exposed to the drug or not (19, 20). In short, pathogenic species which are mostly Gram-negatives, pose an immediate threat to the human/animal health as it is difficult to eradicate those carrying AMR determinants. Gram-positives with AMR consisted mostly of non-pathogenic or opportunistic pathogens (8).

Transfer of resistance to humans through the food chain where livestock harbor foodborne pathogens such as *Salmonella* species, *Campylobacter* species and some strains of *E. coli* have been observed (20). One of the routes for transfer of resistant determinants in bacteria is close contact, which can occur between animals, people, animal products, feces and surface water (5). This is a cause for concern since use of antibiotics in animals carrying these foodborne pathogens may contribute to selection for more resistant populations. Under selective pressure, these pathogens have an advantage that can help in their colonization, which can result in a higher pathogen load (20). There are also suggestions of antibiotic resistance selection in nonpathogenic microflora that could provide additional reservoir of resistance genes. There is the possibility of increased prevalence of resistance factors on integrons, transposons and plasmids since non-target enteric microflora are exposed to the same antibiotic regimens as the foodborne pathogens which are the highest profile risk (13). The outcome of this exposure in microflora includes the establishment of a resistance reservoir which can support transfer of resistance to pathogenic bacteria. (13).

Assessing any threat to public health from AMR in farm animals is crucial and levels of resistance must be determined in those populations. However, passive surveillance is relied on for national level reporting of AMR in farm animals. A complete ban on antimicrobials use in farm animals would cause serious repercussion on animal welfare, health, productivity and also food prices. Hence, negative effects of antibiotic restriction in animal agriculture might be at least partially offset by viable antibiotic alternatives (32). However, if any, few antimicrobial alternatives have demonstrated the same beneficial effect as antimicrobials on infectious bacterial disease in humans or animals.

Intrinsic and Acquired Antimicrobial Resistance

Antimicrobial resistance includes two categories, intrinsic and acquired. Intrinsic resistance is defined as when a particular strain possesses inherent properties that decreases its susceptibility to some antibiotics. In short, it is naturally resistant to certain actions of antimicrobials (10). The second category is acquired resistance where a strain which is originally susceptible to a certain antimicrobial becomes resistant (29). One pathway of acquired resistance is through mutation of the gene into a resistance gene, occurring during a bacterial replication. The rate of mutation is determined by bacterial genetics, population dynamics, cell physiology and environmental factors (21). Another method of acquired resistance involves resistance determinants being incorporated into the recipient bacterium from a donor bacterium via three forms of horizontal gene transfer, which are transduction, transformation and most importantly, conjugation (5). The transfer of DNA fragments during conjugation can vary from large to small chromosomes. The general procedure of a conjugation process is a contact between cells, formation of a mating pair and lastly, the transfer of a plasmid DNA through a pilus or a pore

forming a channel allowing for the plasmids to pass through (14). The spread of mobile genetic elements enables the transfer of resistance through autonomous self-replicating plasmids, transposons or gene casettes/integrons (5). Integrons have the potential as an important part of dissemination of multi-drug resistance (MDR) among bacterial strains. Class 1 integrons, one of the classes of the resistance integrons (RI) carrying gene cassettes that encode resistance to antibiotics and disinfectants, are most commonly found in bacteria associated with livestock (20). Transposons, on the other hand, are the most frequent mechanism that contributes to the spreading of AMR in bacteria. One such example is the conjugative transposon, *Tn916*, carrying the *tet*M gene and exhibiting a wide host range of Gram-positive and Gram-negative bacteria (8).

Natural transformation also allows for the uptake of plasmid and chromosomal DNA from the environment, where bacterial cells take up free DNA and insert it into their genomes. Natural genetic transformation begins with donor cells releasing their DNA, after which it disperses and persists in the environment. The recipient cells then become competent for DNA uptake, followed by taking up the DNA. Through illegitimate or homologous recombination, the competent cells incorporate it into their genome, with the donor DNA genes expressed in the recipient cells (14).

Another mechanism that facilitates transfer of virulence and antimicrobial resistance genes is transduction by bacteriophages. Bacteriophages are viruses that infect bacteria, including lytic and temperate phages. The transfer of antibiotic resistance or virulence genes by phage-mediated transduction has been reported in *Enterococcus faecalis* strains. Another study also demonstrated transfer of genes from staphylococci to enterococci via the same mechanism (9).

Direct-Fed Microbial

Probiotics are "live microorganism which when administered in adequate amounts, confer health benefit on the host". The terms "probiotics" and "Direct-fed microbial" are used interchangeably. However, they are not similar (21). Direct-fed microbial (DFM) products is defined by the U.S. Food and Drug Administration as products that are purported to contain live (viable) microorganisms. FDA requires feed producers to utilize this term, instead of probiotic (16). However, based on consumer understanding, DFM products include live and non-living supplements enhancing bacterial growth as per feed producers. In the twentieth century, DFM products were first used in animal husbandry to reduce *Salmonella* causing intestinal colonization in chickens, to decrease diarrhea in cattle and pigs, increase milk production in cattle and feed utilization efficiency in pigs (3).

Benefits of using DFM include the influence of microorganisms on animal health and their productivity in terms of competitive exclusion of pathogenic bacteria, competition for mucosal attachment and nutrients, production of bacteriocins, acid fermentation lowering intestinal pH, and stimulation of the gut associated immune system. DFM products contain different genera and species, as well as different strains of the same species (30).

Functional and technological characteristics of each strain are the basis for selection of certain strain for inclusion in a DFM product. There are guidelines to be followed, which include identification methods specifications and assessment of safety characteristics of the strain. The strains should be safe in terms of antibiotic resistances in them (6). Generally, probiotic bacteria should be able to adhere to the intestinal mucosa and behave antagonistically towards other pathogens (22).

About 20 microbial feed additives are allowed to be used in the European union (3) The most commonly used species include lactic-acid producing bacteria (LAB) that can convert fermentable carbohydrates into lactic acids, such as *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Pediococcus* species (18, 21). *Bacillus, Enterococcus* and *Lactobacillus* species are more than likely to be efficient in poultry and pigs (3). This particular group has had a long history of safe usage in food production, leading it to acquire the GRAS (generally recognized as safe) status, which was designated by the Qualified Presumption of Safety (QPS) classification by the European Food Safety Authority (EFSA) and the US Food and Drug Administration (FDA) (8). The LAB group also has the ability to limit the activity of *Salmonella* in vitro by producing hydrogen peroxide in the presence of oxygen (21). It has also been shown that *Lactobacillus acidophilus* strain NP51, one of the strains in a DFM for cattle, reduced *Escherichia coli* O157:H7 fecal shedding in cattle (21). Other bacteria used in DFM include *Bacillus* and *Bifidobacterium* which are generally used in poultry, and yeasts such as *Saccharomyces cerevisiae* primarily used in dairy cattle. In the cattle industry, DFM has been used to improve milk production, growth performance and feed conversion efficiency, either mixed in the feed or administered to the animal in the form of an encapsulated bolus (20).

Most DFM or probiotics are common members of the human intestinal tract. Since they are consumed in large amounts, the presence of antibiotic resistance determinants in their genome must be screened (13). It has been acknowledged that LAB plays a role as a reservoir of antibiotic resistance determinants with the potential to transmit this resistance to pathogenic species, posing a potential health risk which has been largely ignored in the past (8). There have been proposed theoretical risks that colonization with DFM/probiotics can have a negative impact on gastrointestinal physiology and function. Other risks include localized and generalized

immunologic effects. Among all, the most worrying concern is the potential for antibiotic transfer from the DFM to commensal or pathogenic bacteria in the gastrointestinal tract (27).

Strains in DFM

Lactobacillus

The *Lactobacillus* genus, a Gram-positive bacterium, is used frequently in DFM. This genus is generally recognized as safe (GRAS) and can be traced back to having a long history of safe usage in dairy products and foods (13, 27). Some lactobacilli have been characterized as having intrinsic resistance, including a vancomycin-resistant phenotype reported in *L. paracasei, L. salivarius* and *L. plantarum* (13, 26). This genus is also generally more resistant to cephalosporins and has higher resistance to aminoglycosides such as kanamycin and gentamicin. However, it is susceptible to antibiotics like penicillin and other β-lactams, as well as to low concentrations of inhibitors targeting bacterial protein synthesis such as lincosamides, macrolides, chloramphenicol and tetracyclines (13). The lactobacilli have also been shown to be intrinsically resistant to aminoglycosides, glycopeptides and fluoroquinolones (18). In *L. acidophilus*, *L. fermentum* and *L. rhaminosus*, there is a higher prevalence of phenotypic resistance to aminoglycoside due to amplicons of resistance genes that encode resistance for aminoglycosides, *aph[3']-III, aaadA, aadE*, as well as tetracyclines, *tet*S (26). The *Lactobacillus* species is also intrinsically resistant to trimethoprim due to a dihydrofloate reductase (7).

There are also several genes contributing towards atypical antibiotic resistance properties detected in lactobacilli such as the chloramphenicol acetyltransferases (*cat* genes), found in *L. acidophilus, L. johnsonii*, and *L. delbrueckii* subspecies *bulgaricus*. Other genes demonstrated in

lactobacilli, but not as often, include the aminoglycoside resistance genes, *aph(3)-IIIa, aac(6)-aph(2)* and *ant(6)* (8).

There are also antibiotic resistance phenotypes in lactobacilli due to chromosomal mutations. Recent studies have confirmed that erythromycin, *erm*B, and tetracycline, *tet*M, resistance genes in lactobacilli represent the most widespread resistance determinants (8). Other erythromycin resistance genes, including *erm*B*, erm*A*, erm*C and *erm*T, which are responsible for the macrolides, lincosamides, and streptogramins (MLS) resistance phenotype are present in a few of the lactobacilli species. Some of the erythromycin resistance genes, *erm*C, *msr*C, and tetracycline resistance genes, *tet*S, *tet*W, *tet*K, *tet*L and *tet*O, encode for ribosomal protection proteins (8, 13).

Tetracycline and erythromycin resistance were also detected in *L. plantarum* 5057, with claims of *L. plantarum* harboring *erm*B and *msr*A/B genes as well as *tet*M genes (26, 27). Other studies have demonstrated transfer of an introduced plasmid encoding erythromycin resistance, such as pAMβ1, from *L. plantarum* and *L. reuteri* to other Gram positive bacteria *in vivo* and *in vitro* (12).

It is important to note there is evidence conjugation transfers from enterococci to lactobacilli in the animal gut. Transfer of resistance to lactobacilli, on the other hand, is not very common (27).

Enterococcus

Enterococci are one of the many inhabitants of the normal intestinal flora, and also an important nosocomial pathogen. One of the major concerns related to the *Enterococci* species includes a dramatic increase in the number of infections in the medical community, causing

infections like peritonitis, endocarditis and bacteremia (11). *Enterococcus faecium* and *Enterococcus faecalis* display a high prevalence in feces of healthy animals and are able to harbor resistance determinants (5). Among enterococci, there are many mechanisms of intrinsic and acquired resistance belonging to the major antibiotic classes of clinical use (11). In some cases, antimicrobial resistance does not add to the safety concern if intrinsic resistance mechanisms or mutations are the cause of the resistance phenotype among strains. However, if the resistance determinants or markers are carried on mobile genetic elements such as plasmids, transposons or integrons that constitute a reservoir of resistance, then there are causes for concern (13).

In general, this genus demonstrates a low intrinsic resistance to β-lactam antibiotics such as ampicillin, penicillin and imipenem. Resistance towards β-lactam antibiotics implies low affinity of penicillin-binding proteins (PBPs). Genes encoding β-lactamases can be located either on the chromosome or plasmids and can contribute to high-level resistance (11). Higher levels of ampicillin resistance in the early studies were observed in *Enterococcus hirae*, achieved through increasing PBP5 expression levels. This case is similar to those in *E. faecium.* In clinical isolates however, the high level of resistance is rarely related to increasing PBP5 levels of expression. It is more common to presume mutations lower susceptibility to the β-lactam antibiotics, which have been identified in highly resistant clinical isolates within *pbp5* genes. (23).

Enterococci have also developed resistance towards aminoglycosides which include gentamicin, streptomycin and kanamycin. In general, this genus is intrinsically resistant towards low-level aminoglycosides due to low cellular permeability. However, they develop high level resistance due to mutations affecting the protein of the 30S ribosomal subunit as well as an

aminoglycoside-modifying enzyme that removes the synergistic bactericidal effect observed when an aminoglycoside is used in combination with a cell wall-active agent (6, 11).

Another form of resistance is a 16S rRNA modification where the rRNA methyltransferase, *EfmM* uses S-adenosyl methionine as a methyl donor to methylate a specific residue on the 16S rRNA. High level resistance to aminoglycosides is also mediated by aminoglycoside-modifying enzymes including acetyltransferases (AACs), nucleotidyltransferases (ANTs) and phosphotransferases (APHs) (11). Some examples of clinically important resistance genes enzymes include the bifunctional gene *aac(6')-Ie-aph(2")-Ia* encoding the enzyme Aac(6′)-Ie-Aph(2″)-Ia, which helps the strain to be resistant to some aminoglycosides like kanamycin, tobramycin, amikacin, netilmicin and gentamicin (MIC $\geq$ 500 µg/mL). It has been reported that *E. faecium* strains produce the aforementioned aminoglycoside acetyltransferase, Aac(6′)-Ii, which is chromosomally encoded. This enzyme eliminates the synergism between tobramycin, netilmicin, sisomicin and kanamycin aminoglycosides and cell wall-active antimicrobials. An aminoglycoside phosphotransferase, Aph(3′)-IIIa, encoded by the *aph(3')-IIIa gene* confers a high-level resistance towards kanamycin and an aminoglycoside nucleotidyltransferase, Ant(4″)-Ia, encoded by the *ant(4')-Ia* gene, confers resistance towards kanamycin, tobramycin and amikacin. There have been cases of the rise of new gentamicin resistance genes, *aph(2")-Ic* and *aph(2")-Id*, with the latter detected in vancomycin-resistant *E. faecium* as well. The prevalence of increasing aminoglycoside resistance genes among clinical enterococcal isolates, especially *aac(6')-Ie-aph(2")-Ia*, limits the choice of using aminoglycosides for synergistic combination therapy. A somewhat promising newer aminoglycoside is arbekacin, a derivative of dibekacin, used in Japan to treat methicillin and

gentamicin-resistant *Staphylococcus aureus* infections. There is a need for development of more potent but less toxic aminoglycosides to aid in combating enterococcal infections (6).

Acquired resistance to glycopeptides such as vancomycin have also been observed and reported based on phenotypic and genotypic criteria (11). Enterococci which are vancomycin-resistant are normally associated with nosocomial infections in the hospitals, and this type of resistance is transferable *in vitro* (22). Vancomycin resistance genes, *van*A and *van*B are the most prevalent with most *van* genes located in plasmids or transposons as well, facilitating their dissemination through horizontal gene transfer. The resistance gene *van*A, encoding for D-ala-D-lac ligase, is mostly transferred by *Tn1546*, a transposon of 10851bp related to *Tn3.* Unlike the *van*A phenotype where it confers resistance to both vancomycin and teicoplanin, the *van*B phenotype confers some resistance of variable degree to vancomycin but most of the strains are still sensitive to teicoplanin in vitro. It seems that most of the *van*B-mediated resistance phenotype is acquired through horizontal dissemination of *van*B2 genes through Tn916, a conjugative transposon (11).

Macrolides such as erythromycin are also important, with it being the first choice to use in patients that are allergic to penicillin. Tylosin is another macrolide commonly used in pigs (11). In European countries, erythromycin resistance ranged from 14 to 82% in *E. faecium* and 86 to 94% in *E. faecalis.* However, a decrease in resistance to 46.6% was observed in Denmark in *E. faecium* and from about 90% to 28.1% in *E. faecalis,* when the usage of tylosin was reduced (15). Mechanisms of resistance include target modification by precise mutations and methylation of the 23S rRNA subunit which prevents binding of macrolides such as *erm*A*, erm*C and *erm*B. Other forms of resistance mechanism includes hydrolysis of the lactone ring of the antibiotic molecule, and efflux pumps removing antibiotic molecules from inside of the bacterial

cells are encoded by genes such as *mef*A*, mef*E*, msr*C*, mre*A *and msr*A. The more frequently occurring macrolide resistance determinants are the *erm* genes, like *erm*B, encoding for methyltransferase acting on specific residues of the 23S rRNA subunit and mostly located on plasmids (11, 15) The functionality of the enzyme then inhibits erythromycin binding by causing a N6-dimethylation of an adenine residue in the 23S rRNA subunit (11). This gene not only mediates resistance to erythromycin but confers resistance to other active macrolides used in human medicine (15). Modification of the ribosomal target causes cross resistance to macrolides or lincosamides, macrolides, ketolides and streptogramin A and B (MKS) or macrolides, lincosamides and streptogramin B (MLSB) (11).

Enterococci have also developed resistance to lincosamide, more commonly described in *E. faecium*. This type of resistance is mediated by lincosamide nucleotidyl transferase, encoded by *linB,* catalyzing 3-(5'-adenylation) of both clindamycin and lincomicin. Another antibiotic combination, quinupristin-dalfopristin inhibits bacterial protein synthesis and is quite effective on *E. faecium*, while *E. faecalis*, is intrinsically resistant to this antibiotic. However, just like with other antibiotics, acquired resistance towards this antibiotic in *E. faecium* is mediated by streptogramin acetyltransferase enzymes that acetylate streptogramin A such as VatB*,* VatE*,* VatG or VatD, hydrolases encoded by *vgb*A, or ATP-binding transporters encoded by *vga*B. ABC transporter, encoded by the gene *lsa*, mediates streptogramin A and lincosamide resistance (11).

There have been frequent cases of resistance of enterococci towards tetracycline. Tetracycline resistance is normally associated with the gene *tet*M, conferring ribosomal protection and associated with *Tn916*-type transposable elements found on both chromosomes

and conjugative plasmids. Other genes that also confer ribosomal protection include *tet*O and *tet*S. Genes encoding tetracycline export pumps include *tet*K and *tet*L (11).

Rifampicin resistance is also frequent in enterococci, arising from commensal microbiota exposure towards the antibiotic while treating other bacterial infections. The resistance is due to mutations in the RNA polymerase B subunit *rpo*B gene. Linezolid, belonging to the group oxazolidinones, also has a high antimicrobial activity towards Gram positive bacteria, with any resistance being due to mutations in the 23S ribosomal subunit. It has been shown that strains which are resistant to linezolid can also demonstrate co-resistance to other antibiotics like ampicillin, macrolides, fluoroquinolones, chloramphenicol, rifampicin, gentamicin, nitrofurantoin, trimethoprim/sulfamethoxazole and vancomycin. (11).

Enterococci have also shown increasing resistance towards quinolones. There have been cases reported where this resistance is due to mutations affecting the GyrA subunit of the DNA gyrase, as well as the ParC subunit of topoisomerase IV. The second quinolone resistance mechanism is mediated by the proteins of the Qnr family, functioning to protect the DNA gyrase and topoisomerase IV from the inhibition of quinolones. Multidrug-resistant efflux pumps like EmeA and EfrAB mediate the third mechanism of quinolone resistance (11).

Resistance towards heavy metals like copper is also on the rise, which can raise opportunities for cross selection of antibiotic resistance. The copper homeostasis was first described in *copYZAB* operon from *Enterococcus hirae.* There have also been reports of a transferable plasmid copper resistance gene, *tcr*B (transferable copper resistance homologous to *copB*), which encodes a putative protein that belongs to the CPx-type ATPase family of heavy metal transporters. This particular gene is genetically linked to genes that encode other

resistances like macrolides, *erm*B and glycopeptides, *van*A, in plasmids that originate from pig isolates (11).

Enterococcus species are a controversial species with questionable usage in DFM or probiotics due to its potential pathogenicity and resistance towards some of the most commonly used antibiotics (18).

Bacillus

The *Bacillus* species is commonly used in probiotic dietary supplements for humans and in animal feed additives, plant production products or vitamin/enzyme production to help encourage stimulation of the immune system. Some *Bacillus* species generally used include *Bacillus subtilis, B. pumulis* and *B. clausii,* with the latter commonly used to prevent infectious bacterial diarrhea. This genus, which is not part of the commensal microbiota, is rod-shaped, aerobic or facultative aerobic Gram-positive bacterium which forms endospores (1, 13). Despite its common usage, there is limited information concerning the antimicrobial susceptibility of *Bacillus* and also its potential for transfer of resistance genes. It has been demonstrated that the *Bacillus* species is resistant to antibiotics like erythromycin, lincomycin, streptomycin, chloramphenicol, tetracycline and penicillin. For instance, *B. clausii* and *B. lincheniformis* isolated from broiler breeds were resistant to both lincomycin and erythromycin. Another study also observed antibiotic resistance to tetracycline, rifampin, chloramphenicol and streptomycin in *B. subtilis* in a probiotic product, Enterogermina (Sanofi-Aventis) (1).

For the *Bacillus* spp., erythromycin resistances genes conferred resistance through methylation of 23S rRNA macrolide binding sites. Some examples include *B. subtilis*, conferring *erm*D *and erm*I, *B. clausii* conferring *erm*34 and *B. anthracis* conferring *erm*J (1). Macrolide-

resistance genes and tetracycline resistance have also been observed in mobile elements like the plasmid-encoded *erm*C and in the plasmid-encoded *tet*L gene in *B. subtilis*. Another tetracycline resistance gene, *tet*M, contained in the conjugative transposon *Tn5397* has also been found in the same species. It has been reported that *tet*K has also been identified in other *Bacillus* strains. Other reports detected the presence of cfr-like genes, encoding ribosome methyltransferases, in some *Bacillus* species. These genes encode resistance to antibiotics like oxazolidinones, lincosamides, pleuromutilins, streptogramin A and phenicols (13).

Due to the limited information and data in regards to the *Bacillus* spp., validated breakpoints for the different species are needed (1).

Propionibacterium

Another strain used in DFM is the *Propionibacterium* genus in the *Actinobacteria* class of bacteria. This genus is a Gram-positive, catalase positive, non-spore forming, anaerobic or aerotolerant, non-motile pleomorphic and rod-shaped bacteria. Typically, the species within this genus are divided into either dairy or classical and cutaneous. *Propionibacterium freudenreichii*, used in the Bovamine direct-fed microbial, is a part of the classical propionibacteria isolated from the dairy and milk environment. Generally, propionibacteria are used to improve the shelf life of food products as their antimicrobial activity helps to decrease the spoilage of food and mold growth. Other benefits of this genus also include the stimulation of the immune system, limiting cancer progression and assisting in reducing mutagen-producing fecal enzyme activities. The mechanism for the latter effect involved is not well-defined. Another positive attribute of the propionibacteria is its ability to adapt and tolerate digestive stress in the human gut, persisting

temporarily for weeks in the gut. An interesting fact about Propionibacteria is that it is able to enhance the growth of *Bifidobacteria* by modulating the intestinal microbiota (6, 22).

There are few published studies describing antibiotic susceptibility of propionibacteria, including breakpoints values (6). However, there are reports of moderate of susceptibility to tetracycline, chloramphenicol, erythromycin, bacitracin, ampicillin and vancomycin (22). Resistance to aminoglycosides like gentamicin, kanamycin and streptomycin is common for anaerobic bacteria since they do not have a cytochrome-mediated drug transport system (6).

Bifidobacterium

Bifidobacterium is one of the main phylogenetic groups of the human gut microbiota Some of the *Bifidobacterium* spp. used in DFM include *B. thermophilum, B. breve*, *B. bifidum* and *B. animalis. B. animalis* subsp. *lactis* is frequently used in fermented dairy products. Although the presence of resistance genes in the *Bifidobacterium* species in the gut does not pose a direct threat due to the lack of infectivity, it is still harmful in the sense that it constitutes a reservoir where resistance genes can be transferred to pathogenic organisms (13). This genus is intrinsically resistant to mupirocin, inhibiting protein synthesis by competing with isoleucine as a substrate for isoleucyl-tRNA synthetase. *Bifidobacterium*'s resistance is due to synthesis of atypical isoleucyl-tRNA synthetase containing amino acid residues accountable for the high resistance of mupirocin. *Bifidobacterium* is also not susceptible to aminoglycosides in high concentrations due to the lack of cytochrome-mediated drug transport. However, this genus is generally inhibited by low concentrations of vancomycin, beta-lactams, rifampicin, macrolides, chloramphenicol and spectinomycin. It has been reported that streptomycin resistance is seen in

some *Bifidobacterium* strains such as *B. breve* and *B. bifidum*, suggesting there is chromosomal mutation on the *rpsL* gene for ribosomal protein (12).

Even in *Bifidobacterium*, there is limited information in regards to antibiotic resistance determinants, with only data on tetracycline and macrolide antibiotics known. However, multi-drug resistance (MDR) has been found in *B. breve* and *B. longum* where the transporters conferred resistance to erythromycin. As a part of the transposon Tn*5432*, *erm*X, a gene that encodes for a ribosomal protection protein, was found in *B. animalis* subsp. *lactis* and *B. thermophilum.* Tetracycline resistance genes, *tet*W, *tet*M, *tet*(O/W), *tet*(W/32/O) and *tet*O, have also been found in some strains of bifidobacteria*,* including *B. animalis* subsp. *lactis, B. bifidum* and *B. thermophilum.* There is a possibility that under suitable conditions, *tet*W resistance genes can be transferred, due to the gene being flanked by either gene coding for transposases or transposase target sequences. Therefore, enzymes catalyzing the movement of DNA fragments between several locations recognize specific target sequences. An example was found in *B. longum*, with a transposase located in the conserved upstream region of the gene as well as being flanked by flawed direct repeats. At low frequencies, there was transfer of the *tet*W gene between *B. longum* and *B. adolescentis* (13). It can be concluded that there is potential for the transfer of antibiotic resistance genes between closely related bifidobacteria.

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Chapter 2 - Antimicrobial Resistance in DFM strains

For more than 60 years, use of antimicrobials in veterinary medicine and animal husbandry produced positive results in healthier and more productive farm animals. This ensured the health and welfare of animals and humans. However, antibiotic-resistant bacteria are increasing and causing infections. Contribution to emergence of resistance has been credited to the usage of antibiotics in farm animals, where reservoir of resistance is transmitted either indirectly or directly through food consumption to humans (6).

Direct-fed microbial (DFM) are feed products containing only live or naturally occurring microorganisms and have been used over 20 years in the cattle industry (9). Commercially used as probiotic supplements and starter cultures, lactic acid bacteria (LAB) are one of the reservoirs for various antibiotic resistance genes, developing from overuse of tylosin, virginiamycin or lincomycin antibiotics as growth promoters. The most concerning mechanism for antimicrobial resistance transfer is conjugation, which contributes to the global spread of resistance through transposons and conjugative plasmids. This includes macrolide resistance transfer from food LAB to intra, inter-generic LAB as well as pathogens. One of the worrying LAB used as strains in DFM is the *Enterococcus,* which has been known to cause increasing frequency of nosocomial infections, especially in immunocompromised patients. This genus has the potential to acquire extrinsic resistance genes, such as erythromycin, vancomycin, tetracycline and chloramphenicol. Besides resistance genes, virulence factors such as enterococcal surface protein (ESP) and aggregation substance (AS) are also found in *Enterococcus*, giving rise to *Enterococcus* as an opportunistic pathogen. With these reports regarding enterococci, the safety of using this strain in DFM must be assured and its benefits analyzed (2). With the decreasing, limited and free usage of antimicrobials in livestock, there has been an urgent call for new interventions and

strategies to decrease the emergence and prevalence of antimicrobial-resistance bacteria in livestock. Resistant strains in DFM used in food systems and feed should be monitored and assessed by proposing guidelines, regulations, standards and criteria (17).

Materials and Methods

Bacterial strains

Twenty-six strains of various genera such as *Enterococcus*, *Lactobacillus*, *Propionibacterium* and *Bacillus* were isolated from ten DFM. The numbers of strains isolated are shown in Table 2-1.

Bacteria isolations

One gram of each DFM was inoculated in 10 mL of peptone water (Thermo Fisher Scientific) or de Man Rogosa and Sharpe broth (MRS, Oxoid, Thermo Scientific) and incubated overnight aerobically at 37°C (*Enterococcus* and *Bacillus* species) or anaerobically (*Lactobacillus* species at 37°C and *Propionibacterium freudenreichii* at 30°C). The inoculum was plated on blood agar and incubated for 4-5 days at 30°C to isolate *Propionibacterium freudenreichii*. Different colonies were picked based on morphology and a positive catalase test indicated the presence of *P. freudenreichii*. For *Bacillus* strains, the same procedure was used but plated on Brain Heart Infusion (BHI) agar and incubated aerobically at 37°C for 16-18 hours. The catalase test result was also positive for *Bacillus* species. *Enterococcus faecium* was isolated and identified by plating the culture mix on a BHI agar and incubating it aerobically at 37°C for 16-18 hours. Based on colony morphology, *E. faecium* was picked and Gram stained. An esculin hydrolysis test was performed to further confirm *E. faecium*. For further identification, the strain was streaked on m-enterococcus agar (Becton Dickinson, BD) and dark pink colonies observed indicated the presence of *E. faecium*. All bacterial species were further confirmed using Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF). *Lactobacillus casei* and *plantarum* were isolated by plating the inoculum on MRS agar with 0.25% L-cysteine

hydrochloric acid (HCl) and incubated anaerobically for 24-48 hours at 37°C. Based on distinct morphologies, different colonies were Gram-stained to confirm that they were *Lactobacillus* species. For biochemical confirmation, fermentation test of *L. acidophilus*, *L. casei* and *L. plantarum*, was prepared and carried out as described by Schreckenberger and Blazevic (1976) (17) with modification. Sugars were substituted with sorbitol (Thermo Fisher) and raffinose (Fisher Scientific). The tests were incubated aerobically at 37°C with negative controls and the results were observed after four to eight hours. A color change from blue to yellow indicated the strain was able to utilize the sugar. *Lactobacillus acidophilus* was confirmed if the sugar test with raffinose was positive, but the test was negative for sorbitol (17, 22). A sugar test positive with sorbitol and negative with raffinose indicated the presence of *L. casei* (11, 23). Both tests were positive as an indication of *L. plantarum* (3, 23).

Species determination

A Polymerase Chain Reaction (PCR) method was employed to identify the gene encoding D-alanine-D-alanine ligase specific for *E. faecium* (*ddl* E. faecium) in each DFM. Cell suspensions of a few fresh enterococci colonies were prepared in 50 µL of 7.5 Chelex 100 Resin (Bio-Rad laboratories, Hercules, California) (16) with slight modifications. The suspension was heated for 15 minutes at 100°C in a heating block and centrifuged in a microcentrifuge (AccuSpin Micro R Filter Benchtop Centrifuge, Fisher Scientific) at 4032 x g for 1 minute. The supernatant liquid was collected and used for the PCR assay. The primers and PCR conditions were based on Kariyama et al., (2000) (9) with some modifications, with the primers designed to amplify *ddl* of 658 bp. A total volume of 25 µL was used in the PCR assay which contained reaction components of 2.5 µL 10x PCR Reaction buffer (Invitrogen), 1.5 µL of 25 mM MgCl2

(Bullseye, MIDSCI), 2 μL of 1mM dNTP (Takara Clontech), 0.2 μL of 1 unit/25 μL *Taq* polymerase (Bullseye, MIDSCI), 0.5 μL of forward and reverse primers (Integrated DNA Technologies) and 2.5 μL of DNA template.

The thermal cycling was carried out in an Eppendorf Mastercycler gradient (USA Scientific) system: initial denaturation at 94°C for 5 min, followed by 30 cycles of amplification of denaturation at 94°C for 60 seconds, then annealing at 54°C for 60 seconds, elongation at 72°C for 60 seconds, extension at 72°C for 60 seconds and a final extension step at 72°C for 10 minutes. The positive control used in the assay was *E. faecium* 7A and the negative control was water. The PCR products were then analyzed on 1.5% agarose gel containing ethidium bromide (0.5 μg/mL) in 1X Tris-acetate-Ethylenediaminetetraacetic acid (TAE) buffer. The DNA size marker used was 1kb (Promega) with 6x Blue/Orange Load Dye (Promega). The gel was viewed under ultraviolet (UV) light. Distinct bands were produced, corresponding to their molecular sizes. All the isolated strains *E. faecium* were submitted to the Kansas State Veterinary Diagnostic Laboratory to further confirm the identification of the species of each genus using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF).

Kirby-Bauer Disk Diffusion

Antimicrobial susceptibility patterns of each strain were determined using the Kirby-Bauer Disk Diffusion Antimicrobial Susceptibility Testing (AST) method. Twenty discs from various antibiotic classes were purchased from Hardy Diagnostics which including ampicillin, 10 μg; chloramphenicol, 30 μg; clindamycin, 2 μg; erythromycin, 15 μg; kanamycin, 30 μg; tetracycline, 30 μg; vancomyin, 30 μg; ampicillin/sulbactam, 20 μg; ceftriaxone, 5 μg; ciprofloxacin, 5 μg; imipenem, 10 μg; levofloxacin, 5 μg; sulfamethoxazole/trimethoprim 25 μg;

and tigecycline, 15 µg, and from Becton Dickinson (BD), gentamicin, 120 µg; linezolid, 30 µg; synercid (Quinupristin and Dalfopristin), 15 µg; metronidazole, 80 µg; rifampin, 5 µg; and streptomycin, 300 µg. *Staphylococcus aureus* strain ATCC 25923 was used as quality control for *E. faecium*. Each bacterial strain to be tested was grown in appropriate broth overnight and adjusted to a 0.5 McFarland standard at 625nm. The bacterial isolates were streaked onto agar plates and ten antibiotics were placed on a 150mm plate, with the other ten on another plate. The plates were incubated aerobically overnight at 37°C for *E. faecium* and *Bacillus* strains. *Lactobacillus* species plates with antibiotics were incubated anaerobically at 37°C and *P. freudenreichii* plates were incubated anaerobically at 30°C. All assays were performed in duplicates for three days on Mueller Hinton agar (Becton Dickison, BD) for *E. faecium*, *Bacillus* (5) and *P. freudenreichii* strains and MRS agar with 0.25% L-cysteine HCl for *Lactobacillus* strains (10), which was determined to be an optimal media for the enumeration of *Lactobacillus* as there were no guidelines in CLSI to perform Kirby-Bauer Disk Diffusion for *Lactobacillus.* The zone of inhibition was measured across the discs in millimeters (mm). The results were interpreted based on CLSI (5) and European Committee on Antimicrobial Susceptibility Testing (19, EUCAST) interpretive standards for *E. faecium*, *Bacillus* strains and *P. freudenreichii.* One-way analysis of variance (ANOVA) and standard deviation were calculated to determine if the results in one day were significantly different to the results in three days.

Broth Microdilution

Broth microdilution assay was used to further confirm the susceptibility of the strains based on the Kirby-Bauer results. The assay was performed according to Clinical and Laboratory Standards Institute (CLSI) (5) guidelines in duplicates for three days. *E. faecalis* strain ATCC 29212 was used as quality control for *E. faecium.* Broth microdilution assay was performed in

96-well plates for *E. faecium, Bacillus* species (5) and *P. freudenreichii* in cation-adjusted Mueller-Hinton broth (Becton Dickinson, BD). A blue dye, resazurin, used to quantify bacterial content, was added (30 µL) at 0.01% (13) the next day and incubated overnight again. Color change from blue to pink indicated resazurin was reduced by *E. faecium*. This positive result indicated bacterial growth (14). The MIC results were determined where color changes were prevented by the lowest drug concentration. EUCAST (20) and EFSA (19) breakpoints were referred to determine the susceptibility of strains if none were found in CLSI. EFSA guidelines recommended performing broth microdilution assay based on CLSI guidelines (5, 19). Both CLSI and EFSA did not provide Kirby-Bauer Disk Diffusion interpretive values for *Lactobacillus*, *Bacillus* spp., and *Propionibacterium freudenreichii.* Hence, based on the results of Disk Diffusion, broth microdilution assay was performed when the strains did not have zone of inhibition around the antibiotic and tested against antibiotics they were not intrinsically resistant to. Broth microdilution assays for *Lactobacillus* strains were performed in cation-adjusted Mueller Hinton broth with 4% lysed horse blood (6), cation-adjusted Mueller Hinton broth for *Bacillus* species, *E. faecium* (5) and *Propionibacterium freudenreichii*. For *Lactobacillus* strains, the MIC was determined by measuring the absorbance at 625nm with a spectrophotometer. Resazurin was used to observe color change for *Bacillus* species *Propionibacterium freudenreichii*. Minimum inhibitory concentrations were determined and the susceptibility of the strains was interpreted based on the breakpoints in CLSI, EUCAST and EFSA (5, 6, 19, 20). MIC_{50} and MIC_{90} of *E. faecium* were calculated, whereby MIC_{50} was taken as the MIC of median value. MIC_{90} represented the concentration of antibiotic that inhibited 90% of the strains tested.

DFM second lots

Our results from Kirby-Bauer Disk Diffusion and broth microdilution from the first lot of DFM led us to question if the resistance would vary from different lots produced at a later time frame. Questions were also raised regarding the variability of resistance profiles between different colonies in a single lot, especially *E. faecium.* Out of the ten DFMs, three second lots of the list of ten DFMs were purchased. Using the same methods, Kirby-Bauer Disk Diffusion and broth microdilution assays were performed as described above for each strain in the DFM. The results were compared to the first lots for *Enterococcus, Lactobacillus* and *Bacillus* strain and also between different *E. faecium* colonies in the second lots. Paired-samples t-test were applied using Minitab, using each strain from each product as the experimental unit, to determine whether there was a statistically significant mean difference in average diameters over three days in strains of the second lot and the strains in the first lot, as well as between *E. faecium* colonies in the second lot.

PCR Assay for antimicrobial resistance genes detection

A PCR assay was used to detect and quantify AMR genes in *E. faecium.* The antimicrobial genes of interest were *erm*B, *tet*M, *van*A and *van*B. The primers and PCR conditions were chosen according to references in Table 2-7, and modified to amplify *erm*B (175bp) (8), *tet*M (156bp) (8), *van*A (1030bp) and *van*B (433 bp) (9). The running conditions were as follows: initial denaturation of 3 min at 94°C, 36 cycles each of 30s at 94°C, 30s of annealing for 30s at 72°C, and a final elongation of 2 min at 72°C using *Taq* DNA polymerase (TaKaRa). The positive control used for *erm*B and *tet*M was *E. faecium* 7A, *E. faecalis* V583PMV158GFP (previously obtained from Dr. Lynn Hancock, Kansas State University) as a

positive control for *van*B and *E. faecium* R2-TX-5034 (previously obtained from Dr. Ludek Zurek, Kansas State University) as a positive control for *van*A. Based on conjugation and whole genome sequencing results, further tests were run to determine if other erythromycin resistance genes such as *erm*A and *erm*C were present in the donors, recipient and transconjugants. Positive controls used for *erm*A was *Staphylococcus aureus* RN1389 (19) and *erm*C was *Staphylococcus aureus* RN4220 (19).The two control strains were kindly provided by Dr. Charlene Jackson (U.S. National Poultry Research center, Athens, GA).

Spotted DNA Microarray Assay

A spotted DNA microarray method was used as it is dependable and able to detect various resistance genes in a single experiment. The complete array contained 489 70mer oligos printed on an Ultra Gap slide (Corning, Lowell, MA) at a concentration of 35 μM in replicates of 3, 10 or 16. The protocol described by Peterson et. al., 2011 (15) was used and adjusted with some modifications. Genomic DNA for microarray was first extracted and labeled with BioPrime Plus Array CGH Genomic Labeling System (Invitrogen, Carlsbad, CA). A random primer was then incorporated and 1.5 μL of 1 mM Cy3 or Cy5-dCTP was spiked in to increase the fluorescence signal. The dye incorporation and amplification were checked using the Nanodrop ND-1000 spectrophotometer (Nanodrop-Thermo Fisher Scientific, Wilmington, DE). The genomic DNA was mixed with 1 μL of 1 nM stock of Cy3 or Cy5-labeled 25 mer and 2X Hybridization solution (GeniSphere, Hatfield, PA). The mixture was heated for 5 minutes at 80°C and carefully added to the array chips, followed by overnight hybridization. The chips were washed for 10 minutes each in the wash buffers 10X SSC + 0.2% sarkosyl, 10X SSC and 0.2X SSC. Finally, the slides were quickly dipped in water, centrifuged in a 50 mL Falcon Tube at

2,200 x g to spin it dry and the fluorescent hybridization signals were visualized using the GenePix 4000B slide reader (Molecular Devices, Sunnyvale, CA) and compared to the GenePix Array List (GAL) file created by the microarray slide printer (15). Microarray was used to detect resistance genes in three DFMs, one *L. acidophilus* and one *P. freudenreichii* strain, and three *E. faecium* strains. The results from the microarray are shown in Tables 2-8-1, 2-8-2 and 2-8-3.

Whole Genome Sequencing

Nine *E. faecium* strains were submitted to the Food and Drug Administration (FDA) for detection and data analysis of resistance genes. The results are shown in Table 2-9.

Conjugation Assay

Donor strains of *E. faecium,* which were resistant towards erythromycin with an MIC ≥ 8 µg/mL and susceptible to linezolid, MIC: ≤ 2 µg/mL, were conjugated with a recipient strain, *E. faecium* 45-25, susceptible to erythromycin but resistant to linezolid. The recipient was kindly provided by Dr. Ludek Zurek (Kansas State University). The assay was repeated three times. Prior to conjugation, donor and recipient strains were streaked on erythromycin and linezolid plates of varying concentration to determine the highest concentration both strains were able to tolerate. The plates with the concentration 3 µg/mL worked best for both antibiotics. Both donors and recipient were grown in Brain Heart Infusion (BHI) broth overnight for 16-18 hours. At 1:5 dilutions, donor and recipient were mixed together. A filter paper was placed on a BHI agar without antibiotics and 1 mL of the mixed culture was pipetted carefully on the filter paper. The plate was incubated at 37°C overnight. After 16-18 hours, the filter paper was immersed in 1 mL of BHI broth and mixed well. The mixture was plated on BHI plates with erythromycin or

linezolid antibiotic plates, and BHI plates with both antibiotics. Transconjugants observed on dual antibiotics were streaked onto agars with erythromycin only or linezolid only antibiotics.

Pulse-Field Gel Electrophoresis (PFGE)

The transconjugants, donors and recipient were subjected to PFGE to observe for genetic diversity (1). The standard used in this assay was *Salmonella*, based on the protocol from CDC (4). The PFGE for *Enterococcus* strains was performed as described by Amachawadi et. al (1). Fresh isolates were incubated overnight in 5 mL of BHI broth at 37°C. The next day, 1 mL of culture was centrifuged down and resuspended in 200 µL of 0.85% saline solution. Plug molds were prepared by mixing 200 µL of 1.6% SeaKem Gold agarose (Lonza, Switzerland) and 200 µL of bacterial suspension, and allowing the mixture to sit at room temperature for 30 minutes. Plugs were transferred into 10 mL of lysis solution (6 mM Tris-HCl, pH 7.4, 1 M NaCl, 100 mM ethylenediaminetetraacetic acid [EDTA], 0.5% sodium lauryl sarcosine, 0.5% Brij, 0.2% deoxycholate, 500 µg/mL lysozyme and 20 µg/mL RNaseA for 4 hours with gentle shaking at 37°C. After 4 hours, the lysis solution was transferred to another 50 mL Falcon tube with 10 mL of EDTA/sodium dodecyl sulfate (SDS)/Proteinase K (ESP) buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% SDS and 50 µg/mL Proteinase K) and incubated overnight at 50°C with gentle shaking. After overnight incubation, the ESP solution was discarded and washed with Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) for 10 minutes for three separate washings at room temperature. The plugs were stored at 4°C until further use. About 1 mm of the plugs were placed in 1X Buffer of *Sma*I restriction enzyme for 10 minutes at room temperature and then 4 hours with the 20U of restriction enzyme at 25°C. The plugs were transferred into a 100 mL of 1% SeaKem Gold agarose agar in 0.5X Tris/Borate/EDTA buffer (TBE) buffer. The

agar was placed in an electric field, CHEF-DR II (BioRad, Richmond, CA). The pulse time was set as follows:

Block 1: Initial time 3.5s, final time 25s, 200V for 12 hours

Block 2: Initial Time 1s, final time 5s, 200V for 8 hours

After 20 hours, the gel image was captured with Gel Doc 2000 system (BioRad). The band patterns were analyzed using BioNumerics software (Applied Maths, Austin, TX). The isolates were arranged in groups based on similar banding patterns.

Results

Kirby-Bauer Disk Diffusion Results

E. faecium

Nine strains of *E. faecium* were isolated and identified from the ten DFM. Six strains were determined to be resistant to ceftriaxone. Five strains were resistant and 1 was intermediately susceptible to clindamycin, 5 strains were resistant to erythromycin and 4 strains were interpreted as intermediately susceptible to erythromycin. One *E. faecium* strain was intermediately susceptible towards synercid, two strains were resistant and 5 strains were intermediately susceptible to ciprofloxacin. Results also demonstrated four *E. faecium* strains resistant to trimethoprim/sulfamethoxazole. Eight strains of *E. faecium* were resistant to rifampin. All the strains were resistant towards metronidazole whether incubated aerobically or anaerobically.

Lactobacillus

Six strains of *L. acidophilus* were isolated. Two strains did not have a zone of inhibition to vancomycin. None of the *L. acidophilus* strains had zone of inhibition for kanamycin, metronidazole, or sulfamethoxazole/trimethoprim. Four strains did not have a zone of inhibition for levofloxacin and five of them lacked zone of inhibition for ciprofloxacin. From the four strains of *L. casei* isolated from four DFM, none had a zone of inhibition zone for kanamycin, vancomycin, ciprofloxacin, and metronidazole. Three strains of *L. casei* did not have zone of inhibition for sulfamethoxazole/trimethoprim. Two strains of *L. plantarum* did not demonstrate any zone of inhibition for kanamycin, vancomycin, ciprofloxacin and metronidazole. One of the two strains showed the same characteristic for sulfamethoxazole/trimethoprim.

Bacillus

Two strains of *B. subtilis* showed no zone of inhibition for both clindamycin and erythromycin in two DFMs. A strain of *Bacillus pumulis* did not have a zone of inhibition for metronidazole.

P. freudenreichii

This strain was susceptible to all antibiotics tested except it did not have a zone of inhibition for kanamycin and levofloxacin.

A one-way ANOVA was conducted to determine if there was a difference in the Kirby-Bauer results for one day and for three days for all the strains. At a 99% confidence interval, there was no statistically significant difference between the two groups as determined by a one-way ANOVA, $p > 0.01$.

MIC results

E. faecium

Six strains of *E. faecium* were determined to be resistant to ceftriaxone, MIC ≥ 0.25 µg/mL. Five out of nine strains were resistant to erythromycin, MIC ≥ 8 µg/mL, and four were interpreted as intermediately susceptible to the same antibiotic (MIC_{50} and MIC_{90}: 8 µg/mL). All *E. faecium* strains demonstrated resistance to clindamycin, MIC > 0.5 µg/mL (MIC_{50} and MIC_{90}: 8 µg/mL). MIC results also showed that only 2 strains were resistant to ciprofloxacin, MIC ≥ 4 µg/mL (MIC_{50}: 1 µg/mL; MIC_{90}: 8 µg/mL) and 8 strains were resistant towards

rifampin, MIC > 4 μg/mL, (MIC_{50}: 8 μg/mL; MIC_{90}: 16 μg/mL) All the *E. faecium* strains were resistant to metronidazole, MIC > 32 μg/mL, whether incubated aerobically or anaerobically (MIC_{50} and MIC_{90}: 64 μg/mL). Four strains were resistant to trimethoprim/sulfamethoxazole, MIC ≥ 1 μg/mL (MIC_{50}: 0.125 μg/mL; MIC_{90}: 2 μg/mL).

Lactobacillus

The MIC results confirmed the resistance of two *L. acidophilus* strains towards vancomycin, MIC > 32 μg/mL. All six strains were resistant to metronidazole, MIC > 32 μg/mL. Similar results were obtained for *L. casei* and *L. plantarum*, where all the strains were determined to be resistant to metronidazole, MIC > 32 μg/mL.

Bacillus

Two strains of *B. subtilis* were resistant to clindamycin, MIC > 4 μg/mL, and erythromycin, MIC > 8 μg/mL. A strain of *B. pumulis* was resistant to metronidazole, MIC > 32 μg/mL.

P. freudenreichii

MIC results confirmed the resistance of this strain to kanamycin, MIC > 64 μg/mL. The MIC for levofloxacin was 16 μg/mL.

Results for second lots of DFMs

Similar results were observed when comparing the data between the first and second DFM lots. A paired-samples t-test was applied and determined there was no statistically

significant mean difference in average diameters of the Kirby-Bauer Disk Diffusion results in the second and first lots, $p > 0.01$, at a 99% confidence interval. Using one-way ANOVA, the susceptibility of four different colonies of *E. faecium* in the second lot was similar and was not significantly different, $p > 0.01$.

PCR Assay Results

The *E. faecium* strains tested did not display any of the target resistance genes: *erm*B, *tet*M, *van*A and *van*B. The presence of an erythromycin resistance gene, *erm*A, was detected in the donors, recipient and transconjugants from the conjugation assay. Another erythromycin resistance gene, *erm*C, was not detected in any of the strains.

Microarray Results

Tables 2-8-1, 2-8-2 and 2-8-3 show the various resistance genes found in a combination of all the strains in three DFMs, *E. faecium* from three DFMs, and *L. acidophilus* and *P. freudenreichii* from one DFM.

Conjugation Results

Transconjugants were observed after four to five days on dual antibiotics (erythromycin and linezolid) plates when two *E. faecium* donor strains were conjugated with an *E. faecium* recipient strain. The transconjugants also grew on agars with either erythromycin or linezolid plates.

PFGE Results

Enterococcus faecium transconjugants, donors and recipient were subjected to PFGE to observe the genetic relationship between the strains with a homology cut-off value of 90%. Antibiogram patterns (Figure 6) did not show distinct similarities between transconjugants and donor, but similar patterns were observed between transconjugants and recipient.

Whole-Genome Sequencing Results

Data analysis of WGS for nine *E. faecium* strains reveald the presence of an intrinsic erythromycin gene, *msr*C. Several plasmids were also detected with functions including potentially mediating resistance to macrolide, lincosamide and streptogramin B alpha (MLS_B) antibiotics (pAMbeta1), pGB354 with rate-limiting initiator protein RepR, and bacteriocin-encoding plasmid pEF1.

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Chapter 3 - Discussion

In this study, it has been demonstrated that commercially available DFM products used in the U.S. feedlot industry carry various AMR genes. It is consequential to note that there is a concern related to transferable resistance by horizontal gene transfer in the gastrointestinal flora, with enterococci having an array of genetic versatility and ability to recruit and express antimicrobial resistance determinants (3, 12). The *E. faecium* strains used in these ten products did not exhibit vancomycin, tetracycline, linezolid or a combination of quinupristin and dalfopristin (synercid) acquired resistance genes, which are of medical importance. Resistance to aminoglycosides results from limited drug uptake related to proteins involved in electron transport or covalent modification inactivation of the hydroxyl or amino groups of the aminoglycoside molecule by naturally occurring enterococcal enzymes. *E. faecium* has a chromosomally encoded 6'-acetyltrasnferase enzyme (AAC(6')-Ii) able to modify kanamycin (12).

Two *E. faecium* DFM strains exhibited resistance to ciprofloxacin. A well-described mechanism of quinolone resistance is through antibiotic externalization through efflux pumps (12). As mentioned previously, rifampin resistance occurs in *E. faecium* strains, arising from various mutations in the *rpo*B gene encoding the β-subunit of RNA polymerase. This was seen in eight of nine *E. faecium* DFM strains. EUCAST zone diameter interpretive values were used instead of CLSI to determine the susceptibility of *E. faecium* strains to trimethoprim/sulfamethoxazole. Generally, enterococci exhibit susceptibility to the compounds when tested *in vitro*. However, four strains were determined to be resistant to those particular antibiotics. If tested in *in vivo*, the compounds are ineffective as enterococci are able to use exogenous sources of folate (12). Enterococci are intrinsically resistant to clindamycin, with its

resistance mediated by the product of the *lsa* resistance gene (11) which was observed in all *E. faecium* DFM strains.

All the strains demonstrated resistance to metronidazole with no zone of inhibition, except *P. freudenreichii* and *B. subtilis* strains. This could be due to metronidazole, a synthetic antimicrobial agent, being mainly effective against anaerobic bacteria and various protozoans. Susceptible microorganisms are affected when intermediates form as a result of the strains' nitroreductase activity, producing a derivative that proceeds to interact with DNA, which can cause damage characterized by helix destabilization, strand breakage and concomitant specific release of thymidine nucleotides (14). However, despite being incubated anaerobically, resistance was still observed in *B. pumulis* and *E. faecium* (MIC_{50} and MIC_{90}: 64 μg/mL) strains. Another study by Delgado et. al. (2005) (6) attributes the resistance towards metronidazole (MIC ≥ 32 μg/mL) in lactobacilli due to the absence of hydrogenase activity in lactic acid bacteria species. A zone of inhibition was observed for *B. subtilis* and *P. freudenreichii* to metronidazole. Susceptibility of *B. subtilis* strains were determined based on the interpretive values for Gram-positive anaerobes in CLSI.

The potential for *E. faecium* as a donor to transfer resistance to other strains makes the resistance profile of this organism in DFM products particularly interesting. As mentioned previously, enterococci increasingly cause nosocomial infections, infecting patients with unrelated underlying illnesses under antibiotic treatment. This results in serious or life-threatening infections and the rise of enterococci as a principal clinical challenge for physicians. One of the concerning antibiotic resistance characteristics of *E. faecium* is vancomycin-resistant enterococci (VRE), which creates problems for disease management. There have been reports of transfer of vancomycin resistance from enterococci to *Staphylococcus aureus.* Nine DFM strains

of *E. faecium* tested did not exhibit any vancomycin or ampicillin resistance. Ampicillin resistance is associated with vancomycin resistance, often being detected before vancomycin resistance in *E. faecium* (13).

Erythromycin resistance with an MIC at 8 μg/mL was observed in five strains from five DFMs. From two DFMs, *E. faecium* strains showed potential for transfer of erythromycin resistance. When plated on agar containing both erythromycin and linezolid at 3 μg/mL, several colonies of transconjugants were observed on the plates. Whole genome sequencing revealed the presence of *msr*C, encoding a putative efflux pump of the ABC transporter family, with the ability to transfer erythromycin resistance. PCR assay also confirmed the presence of *msr*C gene in the donors, transconjugants and recipient. However, Portillo et. al (2000) (14) reported it was an indigenous or intrinsic gene present in *E. faecium* chromosomes, not an acquired resistance due to selective pressure conferring resistance towards macrolides. This intrinsic gene is related to *msr*A and *msr*B in staphylococci, conferring low-level resistance to streptogramin B compounds (7). PFGE results demonstrated similar profiles between transconjugants and recipient, confirming that conjugation occurred. However, *erm*A was detected by PCR assay in the *E. faecium* donors, transconjugants and recipient, which were not identified by WGS.

Based on initial AST results, a question was raised if the antimicrobial resistance profiles for the lots following after would have a variation in susceptibility profiles. Comparing to the first lots, the results of the second lots of three DFMs (*E. faecium*, *L. acidophilus*, *L. casei* and *L. plantarum)* were similar and not significantly different.

Microarray was used to screen various genetic information and to detect many genes harbored by bacteria in a single assay. Compared to traditional PCR, the advantage of using this assay was that target genes were identified based on its internal sequences and not on the length

of the PCR products, with the oligonucleotides probes having greater specificity than PCR primers. Two erythromycin resistance genes, *erm*B and *ereA,* resistance genes were detected in *E. faecium* strains from two DFM. Although it was determined that both strains were resistant towards erythromycin, the presence of *erm*B was not verified through PCR amplification. *Enterococcus faecium* strains were positive for some genes but susceptible to a corresponding antibiotic. For example, tetracycline resistance genes were detected in a strain susceptible to that antibiotic. This could be due to high numbers of false positives causing lower specificity and predictive values of a positive test. This demonstrated weak correlation with the resistance phenotypes (1). However, it was also possible that the genes were nonfunctional due to mutations or open reading frames (ORFs).

Tests were recommended to be performed and results interpreted according to CLSI as it is an internationally recognized standard (17). However, not all strains have interpretive values, especially fastidious bacteria. *Lactobacillus* and *Bacillus* only had breakpoint and cut-off values for certain antibiotics to determine their MIC. *Propionibacterium* did not have susceptibility interpretive values for Kirby-Bauer disk diffusion and broth microdilution in CLSI as there were no adequate studies to develop reproducible, definitive standards for results interpretation (5). In the absence of CLSI breakpoints, the epidemiological cut-off values in EFSA were used. The term "breakpoint" has been used in various ways including referring to the MIC for antimicrobial distinguishing populations of bacteria with acquired or selected resistance mechanisms from the wild-type strains which does not harbor selected or acquired resistance to an examined antimicrobial or others with the same mechanism/site of action. Clinical breakpoints (CLSI) refer to MIC concentrations separating strains where there is a possibility of treatment success from the bacteria where treatment is more likely to fail. These breakpoints are derived from

prospective human clinical studies where the outcomes are compared to the MICs of the infecting pathogen. The term “epidemiological, or wild-type, (EFSA) cutoff value has been proposed by the European Committee on Antimicrobial Susceptibility Testing in an attempt to decrease confusion regarding the meaning of the term “breakpoint” (19). Breakpoints are necessary to categorize organisms into susceptible, intermediately susceptible, and resistant for the antimicrobial agent. This requires understanding of dosing, pharmacokinetics, pharmacodynamics, clinical outcome in clinical situations, and MIC organism distributions without resistance mechanisms. (9). Breakpoints also allow communication from the clinical laboratory to the prescriber in regards to possibility that a particular antimicrobial regimen will be clinically useful to treat patients with infections (17). Other breakpoints used when CLSI values are not available, is the European Committee on Antibiotic Susceptibility Testing (EUCAST) clinical breakpoints. These breakpoints are based on pharmacokinetic-pharmacodynamic (PK-PD) properties, epidemiological MIC cut-offs (ECOFFS) and in part, the data from clinical outcomes (8, 10). EUCAST guidelines are more freely available and a study by Kassim et.al (2016) showed an acceptable level of agreement between the CLSI AST guidelines and EUCAST, specifically *E. coli, S. aureus* and *P. aeruginosa* (10). However, discrepancies have been observed due to different epidemiologies, a lower number of data points in the EUCAST distribution, bias resulting from low number of laboratories contributing data, pooling of results from various sources and differences from ECOFF determination methods. Hombach et. al (2014) reported the importance of determining clinical breakpoints (8). Reliable clinical breakpoints/ECOFFs require species-specific criteria. Antibiotic disk content is also critical to separate resistant from wild-type populations and diameter distributions are also influenced by pooling of data from various sources (8).

With that being said, there is a need for better standardized breakpoints to indicate and confirm these strains' susceptibility for future references.

Conclusion

In the increasing development of antimicrobial resistance, the gut plays an important role and is the center of AMR (2). Alterations in the gut microbiome occur through ingestion of antibiotics, highly pathogenic microorganisms, or just aggressive non-pathogenic colonizers which may or may not carry transferable resistance genes. The presence of possible transfers of resistance genes comprise of a theoretical risk of transfer to less pathogenic organisms in gut microbial community (16). This study has provided results for some of the strains used in DFM that are considered safe and why there is a need for resistance profiles for them including organisms considered as safe like *Lactobacillus* and *Bifidobacterium* species (16). DFM strains used in commercial products in the industry must be evaluated to make sure that there are no transferable resistance genes before going out to the market. Using low cost genomic sequencing technologies to confirm the absence of genes of concern is advisable (16). Established, better interpretive criteria and standardized guidelines are necessary for performing tests to determine the resistance and susceptibility of these strains, especially for lactic-acid bacteria.

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Chapter 4 - Figures and Tables used in Chapter 2

Table 2-1 Strains in ten DFM used in this study

Strains	Number of strains
Enterococcus faecium	9
Lactobacillus acidophilus	6
Lactobacillus casei	4
Lactobacillus plantarum	2
Bacillus subtilis	3
Bacillus pumulis	1
Propionibacterium freudenreichii	1

Table 2-2 List of antibiotics and zone diffusion disc contents used in this study

Antimicrobial Agent	Concentration (µg)
Ampicillin/ Sulbactam	20
Ampicillin	10
Ceftriaxone	30
Chloramphenicol	30
Ciprofloxacin	5
Clindamycin	5
Erythromycin	15
Gentamicin	120
Imipenem	10
Kanamycin	30
Levofloxacin	5
Metronidazole	80
Rifampin	5
Streptomycin	300
Sulfamethoxazole/Trimethoprim	25
Synercid (Quinuprisitn/Dalfopristin)	15
Tetracycline	30
Tigecycline	15
Vancomycin	30

Table 2-3-1 Disk Diffusion: *Enterococcus faecium*

Antimicrobial Agent	Averages (mm) for nine strains	Ranges for nine strains	Quality Control Ranges	Susceptible/Intermediate/ Resistant Strains	Guidelines followed
Ampicillin	25.94	21-30	27-35	S ≥ 15 (n= 9)	CLSI
Amp/Sul	27.61	24-32	29-37	S ≥ 15 (n= 9)	CLSI
Ceftriaxone	11.45	0-24	22-28	S ≥ 18 (n= 3) R < 18 (n= 6)	EUCAST*
Chloramphenicol	22.68	20-25	4-16	S ≥ 18 (n= 9)	CLSI
Ciprofloxacin	19.25	13-26	22-30	S ≥ 21 (n= 2) I, 16-20 (n= 5) R ≤ 15 (n= 2)	CLSI
Clindamycin	15.88	8-33	24-30	S ≥ 19 (n= 3) I, 16-18 (n= 1) R ≤ 15 (n= 5)	CLSI*
Erythromycin	14.93	11-21	22-30	I, 14-22 (n= 4) R ≤ 13 (n= 5)	CLSI
Gentamicin	22.67	20-28	19-27	S ≥ 10 (n= 9)	CLSI
Imipenem	26.38	23-30	n.a.	S ≥ 21 (n= 9)	EUCAST
Kanamycin	4.29	0-14	19-26	n.a.	n.a.
Levofloxacin	18.82	17-24	25-30	S ≥ 17 (n= 9)	CLSI
Linezolid	25.60	22-30	25-32	S ≥ 23 (n= 9)	CLSI
Metronidazole	0	0	n.a.	n.a.	n.a.
Rifampin	13.69	11-27	26-34	S ≥ 20 (n= 1) R ≤ 16 (n= 8)	CLSI
Streptomycin	20.59	18-24	14-22	S ≥ 10 (n= 9)	CLSI
Synercid	21.59	17-27	21-28	I, 16-18 (n= 1) S ≥ 19 (n= 8)	CLSI
Tetracycline	26.46	22-32	24-30	S ≥ 19 (n= 9)	CLSI
Tigecycline	23.21	17-28	20-25	S ≥ 18 (n= 9)	EUCAST
Trim/Sulfa	26.48	20-31	24-32	R ≤ 21 (n= 4)	EUCAST
Vancomycin	20.09	18-22	17-21	S ≥ 17 (n= 9)	CLSI

n.a.: not available

n: number of strains; S: susceptible; I: Intermediately susceptible; R: Resistant

CLSI*: Zone Diameter Interpretive values from *Streptococcus* species **β** –Hemolytic Group

EUCAST*: Zone Diameter breakpoint from *Streptococcus* groups A, B, C and G

Table2-3-2 Disk Diffusion: *Lactobacillus acidophilus*

Antimicrobial Agent	Averages (mm) for six strains	Ranges for six strains	Quality Control Ranges	Absence of Zone of Inhibition
Ampicillin	32.56	28-37	30-36	n=0
Amp/Sul	34.10	32-38	n.a.	n=0
Ceftriaxone	30.44	22-34	30-35	n=0
Chloramphenicol	28.76	27-31	23-27	n=0
Ciprofloxacin	2.54	0-16	n.a.	n=5
Clindamycin	21.49	9-40	19-25	n=0
Erythromycin	28.96	22-34	25-30	n=0
Gentamicin	19.67	15-28	n.a.	n=0
Imipenem	30.35	22-39	n.a	n=0
Kanamycin	0	0	n.a.	n=6
Levofloxacin	4.82	0-17	20-25	n=5
Linezolid	32	28-37	25-34	n=0
Metronidazole	0	0	n.a.	n=6
Rifampin	27.63	21-33	25-30	n=0
Streptomycin	19.93	0-29	n.a.	n=1
Synercid	23.81	18-31	0.25-1	n=0
Tetracycline	27.74	13-34	27-31	n=0
Tigecycline	27.45	20-32	23-29	n=0
Trim/Sulfa	0	0	20-28	n=6
Vancomycin	12.29	0-28	20-27	n=2

Note: No interpretive values found for *Lactobacillus acidophilus*
n.a.: not available
n: number of strains

Table 2-3-3 Disk Diffusion: *Lactobacillus casei*

Antimicrobial Agent	Averages (mm) for four strains	Ranges for four strains	Quality Control Ranges	Absence of Zone of Inhibition
Ampicillin	34.42	28-39	30-36	n=0
Amp/Sul	35.27	31-42	n.a.	n=0
Ceftriaxone	28.46	23-33	30-35	n=0
Chloramphenicol	26.71	26-27	23-27	n=0
Ciprofloxacin	0	0	n.a.	n=4
Clindamycin	25.65	23-29	19-25	n=0
Erythromycin	27	23-30	25-30	n=0
Gentamicin	17.59	15-21	n.a.	n=0
Imipenem	26.67	21-38	n.a	n=0
Kanamycin	0	0	n.a.	n=4
Levofloxacin	11.29	0-17	20-25	n=1
Linezolid	31.67	28-35	25-34	n=0
Metronidazole	0	0	n.a.	n=4
Rifampin	28.48	24-31	25-30	n=0
Streptomycin	18.54	12-28	n.a.	n=1
Synercid	27.13	26-30	0.25-1	n=0
Tetracycline	28.71	12-28	27-31	n=0
Tigecycline	29.19	22-34	23-29	n=0
Trim/Sulfa	3.125	0-13	20-28	n=3
Vancomycin	0	0	20-27	n=4

Note: No interpretive values found for *Lactobacillus casei*
n.a.: not available
n: number of strains

Table 2-3-4 Disk Diffusion: *Lactobacillus plantarum*

Antimicrobial Agent	Averages (mm) for two strains	Ranges for two strains	Quality Control Ranges	Absence of Zone of Inhibition
Ampicillin	38.92	36-42	30-36	n=0
Amp/Sul	42.09	41-44	n.a.	n=0
Ceftriaxone	35.5	31-40	30-35	n=0
Chloramphenicol	28.67	26-31	23-27	n=0
Ciprofloxacin	0	0	n.a.	n=2
Clindamycin	18.88	10-28	19-25	n=0
Erythromycin	27.92	24-26	25-30	n=0
Gentamicin	18.67	16-21	n.a.	n=0
Imipenem	36.75	34-39	n.a	n=0
Kanamycin	0	0	n.a.	n=2
Levofloxacin	3.84	0-8	20-25	n=1
Linezolid	32.08	29-35	25-34	n=0
Metronidazole	0	0	n.a.	n=2
Rifampin	23.83	22-26	25-30	n=0
Streptomycin	13.5	11-16	n.a.	n=1
Synercid	23.83	21-27	0.25-1	n=0
Tetracycline	27	24-30	27-31	n=0
Tigecycline	24.25	23-25	23-29	n=0
Trim/Sulfa	11.59	0-24	20-28	n=1
Vancomycin	0	0	20-27	n=2

Note: No interpretive values found for *Lactobacillus plantarum*
n.a.: not available
n: number of strains

Table 2-3-5 Disk Diffusion: *Bacillus* species

Antimicrobial Agent	Averages (mm) for four strains	Ranges for four strains	Absence of Zone of Inhibition
Ampicillin	23.21	18-30	n=0
Amp/Sul	28.73	26-35	n=0
Ceftriaxone	20.98	12-30	n=0
Chloramphenicol	20.29	13-30	n=0
Ciprofloxacin	31.96	24-37	n=0
Clindamycin	9.19	0-24	n=2
Erythromycin	13.67	0-31	n=2
Gentamicin	33.54	24-40	n=0
Imipenem	43.83	32-46	n=0
Kanamycin	27.46	22-32	n=0
Levofloxacin	33.17	28-39	n=0
Linezolid	34.23	25-39	n=0
Metronidazole	12.21	0-21	n=1
Rifampin	21.46	18-24	n=0
Streptomycin	30.29	23-37	n=0
Synercid	17.63	15-21	n=0
Tetracycline	23.63	21-32	n=0
Tigecycline	25.65	18-29	n=0
Trim/Sulfa	35.40	32-39	n=0
Vancomycin	22.58	21-24	n=0

Note: No interpretive values found for *Bacillus* species
n: number of strains

Table 2-3-6 Disk Diffusion: *Propionibacterium freudenreichii*

Antimicrobial Agent	Averages (mm)	Absence of Zone of Inhibition
Ampicillin	50.67	n=0
Amp/Sul	57	n=0
Ceftriaxone	12	n=0
Chloramphenicol	54.17	n=0
Ciprofloxacin	67.83	n=0
Clindamycin	42.67	n=0
Erythromycin	48.33	n=0
Gentamicin	43.83	n=0
Imipenem	36.17	n=0
Kanamycin	0	n=1
Levofloxacin	0	n=1
Linezolid	36.83	n=0
Metronidazole	56	n=0
Rifampin	55.67	n=0
Streptomycin	49.67	n=0
Synercid	51.33	n=0
Tetracycline	53.33	n=0
Tigecycline	45.5	n=0
Trim/Sulfa	57	n=0
Vancomycin	59	n=0

Note: No interpretive values found for *Propionibacterium freudenreichii*
n: number of strains

Table 2-4-1 MIC: *Enterococcus faecium*

Antimicrobial Agent	Averages (µg/mL) for nine strains	Ranges for nine strains	Quality Control Ranges	Susceptible/ Intermediate/ Resistant Strains	MIC Breakpoints/ Cut-off Values (µg/mL)	Guidelines followed
Ceftriaxone	0.7	0-2	n.a.	S ≤ 0.25 (n= 3) R ≥ 0.25 (n= 6)	0.25	EUCAST*
Ciprofloxacin	2.5	0.5-8	0.25-2	S ≤ 1 (n= 7) R ≥ 4 (n= 2)	4	CLSI
Clindamycin	8.67	2-16	4-16	R > 0.5 (n= 9)	0.5	CLSI*
Erythromycin	5.67	2-8	1-4	I, 1-4 (n= 4) R ≥ 8 (n= 5)	8	CLSI
Metronidazole	32	32	n.a.	R > 32 (n=9)	32	n.a.
Rifampin	12.67	2-16	0.5-4	S ≤ 1 (n= 1) R ≥ 4 (n= 8)	4	CLSI
Synercid	0.5	0.5	2-8	S ≤ 1 (n= 9)	4	CLSI
Trim/Sulfa	0.125	0.125-2	n.a.	S ≤ 0.03 (n=5) R > 1 (n= 4)	1	EUCAST

n.a.: not available

n: number of strains; S: susceptible; I: Intermediately susceptible; R: Resistant

EUCAST*: MIC breakpoint values from *Streptococcus* groups A, B, C and G

Table 2-4-2 MIC: *Lactobacillus* species

Lactobacillus acidophilus

Antimicrobial Agent	Averages (µg/mL) for six strains	Ranges for six strains	Quality Control Ranges	Susceptible/ Intermediate/ Resistant Strains	MIC Breakpoints/ Cut-off Values (µg/mL)	Guidelines followed
Vancomycin	11	0.5-32	0.12-0.5	S ≤ 4 (n= 4) R ≥ 32 (n= 2)	32	CLSI
Metronidazole	32	32	0-32	R ≥ 32 (n= 6)	32	CLSI**

**: Indicating breakpoint interpreted based on Gram-positive Anaerobes' values

Lactobacillus casei

Antimicrobial Agent	Averages (µg/mL) for six strains	Ranges for six strains	Quality Control Ranges	Susceptible/ Intermediate/ Resistant Strains	MIC Breakpoints/ Cut-off Values (µg/mL)	Guidelines followed
Metronidazole	32	32	0-32	R ≥ 32 (n= 4)	32	CLSI**

****:** Indicating breakpoint interpreted based on Gram-positive Anaerobes' values

Lactobacillus plantarum

Antimicrobial Agent	Averages (µg/mL) for six strains	Ranges for six strains	Quality Control Ranges	Susceptible/ Intermediate/ Resistant Strains	MIC Breakpoints/ Cut-off Values (µg/mL)	Guidelines followed
Metronidazole	32	32	0-32	R ≥ 32 (n= 2)	32	CLSI**

****:** Indicating breakpoint interpreted based on Gram-positive Anaerobes' values

Table 2-4-3 MIC: *Bacillus* species

Antimicrobial Agent	Averages (µg/mL) for six strains	Ranges for six strains	Quality Control Ranges	Susceptible/ Intermediate/ Resistant Strains	MIC Breakpoints/ Cut-off Values (µg/mL)	Guidelines followed
Clindamycin	1.13	0.25-2	0.06-0.25	S ≤ 0.25 (n= 4) R ≥ 0.25 (n= 2)	4	EFSA
Erythromycin	4.125	0.25-8	0.25-1	S ≤ 0.5 (n= 4) R ≥ 8 (n= 6)	8	CLSI
Metronidazole	14	8-32	0-32	R ≥ 32 (n= 6)	32	CLSI**

****:** Indicating breakpoint interpreted based on Gram-positive Anaerobes' values

Table 2-4-4 MIC: *Propionibacterium freudenreichii*

Antimicrobial Agent	Averages (μg/mL)	Ranges for six strains	Susceptible/ Intermediate/ Resistant Strains	MIC Breakpoints/ Cut-off Values (μg/mL)	Guidelines followed
Kanamycin	128	0.25-2	R ≥ 64 (n= 1)	32	EFSA
Levofloxacin	4.125	0.25-8	n.a.	n.a.	n.a.

n.a.: not available

Table 2-5 Overall MIC Breakpoints/Cut-off Values based on CLSI or EFSA

Antimicrobial Agent	*Bacillus* (µg/mL)	*Enterococcus faecium* (µg/mL)	*Lactobacillus* (µg/mL)	*Propionibacterium* (µg/mL)
Ampicillin	0.5	16	2**	2**
Amp/Sul	n.a.	16	32/16**	32/16**
Ceftriaxone	64	0.25*	64**	64**
Chloramphenicol	8	32	32**	32**
Ciprofloxacin	4	4	n.a.	n.a.
Clindamycin	n.a.	0.5	4	8**
Erythromycin	8	8	8	0.5
Gentamicin	16	> 500	16	n.a.
Imipenem	n.a.	8	16**	8**
Kanamycin	8	n.a.	16	64
Levofloxacin	n.a.	8	n.a.	n.a.
Linezolid	n.a.	8	n.a.	n.a.
Metronidazole	32**	32**	32**	32**
Rifampin	n.a.	4	n.a.	n.a.
Streptomycin	8	> 1000	16	64
Synercid	n.a.	4	n.a.	n.a.
Tetracycline	8	16	16**	16**
Tigecycline	n.a.	0.5	n.a.	n.a.
Trim/Sulfa	n.a.	n.a.	n.a.	n.a.
Vancomycin	4	32	32	2**

n.a.: not available
*: Indicating breakpoint values interpreted based on *Streptococcus* groups A, B, C and G
**: Indicating breakpoint values interpreted based on Gram-positive Anaerobes' values

Clinical and Laboratory Standards Institute (CLSI)

European Food Safety Authority (EFSA)

European Committee on Antimicrobial Susceptibility Testing

Table 2-6 MIC_{50} and MIC_{90} of *E. faecium* strains from ten DFMs

Antimicrobial Agent	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Breakpoints (μg/mL)
Ceftriaxone	0-2	1	1	0.25*
Ciprofloxacin	0.5-8	1	8	4
Clindamycin	2-32	8	8	0.5*
Erythromycin	2-8	8	8	8
Metronidazole	16-64	64	64	n.a.
Rifampin	2-32	8	16	4
Trim/Sulfa	0.125-2	0.125	2	1*

* Based on EUCAST

Table 2-7 Primers used in antibiotic resistance in *E. faecium*

Primers	**Sequences**	**Annealing Temperatures, °C**	**Product Sizes, bp**	**References**
*erm*A	F 5’ TCTAAAAAGCATGTAAAAGAA 3’ R 5’ CTTCGATAGTTTATTAATATTAGT 3’	47	645	Sutcliffe et. al, 1996
*erm*B	F 5’ GAATCCTTCTTCAACAATCA 3’ R 5'ACTGAACATTCGTGTCACTT 3'	54	175	Modified from Jacob et. al, 2014
*erm*C	F 5’ TCAAAACATAATATAGATAAA 3’ R 5’ CTTCGATAGTTTATTAATATTAGT 3’	47	642	Sutcliffe et. al, 1996
*tet*M	F 5′ CTGTTGAACCGAGTAAACCT 3′ R 5′ GCACTAATCACTTCCATTTG 3′	48	156	Modified from Jacob et. al, 2014
*van*A	F 5’CATGAATAGAATAAAAGTTGCAATA 3’ R 5’CCCCTTTAACGCTAATACGATCAA 3’	54	1030	Modified from Kariyama et. al, 2000
*van*B	F 5’ GTGACAAACCGGAGGCGAGGA 3’ R 5’ CCGCCATCCTCCTGCAAAAAA 3’	54	433	Kariyama et. al, 2000

F: Forward primer

R: Reverse primer

Table 2-8-1Microarray Result

Resistance genes detected in three DFM combinations

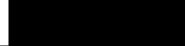
DFM Combinations		**DFM B**	**DFM C**	**DFM F**
Antimicrobial Resistance				
Tetracycline	*tet*M			■
	*tet*32	■		
	*otr*A		■	
	*tet*38	■		
Trimethoprim	*dhfrIX*		■	
Virginiamycin	*vgb*A			■
Miscellaneous AMR				
Aminoglycosides	*aad*E			■
	aphA-3			■
Glycopeptides	*van*Y A	■		
Metal Resistance				
Arsenic	*ars*R	■		■
Cobalt/Nickel	*cnr*B		■	■
Copper	*cop*B	■		
Copper/Silver	*cus*A		■	
Miscellaneous Genes				
Class I Integrons	*Intl 1 2*	■		
Insertional Elements	*M13*			

DFM B, C and F corresponds to the DFM products in our system

Table 2-8-2 Microarray Result

Resistance genes detected in *E. faecium* from three DFM

Enterococcus faecium in DFM		DFM C	DFM D	DFM F
Antimicrobial Resistance				
Chloramphenicol	*flo**			■
Erythromycin	*ere*(A2)			■
	*erm*B	■		
Tetracycline	*tet*C	■		
	*tet*D			■
	*tet*K			■
	*tet*V			■
	*tet*31		■	■
	*tet*39		■	
	*otr*A			■
Virginiamycin	*vgb*B			■
Miscellaneous AMR				
Aminoglycosides	*aad*A1			■
	ANT4-Ia			■
Macrolides	*mphBM*			■
Metal Resistance				
Aluminum	*yba*X		■	
Cadmium	*cad*D4			■
Cobalt/Nickel	*cnr*B	■		■
Copper	*cut*A			■
	*pco*A			■
	*pco*B			■
	*pco*D			■
	*pco*E			■
	*pco*R			■
	*pco*S	■		■
Copper/Silver	*cus*A	■		■
Metal Genes				
Nickel	*ncr*C	■		
Silver	*sil*A			■
	*sil*S			■

Transferable Copper	*tcr*Z			

DFM C, D and F corresponds to the DFM products in our system

Table 2-8-3 Microarray Result

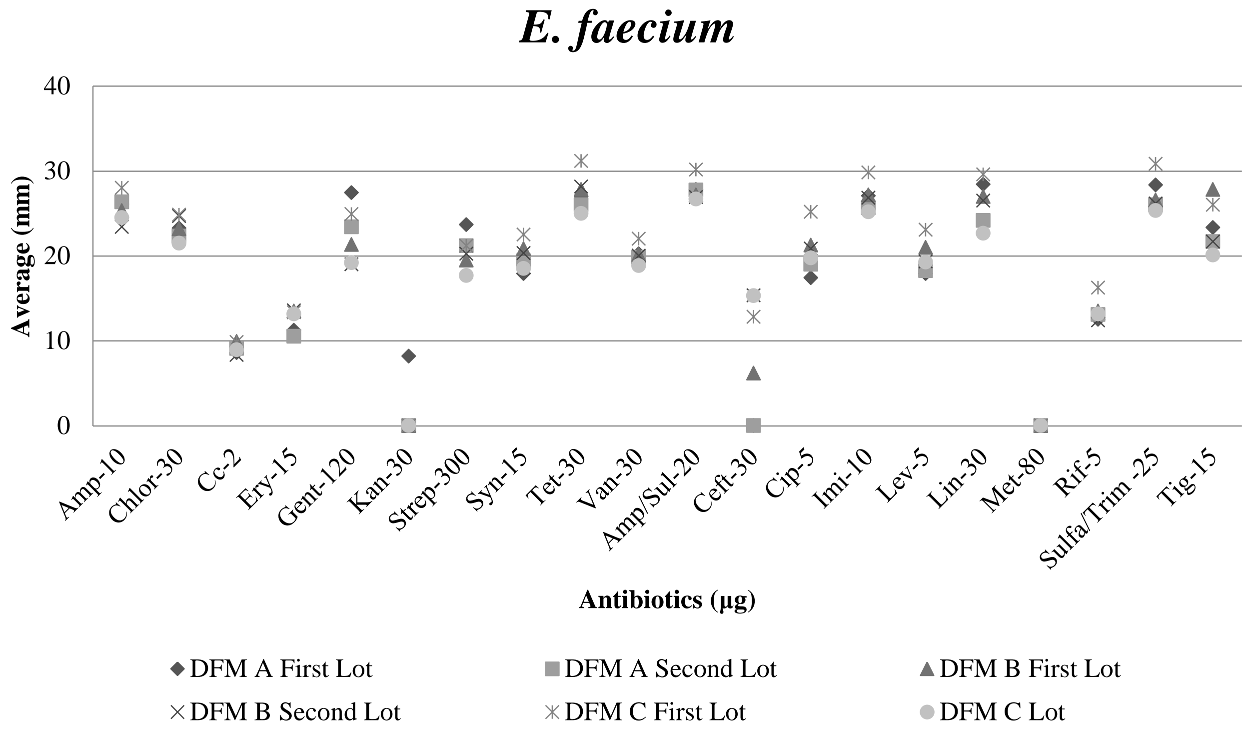
Resistance genes detected from other genera

Strains in a DFM		*L. acidophilus*	*P. freudenreichii*
Antimicrobial Resistance			
Tetracycline	*tet*C	■	
	*otr*C	■	
	*otr*A		■
	*tet*W		■
	*tet(*R)	■	
Trimethoprim	*dhfrI*X	■	
Virginiamycin	*vgb*B		■
Miscellaneous AMR			
Aminoglycosides	*AAC6*-Ii		■
Metal Resistance			
Cobalt/Nickel	*cnrB*	■	■
Copper	*cop*B	■	
Copper/Silver	*cus*A	■	
	*cus*S	■	
Metal Genes			
Copper/Zinc/Cadmium	*czcA 2 copABCD*	■	
Lead	*pbr*A		■

Table 2-9 Whole-Genome Sequencing Results for *E. faecium*

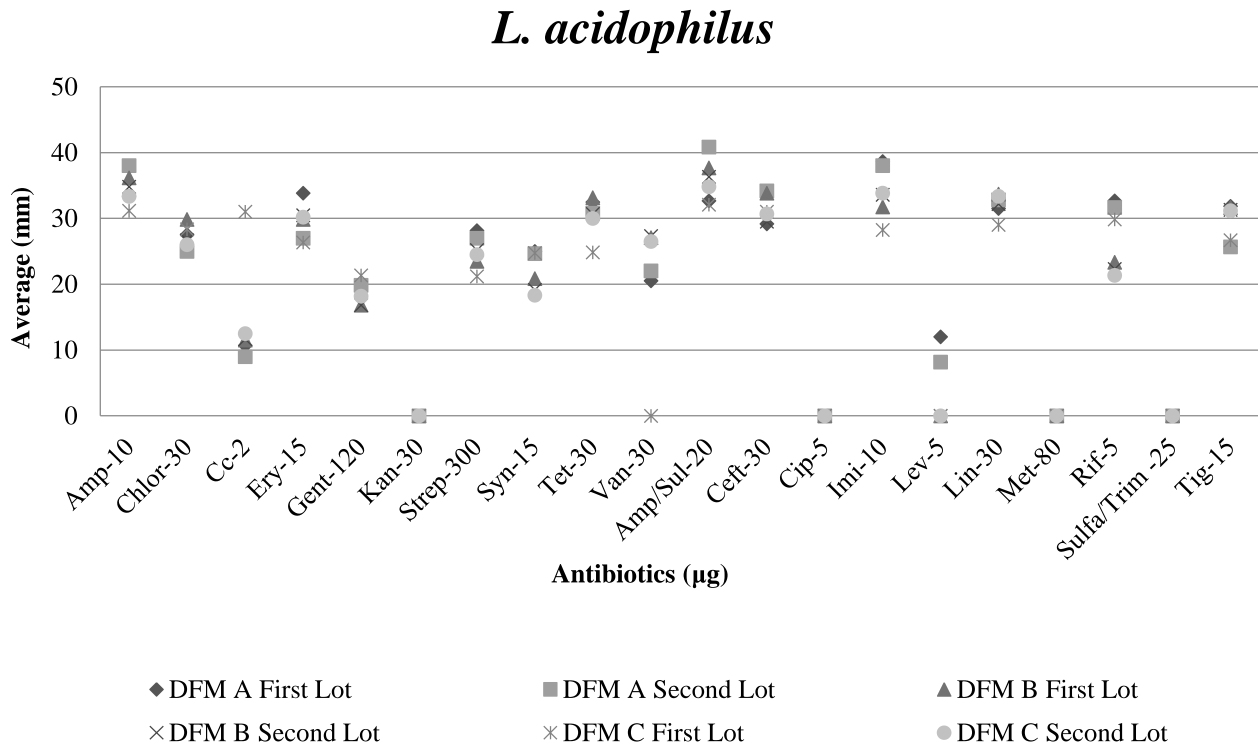
***E. faecium* in different DFM**			**Number of Strains**
Antimicrobial Resistance Group	Macrolide	*msr*C	9
Plasmids	Query ID	pAMbeta1, AF007787	4
		pBT233, X64695	4
		pGB354, U83488	4
		orf17, NC011140	4
		pRE25, X92945	4
		pTEF1, AE016833	4
		p200B, AB158402	4
		E.faeciumContig1183, JDOE	2
		pEF1, DQ198088	2
		E.faeciumContig1258, JDOE	2
		E.faecium287, NZAAAK010000287	2

Figure 1 Comparison of *E. faecium* second to first DFM lots



Note: DFM A, B and C denotes different *E. faecium* strains from different DFM products

Figure 2 Comparison of *L. acidophilus* second to first DFM lots



Note: DFM A, B and C denotes different *L. acidophilus* strains different DFM products

Figure 3 Comparison of *L. casei* second to first DFM lot

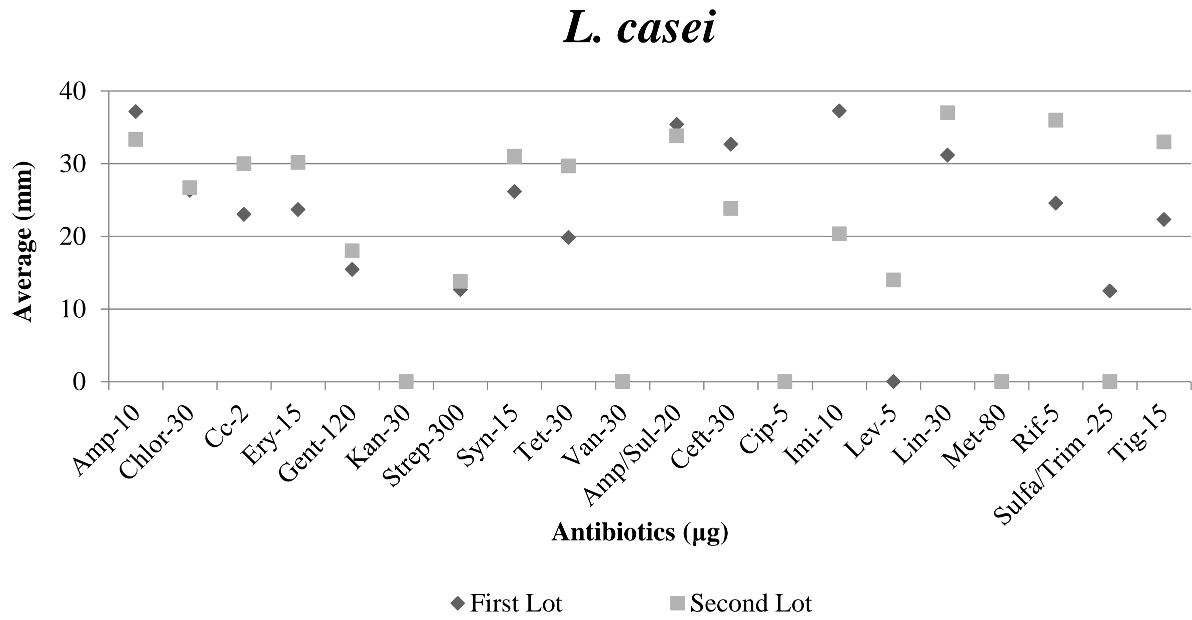


Figure 4 Comparison of *L. plantarum* second to first DFM lot

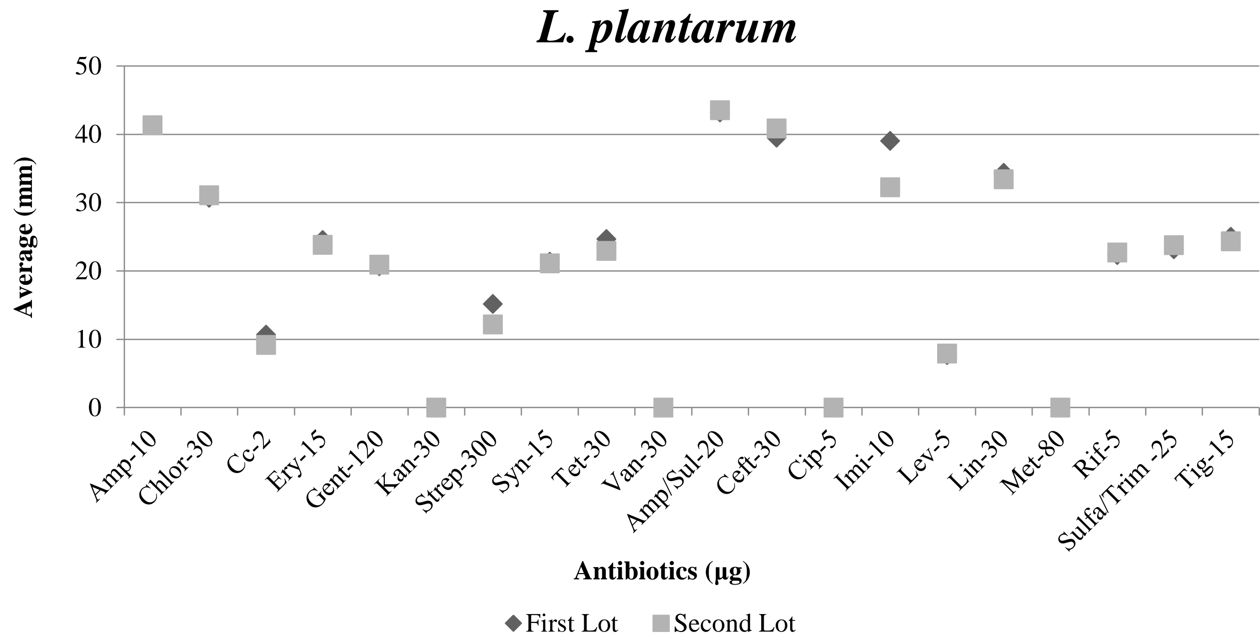


Figure 5 Comparison of *Bacillus subtilis* second to first DFM lot

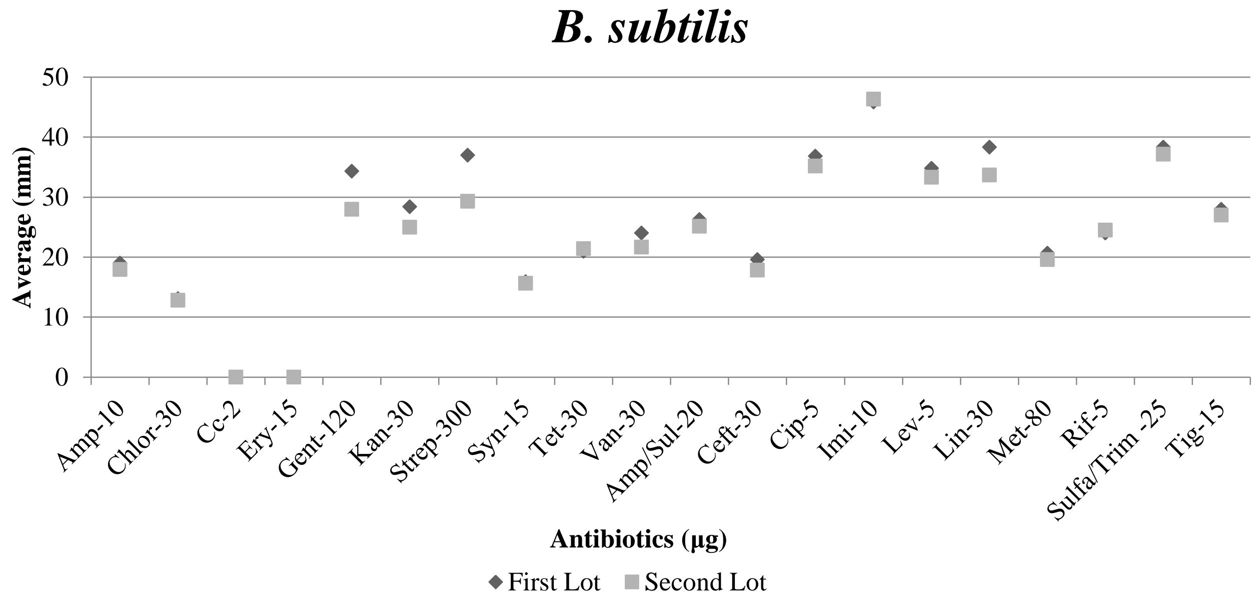
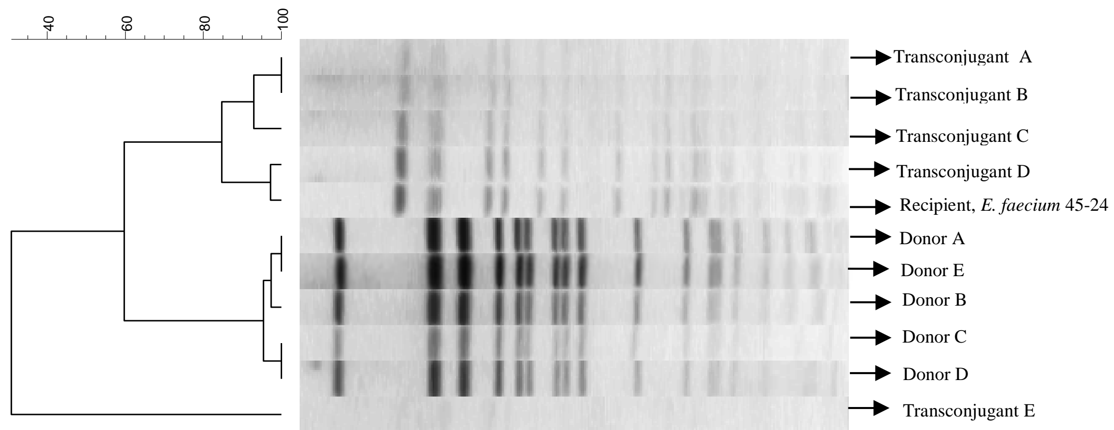


Figure 6 PFGE Antibiogram Patterns



Note: A and E: Different colonies from the same DFM; C and D: Different colonies from the same DFM