Prevalence and Characteristics of Antibiotic Resistant Bacteria in Selected Ready-to-Consume Deli and Restaurant Foods

Thesis

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Abstract

One of the major public health challenges nowadays is the rapid emergence of antibiotic resistant (ART) pathogens. In the past couple of decades, despite the extensive studies on ART pathogens in clinical settings, the lack of proper understanding on major routes and mechanisms of antibiotic resistance (AR) emergence, dissemination and persistence likely contributed to the incompetence in the battle against AR. Particularly, the impact of commensal bacteria, which account for a majority of the microbial population, on the development of AR has been overlooked. Since horizontal gene transfer is crucial to the rapid dissemination of AR, these dominant organisms in microbial ecosystems likely have played a key role in the emergence of ART pathogens. Recently a large amount of ART commensal bacteria were detected in many food items, suggesting that the food chain may serve as an important avenue of disseminating AR to human. Therefore, the objectives of this study were to reveal the AR status of commensal bacteria from selected ready-to-consume (RTC) food products collected from local stores and restaurants and to analyze the genetic elements of ART commensals for an improved understanding of the AR risk associated with the food chain.

ART bacteria, screened by selective media with tetracycline, were detected in 20 out of 26 food samples collected from salad bars from local grocery stores and restaurants.

The total counts varied among samples, with 46% ranging from 10^5 CFU/g to 10^9CFU/g. Tetracycline resistant (Tet^r) bacteria counts of no less than 10^3 CFU/g were detected in seven samples. Noticeably, four samples, including mushroom salad, spicy shrimp, chicken salad, carried Tet^r counts about 10^4 CFU/g. Out of 740 Tet^r isolates examined, 114 (15.4%) carried one or more of *tetM, tetL, tetS, tetK* genes by conventional PCR, 27 harbored more than one Tet^r determinants. The most prevalent genotype was *tetM*, which was detected in 57.0% of the Tet^r gene carriers, followed by *tetL* (37.8%), *tetS* (9.6%) and *tetK* (3.0%). The genera identified by 16S rRNA gene analysis included *Enterococcus, Lactococcus, Staphylococcus, Brochothrix, Carnobacterium, Sphingobacterium*. Out of 15 Tet^r isolates examined, 12 had the Tet^r gene(s) located on both the chromosome and plasmid, and 2 only on chromosome and 1 on plasmid by Southern hybridization. AR determinants from selective samples were successfully transmitted into *Streptococcus mutans* via natural gene transformation and *Enterococcus faecalis* via electroporation. Isolates which carry plasmids with AR determinants were consecutively transferred for more than 400 generations in bacterial media without tetracycline to study the stability of resistance. Result showed that among 13 isolates tested, only 2 lost the Tet^r phenotype in 1% of the progenies. These findings suggest that restaurant and deli foods serve as an avenue in transmitting AR to humans, the AR genes can potentially be transmitted to other bacteria including pathogens, and the AR trait is quite stable even at the absence of the selective pressure. A comprehensive understanding of the AR risk will assist in the development of proper control strategies.

Dedication

Dedicated to PengPeng for her unconditional support and everlasting love

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Antibiotics initially were defined as substances or compounds produced by microorganisms that could kill or inhibit the growth of other bacteria. Modern antibiotics also include synthetic antibacterial compounds [1]. Ever since the advent of modern antibiotics, countless once deadly bacterial infections have been effectively treated, greatly enhancing the life expectancy and quality of human lives around the world. More than 5000 antibiotics are now known and about 100 are currently used to treat human infections [1]. In 2005, the worldwide sales of oral antibiotics totaled $25 billion [2]. In addition, the estimated use of antimicrobials by livestock producers in the United States reached 24.6 million pounds for non-therapeutic purposes, accounting for 70 percent of total antimicrobial use in 2001[3]. Specifically, tetracycline, penicillin, erythromycin and other antimicrobial agents that are important for human therapies are also extensively used for non-therapeutic purpose, such as growth promoter, in livestock production.

The extensive applications of antibiotics, however, had been linked to the pervasive AR problem, which started shortly after the appearance of antibiotics in the 1940s when

sulfonamide-resistant *Streptococcus pyogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA) and streptomycin-resistant *Mycobacterium tuberculosis* were first isolated in hospitals [4]. It is now a general belief that AR will inevitably emerge in almost all species. Accordingly, the usefulness of antibacterial agents has been and will continue to decrease. Taking into account the accelerated emergence of resistance and the slow introduction of new effective drugs, there's a great need to extend the lifespan of these once magical antibiotics and to be aware of the potential recurrence of fatal bacterial infection caused by multi-resistant superbugs. On one hand, the prudent use of antibiotics in clinical treatments should be ensured by providing sufficient education to the public as well as clinic practitioners. On the other hand, the impact of agriculture practices on the AR emergence and dissemination, especially those associated with food production, need to be extensively evaluated.

1.2 Antibiotics

1.2.1 Classes of Antibiotics

Antibiotics can be classified by the mechanisms of action (bactericidal or bacteriostatic), spectrum of activity (broad or narrow), routes of administration (oral or injection), or most of the time, by the chemical structure [5]. The chemical structure of major classes of naturally derived antibiotics (β-lactam, tetracycline, phenylpropanoid, macrolide, glycopeptides and lipopeptide) and synthetic antibiotics are shown in Figure 1.1.and 1.2. Table 1.1 includes a brief summary of the main classes of antibiotics [1].

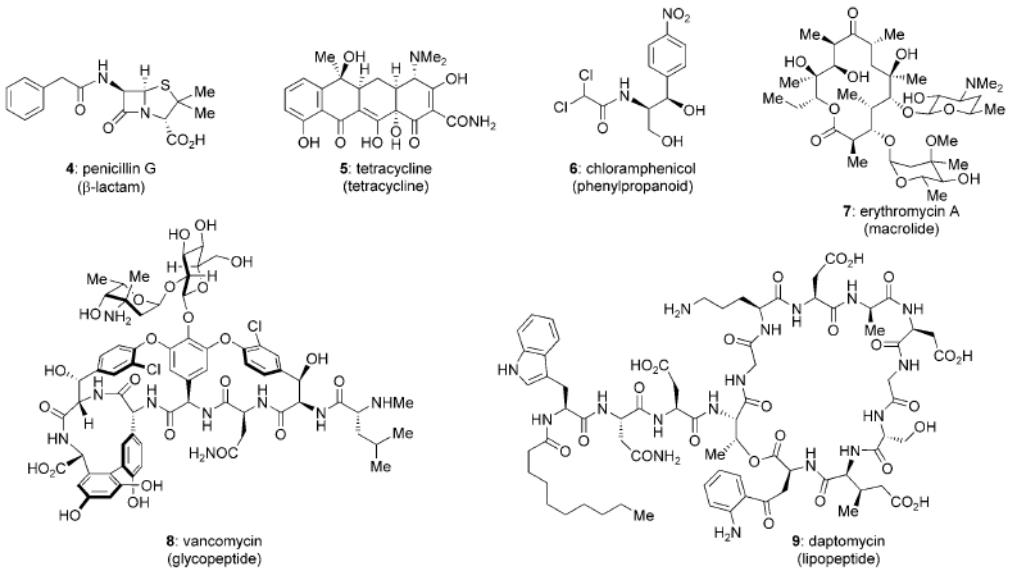

Figure 1.1. Selected antibiotics derived from natural products [6]

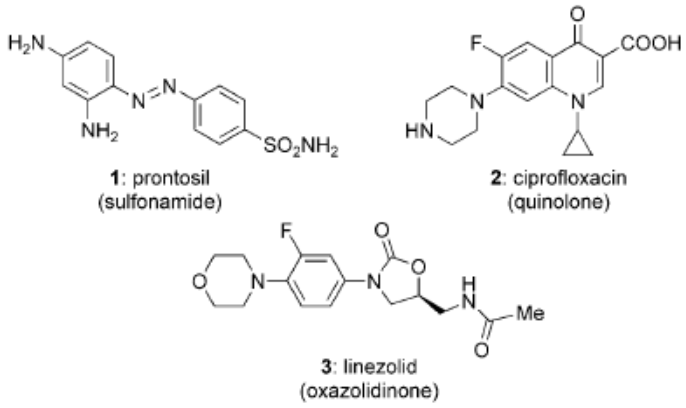

Figure 1.2. Selected antibiotics of synthetic origin [6]

Antibiotic class	Introduction	Derivation	Example	Mechanism
Sulphonamide	1935	Synthetic	Sulfapyridine	Antifolate
β-Lactam	1941	NP-derived	Penicillin	Bacterial cell wall
Bacterial peptide	1942	NP-derived	Bacitracin	Bacterial cell wall
			Polymixin	Bacterial cell membrane
Aminoglycoside	1944	NP-derived	Streptomycin	Protein synthesis
Cephalosporin	1945	NP-derived	Cephalosporin	Bacterial cell wall
Nitrofuran	1947	Synthetic	Nitrofurantoin	Various
Hexamine	1947	Synthetic	Methenamine mandelate	Release of formaldehyde
Chloramphenicol	1949	NP-derived	Chloramphenicol	Protein synthesis
Tetracycline	1950	NP-derived	Chlortetracycline	Protein synthesis
Isoniazid	1951	Synthetic[a]	Isoniazid	Fatty acid biosynthesis
Viomycin	1951	NP-derived	Viomycin	Protein synthesis
Macrolide	1952	NP-derived	Erythromycin	Protein synthesis
Lincosamide	1952	NP-derived	Lincomycin	Protein synthesis
Streptogramin	1952	NP-derived	Virginiamycin	Protein synthesis
Cycloserine	1955	NP-derived	Cycloserine	Bacterial cell wall
Glycopeptide	1956	NP-derived	Vancomycin	Bacterial cell wall
Novobiocin	1956	NP-derived	Novobiocin	DNA synthesis
Ansamycin	1957	NP-derived	Rifamycin	RNA synthesis
Nitroimidazole	1959	Synthetic	Tinidazole	DNA synthesis
Ethambutol	1962	Synthetic	Ethambutol	Bacterial cell wall
Quinolone	1962	Synthetic	Nalidixic acid	DNA synthesis
Fusidane	1963	NP-derived	Fusidic acid	Protein synthesis
Diaminopyrimidine	1968	Synthetic	Trimethoprim	Antifolate
phosphonate	1969	NP-derived	Fosfomycin	Bacterial cell wall
Pseudomonic acid	1985	NP-derived	Mupirocin	Protein synthesis
Oxazolidinone	2000	Synthetic	Linezolid	Protein synthesis
Lipopeptides	2003	NP-derived	Daptomycin	Bacterial cell membrane

[a] Isoniazid is based on the structure of nicotinamide (vitamin B_2).

Table 1.1. Antibiotic classes with approximate year of clinical introduction, derivation, example of drug and mechanism of action [7]

β-lactam antibiotics, such as penicillin, cephalosporin and carbapenem, are usually bactericidal against Gram-positive and Gram-negative bacteria by inhibiting the synthesis of the cell wall. Tetracycline and its derivatives, such as chlortetracycline, inhibit bacterial protein synthesis by reversibly binding to the prokaryotic 30S ribosomal subunit and blocking the interaction of the aminoacyl-tRNA with bacterial ribosome [8]. Aminoglycosides, including streptomycin, neomycin, kanamycin, are bactericidal against aerobic Gram-negative bacilli. Aminoglycoside binds irreversibly to the 30S bacterial ribosome thus inhibiting protein synthesis. By contrast, macrolides inhibit protein synthesis by reversibly binding to the 50S ribosomal subunits of susceptible organisms. Therefore, macrolides antibiotics such as erythromycin, azithromycin, clarithromycin are usually

considered bacteriostatic against Gram-positive cocci and Gram-negative anaerobes. Ketolides, derivatives of macrolides, have similar structure as erythromycin but higher affinity for ribosomal binding, therefore ketolides can be used for macrolides resistant strains. Finally, glycopeptides, typified by vancomycin, are bactericidal agents that inhibit peptidoglycan polymerase and transpeptidation reactions of sensitive Gram-positive bacteria.

1.2.2 Historical Overview of Modern Antibiotics

The early search for antibiotics dated back to the 19th century. In 1890s, Rudolf Emmerich and Oscar Löw found *Bacillus pycyaneus* (now called *Pseudomonas aeruginosa*) and pyocyanin extracted from its culture can be used to destroy bacteria that caused cholera, typhoid, diphtheria and anthrax [9]. Then in 1928, Alexander Fleming discovered penicillin, the first antibiotic in modern medicine produced by *Penicillium notatum* [10]. Penicillin were widely used in the World War II and saved millions of lives ever since. In 1945, Fleming shared the Nobel Prize in Physiology or Medicine with Chain and Florey for “the discovery of the penicillin and its curative effect in various infectious diseases”. Fifteen years after the discovery of penicillin, Selman A. Waksman found streptomycin from a mold that was able to kill tubercle bacilli in 1943 and received the Nobel Prize in 1952 for his discovery [1]. Subsequently, various broad-spectrum penicillins and other aminoglycosides were developed and many new classes of naturally occurring antibiotics were discovered (Table 1.1).

Though most antibiotics in clinic use today can be traced back to their natural sources, synthetic antibacterial agents play an increasingly important role in the expanding antibiotic market. Prontosil, the first general-purpose antibiotic of synthetic origin, was discovered by Domagk in early 1930s and launched in 1935 by the Bayer Laboratories [6]. Prontosil belongs to a large class of antibacterial agents known as sulfonamides. In 1939, Domagk was awarded the Nobel Prize in Physiology or Medicine for "the discovery of the antibacterial effects of prontosil." The second class of synthetic antibacterial agents is quinolone, which was introduced by George Lesher in 1962. Ciprofloxacin is the one example of this class still in market.

The introduction of synthetic sulfonamides in the 1930s and natural penicillins in the 1940s endowed people with effective weapons in the battle against bacterial infections. Most classes of antibiotics used today were discovered between 1940 and 1970. After that, however, the development of both synthetic and natural antibiotics stalled for almost 10 years partly because it was believed that those bacterial infections were no longer a problem. But studies about new antibacterial agents resumed in the 1980s in response to the increasing onset of ART pathogens in clinical settings. However, despite the continuous research in antibiotic development, only three new classes of antibacterial have entered the market since 1980, which are the psudomonic acid antibiotic mupirocin in 1985, the oxazolidinone linezolid in 2000 and the lipopeptide daptomycin in 2003 [7]. Over the last decade, only 22 new antibacterial drugs have been launched. Twelve naturally derived drugs belonging to five structure classes (β-lactam, streptogramin, macrolide, tetracycline

and daptomycin) and ten synthetic drugs belong to two classes (nine from quinolone and one from oxazolidinone) [7].

Fortunately, the recent progress in chemistry and biology greatly enhances the search for novel antibiotics. First, the advanced technologies of compound separation and structure elucidation would help chemists discover more natural products as a starting point for new drug design. By studying the existing antibiotic structures, chemists could synthesize drugs with improved medical properties and reduced production costs. In fact, many new antibiotics are modified from previous ones, and some classes are already in their third or fourth generation [11]. Secondly, the genomes of more than 140 bacteria have been sequenced, revealing many highly conserved, essential bacteria genes that could be used as target of antibacterial drugs. Based on that information, high throughput cell-based screening for inhibitors of transcription, translation, and other biosynthetic pathways seem to offer good prospects in new antibiotics discoveries [12].

1.3 Antibiotic Resistance

1.3.1 History and Prevalence of Antibiotic Resistance(AR)

The history of AR started as early as 1930s when sulfonamide-resistant *Streptococcus pyogenes* emerged in military hospitals. Later on, methicilin resistant *Staphylococcus aureus* and streptomycin resistant *Mycobacterium tuberculosis* appeared shortly after the introduction of corresponding antibiotics [4]. Ever since then, AR spread among almost all pathogens at an accelerating speed. In 2006, studies found that 54.0% of the *Salmonella* Typhi isolates were nalidixic acid resistant and 19.6% of *campylobacter* strains were

resistant to ciprofloxacin compared to 19.2% and 12.9% respectively in 1999 [13]. In 2002, The U.S. Centers for Disease Control and Prevention (CDC) estimated that more than 45000 deaths in the U.S. every year could be attributed to bacterial infection caused by ART bacteria [14].To make things worse, multidrug resistance was initially detected in 1950s among *Escherichia coli*, *Shigella* and *Salmonella* [15] and was blamed for the re-occurrence of tuberculosis in 1980s [16]. Since most of the traditional treatments would fail when confronted with multidrug resistant pathogens, many life threatening outbreaks these days were eventually linked to those "superbugs" [17-19].

The increasing clinical use of antibiotics is usually considered to create a survival advantage of ART bacteria over their sensitive counterparts. Consequently, many previously affordable and effective antimicrobial treatments become unsuitable for their original usage. The growing list of outdated antibiotics includes penicillin and oxacillins against staphylococcal infection, sulfonamides and ampicillin against urinary tract infection, fluoroquinolones against gonorrhea, etc. [20]. Resistance to the third-generation of clinically important antibiotics such as ceftiofur, ceftriaxone, was already found in *Campylobacter jejuni, Salmonella* Newport, *shigella flexneri,* and *Escherichia coli* O157 [13]. At the mean time, the antibiotics applied to the human-consumed-animals are also responsible for the resistance problems in humans [21]. When antibiotics are applied to animals as therapy or disease prevention, or as growth promoter, ART bacteria would likely develop in animals and then could transmit to human via direct contact or food chain.

Ever since 1970s, reducing unnecessary clinical use of antibiotics has been adopted gradually as a strategy of combating AR problem [22, 23]. These practices are based on the

belief that ART traits would gradually disappear, or occur at a lower frequency, when the selective pressure of antibiotics is removed. The results of such strategies, however, are less convincing. According to the data from the UK Healthy Protection Agency and European Antimicrobial Resistance Surveillance System, the suspension of certain antibiotics did not usually lead to the reduction of the prevalence of AR[24]. To be more specific, after a 63% reduction in macrolides use in Finland between 1988 and 1994, erythromycin resistance continued to increase in *Streptococcus pneumonia* isolates [24]. In a similar case, sulfonamide-resistant *Escherichia coli* increase by a 6.2% after a 98% decrease in sulfonamide prescriptions in the United Kingdom during the 1990s [25].

When regarding animal use of antibiotics, the precautionary principle also leads to the discontinuation of using antibiotics both as veterinary therapy and as growth promoter in many countries. For example, the Alliance for the Prudent Use of Antibiotics (APUA) at http://APUA.org aims at curbing improper use of antibiotics in animals globally. Although the prevalence of AR in animal feces was reduced after abolition of certain antimicrobial agents, resistance could persist at a low but detectable level for many years [26]. In some case, resistant enterococcal infection in humans remained undiminished after the abolition of antibiotics used as growth promoters in the European Union since 1999[27]. Another study showed that the frequency of AR in organic farms was found not different from conventional farms despite the restricted use of antibiotics in the former [28].

1.3.2. Measurements Taken by ART Bacteria against Antibiotics

The most common survival strategy of ART bacteria at the presence of environmental antibiotics is to reduce the concentration of the inner cellular antibiotic to the sub-lethal level. The three major mechanisms of this strategy are reduction of permeability of the cell wall to antibiotics, expulsion of antibiotics, and destruction of antibiotics by upgrading an antibiotic-inactivating enzymatic pathway [24, 29]. Another less frequent strategy, named target-mediated AR, is to produce a variant target molecule of certain antibiotics that have lower binding affinity with the antibiotics but normal or near normal metabolic function [30, 31].

Bacteria develop AR mainly through three routes: develop intrinsic resistance due to a specific natural cellular property [32], accumulate mutations of target gene under strong selective pressure and transmit the gene vertically to the offspring, and acquire resistance within or between genus through horizontal transfer [29]. Compared to the limited cases of intrinsic resistance and the low frequency of mutation (around 10^{-8}-10^{-9}), horizontal transfer of antibiotic resistant determinants seems to play a key role in the development of AR.

1.3.3. Horizontal Gene Transfer of Antibiotic Resistance Determinants

Horizontal genetic exchange, also called lateral gene transfer, is the movement of genetic material between bacteria other than by descent. Gene comparison, phylogenetic study, together with complete genome sequence analysis provide clues that horizontal gene transfer plays an integral role in the evolution of bacteria and contributes to the rapid spread of AR gene[33, 34]. For example, laterally transferred sequence is estimated to

account for 12.8% of the total genetic material in *Escherichia coli* K12 [34]. Horizontal gene transfer occurs in bacteria through three main avenues: conjugation, transformation and transduction. Conjugation happens when two microorganisms conjoin via a proteinatious structure such as pilus in Gram-netative bacteria and then recipient bacterium acquires a copy of double-stranded circular piece of DNA that has the autonomous inner cellular replication system. In addition, conjugation of many Gram-positive bacteria is often initiated by the production of sex pheromones, which induces the clumping of donor and recipient organisms and the exchange of DNA [35].

Similar to conjugation, transformation involves the uptake of naked DNA via the cell wall of a competent recipient and integration of that DNA into genome or plasmids. The permanent incorporation of recruited DNA is usually mediated by *RecA* through homologous recombination [36]. Recombination of large fragments of DNA tends to results in gene replacement while small fragments the production of mosaic genes [37]. Transformation has been found in species that are perpetually competent such as *Neisseria gonorrhoeae*[38], *Campylobacter* [39], and *Haemophilus influenza*; and species that become competent in certain stage of life cycle such as *Streptococcus*[40], *Bacillus subtilis* [36].

Unlike conjugation and transformation, AR genes can also be packaged into bacterial phage and then released into a new strain by infection during transduction. Transduction requires the microorganisms that can be transduced to contain receptors recognized by bacterial phage. The DNA that can be transferred in a single event depends on the size of phage, which can be as much as 100 kilobases[34].

Movable genetic elements can be found on self-transmissible plasmids, conjugative transposons, or integrons. Plasmids are extra-chromosomal DNA that is capable of replicating independently of the genome DNA. Self-transmissible plasmids usually contain *tra*-genes that initiate the complex process of conjugation or transduction. Conjugative plasmids are also found to facilitate the mobility of chromosomal genes, smaller non-transferable plasmids or antibiotic mediated integrons [37, 41]. Transposons are mobile genetic elements within the chromosomal DNA. Transposons can be divided into two groups: compound and complex, represented by IS257 and Tn21 respectively. The former group is made up of two identical insertion elements flanking a functional gene such as drug resistance genes. The movement of these transposons is induced by transposases encoded by the insertion elements. In the latter group, transposase is encoded together with functional gene in a transferable structure flanked by short inverted repeats. [42]. Many conjugative transposons can mobilize co-resident plasmids, and some can even mobilize unlinked integrated elements [43]. An integron is a gene capture system that can be found in plasmids, chromosomes, and transposons. Integrons are composed of a gene encoding a site-specific integrase and a recombination site for insertion of gene cassettes. Class 1 integrons are usually related to AR [42].

The rate of horizontal gene transfer can be affected by several factors. First of all, prolonged antibiotic use could select for novel gene variants or recombinants that have higher minimum inhibition concentration (MIC). One example is the mosaic recombinant of *tetW* and *tetO* isolated from *Megasphaera esldenii* from tetracycline treated swine [33]. Secondly, the use of antibiotics at sub-therapeutic level has been reported to accelerate horizontal

gene transfer. In a study, the sub-inhibitory concentration of tetracycline was found to enhance the transfer of resistance plasmids in *Staphylococcus aureus* by up to 1000 fold[44]. The similar stimulatory effect was also demonstrated in the transfer of Tet^r conjugative transposons Tn1545 and Tn916[45]. In addition, the co-selection of AR and heavy metal resistance was noticed four decades ago and has been extensively studied since then [46]. Finally, Stress could induce the mobility of transposons. Some antibiotics (mitomycin and ciprofloxacin) are found to induce the SOS response and higher efficiency of conjugative transfer of AR in *Vibrio* and *Escherichia*[47]. Last but not least, the rate of horizontal gene transfer is also affected by the size of the gene pool as well as the constitutions of donor and recipient.

1.3.4. Maintenance of Antibiotic Resistant Traits

The carriage of AR on plasmids or transposons is generally thought to impose additional metabolic cost on the host strain thus reducing bacterial fitness to the environment. Based on this concept, ART bacteria are expected to gradually disappear once the use of certain antibiotics is banned or restricted. The so called “easy-to-get, hard-to-lose phenomenon”, however, are observed in many cases contrary to our expectation [33]. In order to better understand this unexpected phenomenon, researchers started to investigate the internal mechanism of AR persistency. Studies indicated that the negative effects of AR genes may be eliminated by compensatory mutations or counteracted by the beneficial effects of AR determinants [48-50]. For example, *mdfA* and *tetL* would raise host’s adaption to elevated pH in the environment [51]. Apramycin resistance plasmids would confer fitness advantage

to the new host [52]. In these cases, ART isolates would not disappear but rather gradually replace antibiotic susceptible isolates in the absence of any antibiotic selective pressure.

Unlike resistance encoded on plasmids or transposons, there's little or no cost on resistant strains with mosaic resistance genes since no addition proteins need to be synthesized. Therefore the resistance phenotype can be considered selectively neutral in these cases, which may also accounts for the persistency of ARs after the removal of selection pressure.

It is also worth noting the impact of constant horizontal gene transfer between commensal and pathogenic microorganisms. The basic cellular biology, including the mechanisms of dissemination, transmission and maintenance of AR, differs little between commensal and pathogenic bacteria. Since commensal microorganisms can present in and on surfaces of a host harmlessly, commensal microorganisms would serve as a relatively stable reservoir of resistance gene. For example, high level of acquired resistance to the oldest antibiotics such as tetracycline, ampicillin can be detected in commensal bacteria even in remote area with minimal antibiotic exposure [53] or in animals that never treated with antibiotics such as organic pig[54].

1.4 Tetracycline

1.4.1 Introduction

Tetracyclines, the first broad-spectrum antibiotics, were discovered in 1945 and put into clinical use in 1948 by Duggar as chlortetracycline [55]. Because of its wide spectrum, low cost and few major side effects, tetracyclines remain the most widely prescribed antibiotic

class in the world. Attempts have been made to determine the annual consumption of tetracycline all over the world and the quantity of tetracycline used per year has been estimated as much as 5000 metric tons [6]. However, the heavy use of tetracycline in the past sixty years brings about severe AR problems.

1.4.6. Application of Tetracycline

Tetracyclines are effective against a broad spectrum of Gram-positive bacteria, Gram-negative bacteria, protozoan parasites and apply to a variety of noninfectious conditions. [6]. Tetracyclines inhibit bacterial protein synthesis by reversibly binding to the prokaryotic 30S ribosomal subunit and by blocking the interaction of the aminoacyl-tRNA with bacterial ribosome [8]. However, Tetracyclines interact weakly with the eukaryotic 80S ribosomal subunit, which accounts for the selective antimicrobial properties and limited side effects. Tetracyclines also inhibit mitochondrial protein biosynthesis by binding to 70S ribosomes in mitochondria, which may be related some of their anti-parasitic activity [8].

Up to date, at least ten members of the tetracycline family have been used in human medicine. Tigecycline, the latest derivative of the tetracyclins, entered the U.S market in 2005 [56]. All of them can be given orally, and four (oxytetracycline, limecycline, doxycycline and minocycline) can be given parenterally. oxycycline and minocycline are the most commonly prescribed tetracyclines for bacterial infections [57]

Even though the emergence of resistance led to its declining medicinal usage, tetracyclines remain first-line medicine for a variety of applications, including acne vulgaris, cholera, Lyme disease, and pneumonia; and alternative medicine for some protozoan diseases such

as malaria [58]; and part of a triple therapy for gastritis and peptic ulcer disease[8]. They are also important for provetion or treatment against infections caused by bacteria that could be used as biological weapons such as *Bacillus anthracis*, *Francisella tularensis*, and *Yersinia pestis* [57]. Besides, tetracyclines have a number of non-antibacterial effects, including anti-inflammation, immune-suppression, inhibition of lipase and collagenase activity, enhancement of gingival gibroblast cell attachment, and wound healing. These properties extend the usage of tetracyclines to a variety of noninfectious conditions, such as acne and rosacea [57].

In addition to human medicinal use, tetracyclines are also used as veterinary medicine for infections treatments or as growth promoters. Growth promoter is defined in the United States as a feed of less than 200g/ton to animals to improve their growth efficiency [59]. Tetracycline are also used to prevent and cure infections of fish, trees, and insects of commercial value [60]. The usage of tetracycline as growth promoters was first reported in 1949 and later approved by FDA in 1951[61]. This application of tetracycline was banned in Europe in the early 1970s (EC directive 70/524) due to the growing concern that sub-therapeutic application of tetracycline might contribute to the resistant isolates in humans. According to DANMAP annual report (http://www.vestinst.dk/), both human and animal consumption of antibiotics, including tetracycline in Scandinavia area decreased during 1989-1999[57]. In the United States, however, tetracycline and many other clinical important antibiotics are still used extensively in livestock production for nontherapeutic purposes[8].

1.4.3. Tetracycline Resistance

The majority of commensal and pathogenic bacteria were susceptible to tetracyclines before mid 1950s [61]. The first tetracycline resistant (Tet^r) bacteria, *Shigella dysenteriae*, were isolated in 1953 shortly after the therapeutic use of tetracycline. Two years later, the first multidrug-resistant *Shigella* was identified resistant to tetracycline, streptomycin, and chloramphenicol [62]. Since then, Tet^r isolates have been found in most genera although the percentage of Tet^r bacteria varies among different species and sources. For instance, increased tetracycline resistance was found in *Enterobacteriaceae* and *Staphylococcus*, *Streptococcus* and *Bacteroides* in 1970s and in *Neisseria gonorrhoeae* in 1980s [57]. Many Gram-positive bacteria such as *Streptomyces* were known for long to carry multiple Tet^r genes of the same of different mechanism of resistance [63, 64]. Recent studies of *Escherichia coli* and *Salmonella* from food, animals and human suggested that multiple Tet^r genes can be present in >10% of the Tet^r Gram-negative population [65-67].

Currently, 38 different tetracycline (tet) and oxytetracycline (otr) resistance genes have been characterized and the resistance mechanisms are divided into four categories: efflux pumps, ribosomal protection proteins, inactivating enzyme and unknown (Table 1.2.)[61]. As shown in table 1.2., twenty three Tet^r genes code for efflux pumps, a super family of energy-dependent, membrane-related proteins that export tetracycline out of the cell [68]. These efflux pumps reduce the intracellular concentration of tetracycline and thus protect bacterial ribosomes from tetracycline [60]. Nineteen genes of this group have been found in Gram-negative bacteria, whereas four (*tetK* and *tetL*, *tet33*

and *tet38*) are found first in Gram-positive isolates and then in some Gram-negative species[60]. Among all efflux genes, *tetB* has the widest host spectrum in Gram-negative bacteria and some Gram-positive bacteria including *Nerssinia gonorrhoeae*, *Campylobacter, Yersinia,* and *Pseudomonas* [61].

Efflux	Ribosomal protection	Enzymatic (3)	Unknown[a]
$n = 23$	$n = 11$	$n = 3$	$n = 1$
$n = 18$ *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E) *tet*(G), *tet*(H), *tet*(J), *tet*(V), *tet*(Y) *tet*(Z), *tet*(30), *tet*(31), *tet*(K), *tet*(L), *tetA*(P) *otr*(B), *tcr3*	$n = 9$ *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(Q), *tet*(T), *otr*(A), *tetB*(P)[b], *tet*	$n = 1$ *tet*(X)	$n = 1$ *tet*(U)
$n = 5$	$n = 2$	$n = 2$	
tet(33), *tet*(35)[d], *tet*(38), *tet*(39) *otr*(C)	*tet*(32), *tet*(36)	*tet*(34), *tet*(37)[c]	

Table 1.2. Mechanism of resistance for characterized Tet^r and Otr^r genes[61]

[a] *tet*(U) has been sequenced but does not appear to be related to either efflux or ribosomal protection proteins.
[b] *tetB*(P) is not found alone and *tetA*(P) and *tetB*(P) are counted as one operon.
[c] *tet*(X) and *tet*(37) are unrelated but both are NADP-requiring oxidoreductases: *tet*(34) similar to the xanthine–guanine phosphoribosyl transferase genes of *V. cholerae.*
[d] Not related to other *tet* efflux genes.
*Underlined are genes used in my study.

Eleven genes code for ribosomal protection proteins, a group of 72.5kDa cytoplasmic ribosomal protection protein (RPP) that has GTPase activity. The study of *tetM* may provide typical mode for the proteins in this group. When tetracycline binds to the ribosome, *tetM* protein binds to GTP and the *tetM*-GTP complex binds and pulls the tetracycline off the ribosome. This reaction cleaves GTP from *tetM*-GTP complex and creates *tetM*-GDP, which is released from ribosome [69].Genes in this group have been found in Gram-positive bacteria, anaerobes and non-enteric Gram-negative bacteria. Sometimes they are found on the same transferable genetic elements with other AR genes, such as *erm* genes, which code

for resistance to macrolides, lincosamides, and streptogramin[31]. This may explain the maintenance of multi-resistance, such as tetracycline and chloramphenicol even after the latter has been abandoned for a long time. The *tetM* gene is the most widely spread Tet^r gene in this group [61].

Only three genes (*tetX, tet34, tet37*) code for enzymatic inactivation protein, a cytoplasmic protein of approximately 44kDa that chemically modifies tetracycline in the presence of both oxygen and NAPD [70]. However, limited work has been done in terms of the evolution and distribution of these genes. Finally, the *tetU* codes for a 11.8kDa protein that confers low-level tetracycline resistance. However, the resistance mechanism of *tetU* is still under investigation [61].

1.4.4. Prevalence of Tetracycline Resistance in Bacteria

The distribution of Tet^r gene is highly variable even for genes within the same resistance mechanism group. The total list of the identified Tet^r gene can be found on website http://www.faculty.washington.edu/marilynr, updated every two years. Since two efflux pump gene, *tetK*, *tetL* and two ribosome protection gene, *tetM*, *tetS* are chosen in our study, the information of these four Tet^r genes has been summarized below.

Among the entire Tet^r genes, the *tetM* gene has the widest host range, which may be associated with the prevalence of *tetM* conjugative transposons. Chopra summarized in 2001 that *tetM* has been found in 25 Gram-positive genera: *Abiotrophia, Bacterionema, Gemella, Mycoplasma, Ureaplasma, Nocarida, Actinomyces, Aerococcus, Bifidobacterium, Garadnerella, Corynebacterium, Eubacterium, Bacillus, Listeria, Staphylococcus, Clostridium,*

Peptostreptococcus, Enterococcus, Streptococcus, Lactobacillus, Lactococcus, Mycobacterium, Streptomyces, Vibrio, and *Erysipelothrix*; and 22 Gram-Negative genera: *Eikerella, Kingella, Neisseria, Bacteroides, Fusobacterium, Haemophilus, Veillonella, Pasteurella, Escherichia, Pseudomonas, Neisseria, Prevotella, Klebsiella, Acinetobacter, Afipia, Enterobacter, Mitsuokella, Porphyromonas, Ralstonia, Photobacterium, Selenomonas,* and *Megasphaera* [60].

Different from *tetM*, the original transposons associated with *tetS* have restricted host range. However, recently a *tetS* gene was identified to replace the *tetM* gene in Tn916 element [71]. This new functional conjugative transposon of *tetS* may accelerate the spread of *tetS* in the future. Until 2001, *tetS* has been found in 4 Gram-positive genera: *Lactococcus, Lactobacillus, Listeria,* and *Enterococcus*; and only one Gram-negative genera*: Veillonella* [60].

According to Chopra, *tetK* has been found in 12 Gram-positive genera: *Staphylococcus, Nocarida, Bacillus, Listeria, Clostridium, Peptostreptococcus, Enterococcus, Mycobacterium, Streptomyces, Lactobacillus, Norcardia,* and *Streptomycies*; and one Gram-negative genera: *Eubacterium* [60]. The *tetK* gene is most commonly found in *Staphylococcus aureus* and in other *Staphylococcus* species except *Staphylococcus intermedius* [72].

Compared with *tetK*, *tetL* has more Gram-negative host. Uptill 2001, *tetL* has been found in 11 Gram-positive genera: *Acinomyces, Bacillus, Listeria, Staphylococcus, Clostridium, Peptostreptococcus, Enterococcus, Streptococcus, Mycobacterium, Streptomyces,* and

Norcardia; and 4 Gram-negative genera: *Actinobacillus, Veillonella, Salmonella,* and *Morganella* [60].

1.4.5. Development of Tetracycline Resistance

Theoretically, spontaneous mutations that alter the permeability of membrane porin, activity of innate efflux pump or the affinity of tetracycline to the 30S ribosome would give rise to tetracycline resistance. However, only a few examples have been found that bacteria develop Tet^r phenotype mutations [73-75]. Compared to mutations, clinically important tetracycline resistance usually results from acquired resistance genes. As early as 1976, Levy reported the transfer of Tet^r genes between chicken *Escherichia Coli* strains, from chicken to chicken and from chicken to humans [76].

In general, tetracycline efflux genes in Gram-negative bacteria are usually associated with transposons inserted into a variety of plasmids or integrons, whereas in Gram-positive bacteria are associated with small plasmids [77]. For instance, *tetK* and *tetL* genes are usually found on small transmissible plasmids ranging in size range from 4.4kb to 5.3kb. Specifically, *in vitro* transmission of the *tetK* plasmid pT181 *Bacillus*[78] and pLS55 in starter culture strain *Lactobacillus sakei* was mediated by the IS257 element [79]. In another research, *tetL* plasmid pCCK3259 in *Mannheimia* and *pasteurella* was demonstrated the function of lateral gene transfer [80].

Different from the efflux genes, the ribosomal protection genes are usually found on conjugative or non-conjugative transposons inserted into the chromosomes [81, 82], or sometimes on plasmids [83, 84]. For example, *tetM* related Tn916-Tn1545 are the most

common conjugative transposons found in both Gram-positive and Gram negative hosts [85]. The Tn916S transposon found in an oral *Streptococcus intermedius* is the first report of transposon associated with *tetS* gene transfer [71]. In addition, transposons often carry multiple AR genes, which may explain the selection of multidrug-resistant bacteria with tetracycline [76]. For example, the Tn2009 and Tn2010 transposons have a macrolide rRNA methylase coded by *ermB* gene inserted downstream of *tetM* gene. These elements link the macrolide resistance efflux genes, *mefA* and *msrD* with *tetM*, which may account for the multi-resistance of these genes [86].

1.5 Antibiotic Resistance in Food

1.5.3. Food Microbiota

Among all the microorganisms in food, pathogenic bacteria receive the most attention because of their direct destructive effect to public health. It has been reported that in the U.S., about 76 million cases of foodborne diseases occur each year, resulting in 325,000 hospitalizations and 5000 deaths [87]. Since most foodborne diseases are sporadic and not reported, this number of incidence is underestimated. The majority of foodborne diseases are caused by *Salmonella, Campylobacter, Listeria, Vibrio* and enterics especially *Escherichia coli* O157: H7 [87].

Nevertheless, pathogens only represent for a small portion of the food microbiota. Most foodborne microorganisms usually do not cause human diseases and are referred to as commensal bacteria. While some commensal bacteria cause food spoilage, which is one of the greatest burdens to the food industry; others are of great economic value by playing an

integral role in food fermentation (Lactic acid bacteria), food preservation (*Lactococci.*) or functional food production (probiotics). For example, the total count of commensal bacteria could be high in fermented food [88]. Though commensal bacteria are generally considered safe, some of them, such as *Pseudomonas, Enterococcus*, and *Staphylococcus*, are opportunistic pathogens and can cause disease to immuno-compromised individuals.

Compared to raw food materials such as meat, sea food, eggs, etc., ready-to-consume (RTC) products including dairy product, restaurant food are usually ingested directly without further processing. Therefore, the ART microorganisms in RTC products reflect the actual risk associate with food intake. Bacterial loads in RTC products depend on different processing methods and storage conditions. Although RTC deli and restaurant foods are supposed to be safe, pathogenic outbreaks associated with these foods, especially the minimal processed vegetables such as salad greens, occasionally occur [89-91]. In addition, previous studies also found that commensal bacteria were prevalent in selected RTE food samples[92]. Study showed that cleaning and food handling implements [93], houseflies[94], and undercooked food [95] all contributed to the contamination problem of RTC food. Therefore, good hygiene practice and proper food handling processes in the kitchen are the effective ways to reduce cross-contaminations of RTC products.

1.5.4. Prevalence of AR in RTC Food

The resistances in raw food material at retail level more or less supports that resistance could be transferred to humans through the food supply, although the extent to which resistant bacteria could survive under antibacterial treatment such as cooking is not fully

clear. The ART bacteria detected from RTC foods, however, would enter the intestinal tract of consumers and exchange AR genes with intestinal colonized microbiota [96]. Therefore, the prevalence of ART bacteria in RTC food may represent more directly the real exposure of humans to ART bacteria through daily consumption of conventional food. To date, ART bacteria has been detected in many different categories of RTC foods.

Meat and seafood: In a study of imported pig and pork product in Denmark, antimicrobial resistance was observed in *Samonella* Rissen isolates recovered from cooked pork originating from Thailand [97]. In another study, *Listeria monocytogenes* were detected in 2.97% of more than three thousand RTC food samples (deli style sandwich, smoked turkey, beef, ham) in Florida, and 78% of the *Listeira* isolates exhibited multidrug resistance to ciprofloxacin, tetracycline and etc. [98]. In Marshall's study of imported RTC shrimp originating four different countries, numerous resistant isolates representing 162 bacterial species were recovered during screening of resistance to 10 antibiotics including tetracycline, vancomycin, erythromycin, etc. [99]

Vegetable: In 2000, the outbreak of 361 cases of *Salmonella Typhimuruim* DT104, resistant to multiple antibiotics was associated with consumption of lettuce [100]. In an assessment of fresh salad vegetables in Canada, the existence of resistance genes and self-transmissible plasmids were common, mainly in oxidase-positive, Gram-negative isolates such as *Pseudomonas*, *Sphingobacterium*, and *Acinetobacter* [101]. In some other experiments, streptogramin resistance was found in 17% *Enterococcus faecium* from deli salads by Multiplex PCR [102, 103]. 43% of the 92 Gram-negative *Enterobacteria* isolates from sprouts were resistant to tetraycycline [104]. In the study by Lee, 27% of the RTC vegetable samples

from Korea were positive for *Yersinia* strains are highly resistant to ampicillin, cephalothin, and carbenicillin[105]. Considering vegetables used in salad recipes usually undertake minimum process before consumption, these ART bacteria from vegetable product likely will be transmitted to human GI track via food intake, thus contributing to the development of AR in human intestine.

Fermented food: Fermentation food is of great concern because the growth of bacteria is actually encouraged in the production of such food and thus the total bacteria count is relatively high in the final product. The presence of ART bacteria in starter culture may lead to a resistance problem in food product. Previous study in our lab showed that 20 out of 23 cheese samples contained Tet^r determinants, although no detectable ART microbes were found in processed cheese and yogurt samples in limited culture conditions[92]. In fact, the *Bifidobacterium lactis* supplemented to yogurt products before 2006 were found containing Tet^r determinants[106]. In addition, a vast prevalence of Tetracycline resistance was found in *Enterococcus* strains from cheese samples, with the most common determinant being *tetL* (94% of the strains), followed by *tetM* (63%) and *tetK*(56%)[107]. Another study showed that all of the 14 Lactic acid bacteria isolated from Armada cheese were resistant to one or more antibiotics tested [108]. Tetracycline resistant was also found in *Lactobacilli* in RTC pre-packed dry sausage[109]. Consider the abundance of lactic acid bacteria in fermented food, the resistance problem related with fermented food is quite startling.

Others: Preliminary study in our lab has revealed the existence of AR in shrimp, pork chop, deli turkey, mushroom and spinach samples from retail stores [92]. In another study, the influx of Tet^r genes to the human digestive tract was estimated conservatively to be $3.8x10^5$

CFU per meal through *Enterococci* in chicken salad alone [110]. About 39% of *Bacillus cereus* isolated from honey exhibited resistance to tetracycline by disk diffusion method, 77% of which contain one of the resistant determinants (*tetK*, *tetL*, *tetM*, *tetO, tetW, otrA and otrB*) according to PCR results [111]. *Klebsiella pneumonia* contamination was detected in 32% of the street food in Malaysia, and 100% of the pathogenic isolates displayed multidrug resistant to ampicillin, erythromycin, and rifampicin [112].

1.5.5. Importance of Antibiotic Resistance in Food

AR in food borne microorganisms becomes a threat to public health for two reasons. First, AR in foodborne pathogens or opportunitistic bacteria diminishes the efficiency of therapeutic treatment to their corresponding diseases in human. Since early 1990s there has been a dramatic increase in outbreaks that can be traced to ART pathogens such as *Salmonella enteric* [113], *Salmonella Typhimurium* [91], *Camylobacter*[95], and to *Escherichia coli* O157: H7[114]. What's more, prolonged outbreaks are often related to multi drug resistant strains of *Salmonlla Typhimurium* [91], *Shigella* [19], *Acinetobacter* [115], *Streptococcus* [18], and *Staphylococcus*[90]. Second, AR determinants in commensal bacteria can be transmitted to pathogens through horizontal gene transfer when foods pass through human gastrointestinal track. Since the pathogens only account for a small amount of food microbiota, the contribution of commensal bacteria to AR dissemination should be emphasized.

There's no doubt the medicinal and additive use of antibiotics in farm animals[116], aqua-culture[117] and even plants [118] should take credit for the increase of ART bacteria in raw

food material [119, 120]. However, food products such as farm animals or crops could also acquire resistance gene through horizontal gene transfer from various sources such as animal feces, irrigation water or soil [121]. In addition, the lateral transmission of AR genes among foodborne microorganism communities can be accelerated by antibiotic residue in food [122] or by sub-lethal stress imposed by certain food processes [123]. The complex transmission routes of AR gene to humans through food chain are summarized in figure 1.3.

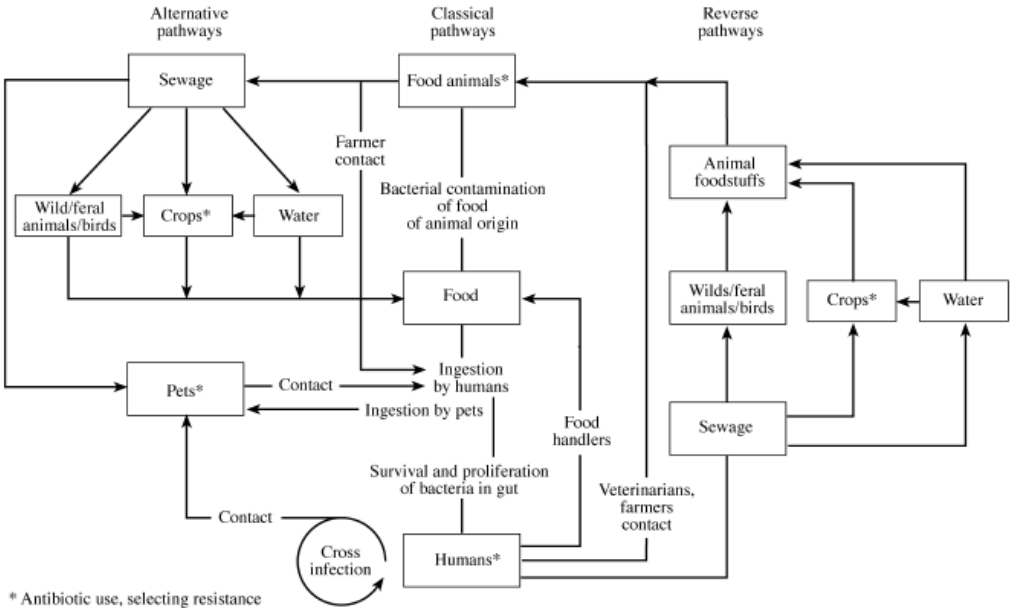

Figure 1.3. Some routes of transmission of antibiotic resistance between food and humans [124]

1.5.6. Horizontal Gene Transfer and Human Health

Horizontal elements such as transposons, and conjugative plasmids were discovered in the stains recovered from food and the transmission mechanisms were studied as mentioned

before. Natural transformation studies show that the AR gene can be transferred intra-genera or between different species that were isolated from food samples *in vitro* by filter mating[125], or in simulated food environments such as broth, milk, ground beef at 25°C and 37°C [126]. Previous study in our lab showed that the *ermC* gene from a salad *Staphylococcus epidermidis* isolates can be transferred to *Streptococcus mutans* UA159 by natural transformation [92]. Lateral gene transfer of AR genes were also demonstrated in numerically predominant groups of human intestinal bacteria, such as *Enterococcus* [110], *Lactococcus* [83] and *Bacteroides* [127]. Take Tet^r genes for example, the widespread distribution of *tetM* in many bacteria genera, including *Lactococcus* is often linked to the presence of conjugative transposons of Tn916-1545 family [128]. Mating experiments have demonstrated the ability of some *Enterococcus faecalis*, *Enterococcus faecium* strains to transfer Tet^r determinants by conjugation [107, 129].

The success of *in vitro* transformation of AR determinants suggested that *in vivo* lateral transformation could possibly happen as well, especially regarding the fairly high bacterial counts in human intestines. Nevertheless, the frequency of horizontal gene transfer is low (usually less than 10^{-6}) even under optimized lab settings. In addition, enterics from animal source may not survive human intestine because of the host preferences. In fact, only a few *in vivo* experiments were conducted to analyze whether similar transfer is a common scene in human gut. Recently ART plasmids were transferred from *Lactobacillus plantarum* to *Enterococcus faecalis* using gnotobiotic rats as model [130]. Although there's little evidence that human infections can be caused by certain resistant strains that is exclusively animal origin, the prevalence of Tet^r microorganisms in oral samples [131] as well in gut microbiota

of exclusively breast-fed infants who have no former exposure to tetracyclines [132, 133] suggested that the horizontal transfer of AR genes could be facilitated through food chain.

1.5.7. Research Trend and Knowledge Gap

AR in foods has been and probably will always be one of the hot topics in the battle against ART pathogens. The evaluation of previous research trends and the efforts to fill the current knowledge gaps would not only contribute to a more comprehensive picture but also provide potential solutions to the exacerbating AR problem.

First of all, most antibiotic screening experiments chose fecal samplings from farm animals or other raw food materials especially various meat and seafood samples. However, a significant part of these ART bacteria are usually destroyed by adequate subsequent cooking. A better understanding of the influx of AR through food chain would be obtained by studying RTC food. Second, the focus on ART foodborne bacteria has been concentrated on several “star” organisms, such as pathogens or opportunistic pathogens. However, pathogens only account for a scant portion of the bacteria involved in the AR dissemination. Thus more studies should be applied to a wide range of commensal bacteria predominant in food chain. At the meantime, lateral transmission studies of AR from food to human gut should also be considered. Third, once standard enrichment technique (ISO1981) is used in enumerating ART bacteria, the result won’t be able to reflect true magnitude of ART bacteria in the foods. A more precise estimation of resistance prevalence could be obtained by using total plate count without enrichment step and other new methods. Fourth, the

advantages and limitations of different detection methods need to be analyzed before any conclusions are made.

Convention PCR was first developed by Roustam Aminov to identify Tet^r gene, although contamination has always been a practical problem that relates with the validity of PCR result. Nowadays, PCR has been adopted by many scientists as a fast, accurate, and sensitive detection of AR genes in pathogenic as well as commensal bacteria [134-136]. However, mosaic genes [137] cannot be distinguished from non-mosaic ones by traditional PCR, which only cover a small region of the gene. Sequencing the entire Tet^r gene might be necessary to solve this problem. Real-time PCR could be used in the quantitative study of a particular gene in a microbial population. Meanwhile, microarray would be a useful tool in an epidemic study of resistance to multiple antibiotics or drug screening for new antibiotics. Generally speaking, the combination of different research methods is usually adopted in the study of AR.

Besides the entire knowledge gaps in the previous studies mentioned above, several critical questions to AR have not been fully answered and need to be considered in the future. First, to what extent the food chain contributes to the emergence of AR? And to what extent the increase of AR are ascribed to antibiotic use in agriculture rather than in clinics? Second, does significant amount of horizontal gene transfer take place in the colon? Even though intra or cross genus antibiotic gene transfer has been achieved under lab settings or even in an animal model, the role played by lateral transformation in the development of AR in human gut is not clearly identified. Third, would present methods provide convincing evidence of horizontal transfer? To be more specific, would 80% of the DNA be identical

from hybridization experiment be sufficient to confirm the existence of gene transfer rather than through mutations [138]? Finally, what is the practical approach to eliminate or reduce current AR based on the study of horizontal gene transfer of AR?

CHAPTER 2

CHARACTERIZATION OF ANTIBIOTIC RESISTANT BACTERIA FROM SELECTED DELI AND RESTAURANT FOODS

2.1. Introduction

The rapid emergence and spread of ART pathogens is becoming a major public health concern due to the increasing financial costs and failure in therapeutic treatments towards severe infections. Besides the selection and enrichment of ART pathogens due to mutations or lateral exchange of AR gene in clinical settings, the impact of other factors on the dissemination and persistence of AR genes have been focused in recent years [33]. The prevalence of ART commensal microbes in various natural and human ecosystems [96, 131, 132, 138-140] suggest that commensal microorganisms might have played a much more important role in the dissemination of AR genes than previously thought. The recent discovery of the large pool of ART commensal bacteria in many retail food items suggest that general public is constantly exposed to AR through daily food consumption, and that the food chain may serve as an crucial avenue connecting the AR between the environment and human beings[5, 92, 141-143]. This interpretation is in fact in agreement with the

recent findings that a large percentage of the microbial in the digestive track of human subjects are already resistant to selected antibiotics, even without prior clinical exposure [132, 133, 144]

In the past decade, studies on AR in food have mainly focused on food-borne pathogens such as *Campylobacter*[141], *Salmonella*[145], *Clostridium*[146], *Listeria*[147], etc., or opportunistic pathogens such as *Enterococcus*[103, 107]. AR in commensal and beneficial bacteria, which account for majority of the microbial population associated with foods, regarding both the magnitude of the AR and the impact on horizontal AR gene transmission to pathogens, was essentially missed in the scope. Moreover, the adoption of the standard laboratory enrichment procedure (ISO1981) in some studies made it impossible to evaluate the magnitude, and thus the real risk of the AR associated to these food products. In addition, most studies aimed at AR in raw food materials such as meat and poultry, with very few mentioned ready-to-consume (RTC) products. Since these raw food materials are susceptible for further processing treatments, the ART bacteria associated are not equivalent to those introduced into humans through food consumption. To address the correlation between AR in foods and AR in humans, further studies are needed to fill in the knowledge gaps regarding the magnitude of AR intake from conventional food consumption, molecular characteristics of the foodborne ART isolates, and their potential involvement in HGT in vitro and in vivo.

2.2. Objectives

The objectives of this study were to investigate the prevalence of ART bacteria, using tetracycline as an example, in selected RTC foods collected from grocery stores and restaurants, and to assess the molecular characteristics of the foodborne ART isolates as related to HGT. The study was conducted by 1) analyzing the prevalence of ART bacteria in selected RTC foods from restaurants and grocery stores using conventional culture and molecular biology methods; 2) characterizing the ART isolates and demonstrating the horizontal transfer of the AR determinants in laboratory settings. Results from this study will enhance the understanding of the impact of food chain on the dissemination of AR to the hosts. The knowledge is essential for the development of strategies to minimize AR in both the hosts and the environment.

2.3. Materials and Methods

2.3.1. Preparation of Food Sample and Enumeration of ART Bacteria

Food samples were obtained from salad bars in popular grocery stores and restaurants in the Columbus, OH area. All samples were analyzed within 48 hrs after purchasing following the procedures previously established in our laboratory [92] with minor modification. Five grams of each sample was aseptically removed to disposable plastic bags containing 10ml of sterile 0.1% peptone water. Bagged samples were homogenized for 5 min and the rinsing liquids were serially diluted and plated on nonselective Brain Heart Infusion agar (BHI, Bacto™ Brain and Heart Infusion, Becton, Dickinson and Company) for total aerobic mesophilic bacteria count and on BHI plates containing 16μg/mL of Tetracycline (Fisher

Biotech, Fair Lawn, NJ) for Tet^r bacteria count. All plates contained 100µg/mL of cycloheximide (Fisher Biotech) to inhibit the growth of molds and yeasts. The average bacterial counts of duplicated plates were collected after aerobic incubation at 37^{o}C for 48 hours.

2.3.2. Total DNA Extraction from Isolates for PCR

This procedure was modified from the method of Yu and Morrison [148] to extract total DNA for PCR amplification from isolates that grew on selective media. The whole colony was picked from plate and removed to a sterile 2ml screw cap tube containing 0.3g of 0.1mm zirconia beads and 120µl distilled water. The tube was homogenized for 3 min at maximum speed on a mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA), followed by boiling for 15-20 min and ice-bathing for 15 min. The DNA extract was stored at -20°C before application.

2.3.3. Detection of AR Genes and Identification of ART Isolates

Conventional PCR was used to detect the presence of Tet^r genes (*tetM, tetL, tetK, tetS*) in the Tet^r isolates and to amplify a 1.5 kb 16S rRNA gene fragment from isolates containing the Tet^r genes. The Tet^r gene carriers were further identified by analyzing the sequence of the corresponding 16S rRNA gene fragment. The PCR amplification reaction was performed in a total volume of 50µl containing 10ng DNA and the following mix components: 1×reaction buffer, 0.4U *Taq* polymerase (New England Biolabs), 0.2mM deoxynucleoside triphosphoate, and 1.5mM $MgCl_2$. Initial denaturation

was performed at 94°C for 5min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 50 s, and a final extension at 68°C for 10 min. The PCR was carried out in a thermo cycler (MyCycler, Bio-Rad). Tetracycline sensitive strain *Streptococcus mutans* UA159 was used as negative control. Strains proved to contain *tetM*, *tetL*, *tetS*, *tetK* in previous studies in the lab were used as positive controls. Primers were designed in our lab following procedures described previously[92, 144] . The information of primers used in the study was summarized in table 2.1.

Primer Name	Sequence (5'-3')	Size (bp)	Tm (°C)
tetM-FP	GTGGACAAAGGTACAACGAG		59.6
tetM-RP	CGGTAAAGTTCGTCACACAC	406	60.6
*tetM*FR600	CGAACAAGAGGAAAGCATAAG		60.4
*tetM*RP1573	CCAATACAATAGGAGCAAGC	974	58.8
*tetS*F160	GAACGCCAGAGAGGTATT		57.8
*tetS*R1209	TACCTCCATTTGGACCTCAC	1050	61.3
*tetK*F65	AGGATAGCCATGGCTACAAG		60.6
*tetK*R1045	ACAAGGAGTAGGATCTGCTG	981	58.4
*tetL*F431	TTGGATCGATAGTAGCC		63.0
*tetL*R1340	GTAACCAGCCAACTAATGAC	910	60.2
16S-up	AGAGTTTGATCCTGGCTCCG		
16S-down	TACCTTGTTACGACTT	1498	

Table 2.1. Sequences, amplicon size and melting temperature of gene specific primers used for PCR screening and identification of Tet^r isolates.

Amplicons of 16S rRNA, *tetM, tetK, tetL, tetS* were analyzed on 1% agarose (BioScientific, inc.) gels, using a 1kb Plus DNA Ladder (Invitrogene™) for molecular weight standards. Gels were run at 9 V/cm in 1× TAE buffer (0.04M Tris, 0.02M acetic acid, 1mM EDTA, pH 8.0) and stained with 0.5 μg/mL ethidium bromide. Selected positive amplicons were purified using

QIAquick® kit (Qiagen Inc.Valencia, CA) and the DNA sequence of the purified fragment were then determined by a DNA analyzer (ABI PRISM® 3700, Applied Biosystems, Foster City, CA) at the Plant Genome Sequence Facility, The Ohio State University. The ART isolates were identified by comparing the analyzed DNA sequence with NCBI database.

2.3.4. Minimum Inhibition Concentration (MIC) Values of ART Isolates

For each food samples that carried ART bacteria, five to ten representative isolates with different Tet^r determinant(s) were selected and detected for the tetracycline MIC values according to NCCLS standard guidelines with small modification. The isolates were first grown in BHI broth at 37°C until the cell density reached 0.5 at OD_{530} by spectrophotometer (MiltonRoy, Spectronic GENESYS 5). The original culture was diluted to 1:2000 with BHI broth medium before application. A 0.1ml of the diluted culture was dispensed into each well of the 96 well plate containing 0.1ml of the various 2x concentrations of the antibiotics to be tested. The final Tetracycline concentrations in the wells tested were 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25µg/mL. Positive control wells contain growth media without antibiotics and were inoculated with working suspension of specific isolates. Negative control wells contain 16µg/mL of Tetracycline and were inoculated with Tetracycline sensitive strain *Streptococcus mutans* UA 159. The 96 well plates were incubated at 37°C for 24h. The MIC results were reported as the minimum concentration of the antibiotic that inhibited visible growth, which is indicated by increased turbidity or deposition of cells at the bottom of the wells.

2.3.5. Stability Assessment of the Antibiotic Resistance Determinants

ART isolates were consecutively inoculated 1:100 into new BHI broth media without tetracycline every 8 hours and were incubated at 37°C. After being transformed for about 400 generations (twice a day for 30 days), cultures were diluted and plated on BHI plate. One hundred progenies were randomly chosen from the BHI plate and each isolate were spotted on both nonselective and selective media to determine the stability of the AR determinants in the ART isolates at the absence of the antibiotic selective pressure.

2.3.6. Southern Hybridization

The plasmid of ART isolates were extracted following the method of Anderson and Mckay [149], and the Southern blotting was performed as described by Wang [92].The Tetracycline-susceptible *Lactococcus Lactis* 2301 was used as a negative control. Supercoiled DNA Ladder (Invitrogen™) was used to measure the size of the plasmids.

The hybridization probes were generated by PCR amplification of strains containing different Tet^r genes using primers described before. The PCR products were resolved by electrophoresis at 9 V/cm for 20 min on 1% agarose gel and strained with ethidium bromide. The probes were labeled and detected using the DIG DNA Labeling and Detection Kit (Roche™).

2.3.7. Electroporation and Natural Transformation

Electroporation: The protocol previously described by Luchansky et al [150] was followed with slight modification. A Gene Pulser ® (Bio-Rad) was used for all electroporation

experiments in this study. *Enterococcus faecalis* OG1RF (a plasmid cured derivative, resistant to rifampicin and fusidic acid and susceptible to tetracycline) was used as recipient strains. An overnight culture of recipient cells was prepared by inoculating 2ml of M17 medium using a single colony from a fresh M17 plate containing 25µg/mL of rifampicin. The overnight culture was diluted 1/100 into 10ml fresh SGM17 (M17 medium supplemented with 0.5M sucrose and 2%-3% glycine) and incubated for 21-24h at 37^{o}C until reached an optical density of 0.6 at OD_{600}. The cells were harvested by centrifugation, washed twice in electroporation buffer (0.5M sucrose and 10% glycerol), and then resuspended in electroporation buffer at 1/20 of the original culture volume divided into 40µl aliquots and frozen at -70^{o}C until application. Plasmid DNA (0.15 to 1 µg in a volume of less than 5µl) was added to 40 µl of thawed competency cells (about 10^9 CFU/mL) and the mixture was placed in sterile electroporation cuvettes (0.1 cm inter-electrode gap). Following the application of a high-voltage electric pulse (2.5kV peak voltage, 25µF capacitance, and 200 Ω resistance), the DNA-cell mixture was mixed with 0.98ml of ice-cold SGM17MC (SGM17 medium supplemented with 20mM $MgCl_2$ and 2mM $CaCl_2$) and allowed to incubate on ice for an additional 5 min before being plated onto selective media. Aliquots of 0.1 ml from the controls and samples were plated directly from cuvettes with or without prior dilution. The total viable recipient cell counts were determined by plating negative control on SR plates(10g tryptone, 5g yeast extract, 200g sucrose, 10 glucose, 25g gelatin, and 15g agar per liter and 2.5mM MgCl2, 2.5mM CaCl2, pH 6.8). Transformants were determined by plating the undiluted samples and positive control on selective SR plates containing appropriate antibiotics (25µg/mL of rifampicin, 16µg/mL tetracycline).

Natural transformation: Natural transformation was conducted following previously described procedure with slight modification [151]. The overnight culture of recipient strain *Streptococcus mutans* UA159 was incubated 1:40 in BHI broth at 37°C for 2-4h until the OD value reached 0.1-0.3 and then 1µg of plasmid DNA/ml was added. The mixtures were incubated at 37°C for an additional 2h. Then the mixture were diluted and plated on selective media.

In both natural transformation and electroporation experiments, transformants were counted after incubation at 37°C for 48 h and confirmed by conventional PCR using corresponding Tet^r gene specific primer sets and by tetracycline MIC. Southern hybridization was applied to representative transformants to determine the location of Tet^r. Stability analyze were used to determine the persistency of transferable Tet^r determinants in the new host. Transferable Tet^r plasmid that previously identified in our lab was used as positive control. Recipient cell without plasmid DNA was used as negative control.

2.3.8. Nucleotide Sequence Accession Numbers

The nucleotide sequences of the 16S rRNA genes of all the Tet^r isolates in this study were deposited in the Genbank database under accession No., respectively.

2.4. Results

2.4.1. Prevalence of ART Bacteria in Food Samples

A total of 26 ready-to-consume food samples (4 cheese, 2 pickles, 4 meat, 4 seafood, 6 vegetables and 6 others) were collected from local salad bars in local grocery stores and restaurants. Total bacterial counts and Tet^r bacteria counts were examined on BHI plate and BHI+tet plate respectively (Fig. 2.1a and Fig. 2.1b).

It was found that the total counts varied among salad samples, with 46% of the samples ranging from 10^5 CFU/g to 10^9CFU/g. Among all samples, spicy shrimp, muscles, and spicy season noodle had relatively high bacterial loads (>10^8CFU/g), whereas the total count of boiled shrimp, three-bean salad, and feta cheese were below detection limit. ART bacteria were found to be widely distributed in 20 samples (78%) (Fig 2.1a and Fig2.1b), except that in boiled shrimp, three beans salad, feta cheese, coleslaw and all pickle samples, ART bacterial counts were below detection limit. In most food samples (boiled egg, cereal salad, macaroni salad, blue cheese I, blue cheese II, vegetable salad III, chicken salad II, chicken salad III, spicy shrimp, muscles, tuna salad II, mushroom salad, red cabbage salad, vege salad I) with a detectable presence of ART bacteria, ART bacteria counts were more than 0.1% in total bacterial population. It is noticeable that tetracycline resistant (Tet^r) bacteria counts of no less than 10^3 CFU/g were detected in seven samples, among which mushroom salad, spicy shrimp, and spicy season noodle carried Tet^r counts above 10^4 CFU/g. These results suggested that consumers can be easily exposed to millions of ART bacteria via the daily intake of deli and restaurant food.

2.4.2. Detection of AR Genes and Identification of Isolates

A total of 740 representative Tet^r isolates from 12 food samples were recovered and examined for the presence of *tetM, tetL*, *tetS*, and *tetK* gene by conventional PCR (Fig.2.2a,b,c,d). It was found that 15.4% (114 isolates) of the selected Tet^r isolates contained one or more Tet^r genes, with *tetM* as the most predominant gene (in 57.0% of *tet* carriers), followed by *tetL* (37.8%), *tetS* (9.6%) and *tetK* (3.0%). Twenty seven isolates (24%) were found to carry more than one Tet^r genes. Representative Tet^r gene carriers were

further identified by 16S rRNA gene sequence analysis (Table 2.3). It was found that *Enterococcus* is the most common genus among the Tet^r gene carriers in RTC foods, as they were detected in red bean with rice, vegetable salad, Italian meat ball, chicken salad, muscles, blue cheese, spicy season noodle, and mushroom salad samples. *Lactococcus* sp. (Chicken salad), *Staphylococcus* sp. (red cabbage salad), *E.coli or Shigella* sp.(vegetable salad), *Lactobacillus or Entericoccus* sp. (blue cheese), *Stenotrophomonas or Pseudomonas* sp.(green salad), *Sphingobacterium* sp.(mushroom salad), *Carnobacterium* sp.(chicken salad), and *Brochothrix* sp.(chicken salad) were also identified as Tet^r gene carriers in tested food sample. Among studied Tet^r genes, *tetM* was widely distributed in most genus (*Enterococcus, Lactococcus, Staphylococcus, E.coli/Shigella, Brochothrix, Sphingobacterium, Carnobacterium,*), followed by *tetL* (*Enterococcus, E.coli/Shigella, Lactobacillus/Enterococcus, Sphingobacterium, Brochothrix*), *tetS* (*Enterococcus, Lactobacillus/Enterococcus, Carnobacterium*), and *tetK* (*Staphylococcus*). It was also found that some isolates contained more than one Tet^r genes, such as *Sphingobacterium* sp. V2-1(*tetM* and *tetL*) from mushroom salad and *Enterococcus* sp.W-20 (*tetL* and *tetS*) from vegetable salad, etc.

2.4.3. Minimum Inhibition Concentration (MIC) Values of ART Isolates

The MIC test was performed in eighty one representative Tet^r food isolates and one tetracycline sensitive control strain, with tetracycline concentration ranging from0.25 μg/mL to 512 μg/mL. While all Tet^r isolates were found to survive at 16 μg/mL of tetracycline, the growth of susceptible strain was inhibited with at0.5 μg/mL. Besides, 90% of Tet^r isolates were found to survive at 64μg/mL of tetracycline, 11% even at 128μg/mL (data not shown).

No correlation between the MIC values of the isolates and the number of resistance genes or the presence of different resistant mechanisms (ribosome protection or protein efflux) was found.

2.4.4. Distribution of Plasmids and Location of Tet^r Gene

A total of 15 identified Tet^r isolates with high MIC (>32μg/mL) were examined for the location of Tet^r determinants (Table 2.4). Plasmid DNA was extracted according to Anderson and McKay [149]. Plasmids of various sizes were observed in all isolates, ranging from 2kb to 20kb. The location of Tet^r genes were determined by southern blotting analysis. It was found that in 12 (80%) isolates, Tet^r genes reside on both chromosome and plasmids, while isolate S1-1 and XR-26 contained chromosomal Tet^r gene only, and W-6 contained plasmid Tet^r gene only.

2.4.5. Stability of the Tetracycline Resistance Phenotype

To assess the persistency of tetracycline resistance phenotype, 13 isolates with AR-encoding plasmids were chosen for AR stability analysis. After being consecutive transferred in bacteria media broth without tetracycline for more than 400 generations, a majority of the isolates (83%) retained the Tet^r trait with little change in tetracycline MIC value (data not shown). *Lactococcus* sp. A2-47 (chicken salad) and *E.coli/Shigella* sp. EE-3 (vegetable salad) were found to lose Tet^r trait in one out of a hundred progenies. All these results showed that AR trait is quite stable, even without the presence of corresponding antibiotic selection pressure.

To further study the mechanism of AR maintenance, tetracycline MIC test and Southern hybridization analysis were performed on the Tet^r and tetracycline sensitive (Tet^s) progenies of *Lactococcus* sp. A2-47 and *E.coli/Shigella* sp. EE-3 (Figure 2.3.). Tetracycline MIC value of A2-47L1 (Tet^s progeny of A2-47) and EE-3L1 (Tet^s progeny of EE-3), dropped below 0.5μg/mL, whereas Tet^r counterparts withstood a minimum of 32μg/mL of tetracycline. The loss of *tetM* in A2-47L1 and EE-3L1 was confirmed by PCR using corresponding primer. Gel electrophoresis analysis revealed that A2-47L1 and EE-3L1 retained a smaller plasmid compared to their Tet^r ancestor (Figure 2.3.). Southern hybridization showed that the smaller plasmid and chromosome of both Tet^s progenies do not contain Tet^r gene as Tet^r ancestors. The loss of resistance gene may be associated with transposon, as *tetM* was commonly found to be associated with transposons family Tn916 in previous studies. The loss of entire plasmid was also discovered in this study. For example, two *tetM*-containing plasmids in EE-3 ancestor (20-30kb) were found to be lost in EE-3L1 and some Tet^r progenies (data not shown).

2.4.6. The Electroporation of Tet^r Genes

Plasmids (*tetS*) in *Enterococcus* sp. W-6 from vegetable salad II and a plasmid (*tetM, tetL*) in *Enterococcus* sp. EE-11 from vegetable salad III were used in electroporation experiment, during which a tetracycline sensitive strain *Enterococcus faecalis* OG1RF served as recipient (plasmid-free, Rif^r, Fus^r) (table 2.4, Figure 2.6). It was found that the recipient was able to acquire and express Tet^r genes from both W-6 and EE-11 at relatively low frequency ($<10^{-8}$ transformants per recipient cell). The presence of Tet^r genes in the transformants was verified by PCR amplification. The transformants SRW-6 and SREE-11 were endowed with

tetracycline MIC of 128μg/mL and 64μg/mL, the same level as its donor strains W-6 and EE-11(table 2.4). Gel electrophoresis analysis of plasmid extracts of the transformants showed the presence of new plasmids, and the size of plasmid were identical to the donor plasmids (Figure 2.4.). Southern hybridization confirmed that the new plasmids in recipients contained corresponding Tet^r genes, indicating the dissemination of Tet^r plasmid may contribute to the acquisition of tetracycline resistance (Figure 2.4.). After consecutively transferred in bacteria media for 400 generations in the absence of tetracycline, all of the 50 randomly chosen progenies of SRW-6 and SREE-11 retained the Tet^r phenotype. The result strongly suggested that under laboratory conditions, Tet^r genes on mobile gene elements can be transmitted to different microorganisms, and once the recipients were transformed, some of the resistance genes can be quite stable in the new host (only apply to these particular ones) without the presence of corresponding antibiotics.

2.4.7. The Natural Transformation of Tet^r Genes

The capability of transferring Tet^r gene through food chain was further investigated by natural transformation. It was found that 2 out of 13 donors were able to transfer their tetracycline resistance gene to *Streptococcus mutans* UA159 strain through natural transformation. Transformation efficiency was less than 10^{-8} (Table 2.5.). The transmission of Tet^r genes to the recipient was verified by MIC test and conventional PCR. All transformants displayed an MIC of more than 32μg/mL (Table 2.5.). The result demonstrated that the acquisition of tetracycline resistance in human pathogenic and residential bacteria through horizontal gene transfer from food isolates is possible under laboratory settings.

The locations of the transferred Tet^r genes were further characterized by Southern hybridization (Fig.2.5). The *tetM* and *tetL* were found on both chromosomal DNA and plasmids (20kb) in donor EE-11. However, only *tetM* was detected on chromosome of the transformant SMEE-11. No plasmid was found after successful transfer of Tet^r genes from EE-11 to the plasmid-free recipient (Figure 2.5.). Similarly, although *tetL* and *tetS* were detected on both chromosomal DNA and plasmid in XR-37; only *tetS* was found on chromosomal DNA in the transformant SMXR-37. These data indicated the insertion of *teM* and *tetS* gene into chromosome during horizontal gene transfer. These results also showed that Tet^r harboring plasmids can transmit cross species through natural transformation.

2.5. Discussions and Conclusion

We chose to examine AR in RTC foods because they are very popular in the United States and represent a potentially common avenue of public exposure to food microbiota without further mitigation. According to our results, the estimated influx of Tet^r determinants can be up to 10^5 CFU per meal (calculated based on 100g chicken salad and 100g vegetable salad and for conventional serving size), which is consistent with results from previous report [110]. Because the culture condition was only suitable for a small portion of the Tet^r bacteria, the real AR exposure through RTC food consumption would be much higher than the reported numbers.

Although other factors, including hygiene of food handling facilities, time and storage condition of food samples before study, etc., may vary among different samples, the numbers of AR bacteria in cooked foods (boiled shrimp, boiled egg, three bean salad) were

generally lower than others especially some salad with minimum processed vegetables (bean sprout salad, mushroom salad, etc.). This finding suggested that heat treatment of raw food is an easy and effective way to reduce total bacteria load as well as ART bacteria counts. Besides, it is worth noting that the ART bacterial numbers were also low in pickles, feta cheese and coleslaw samples, although we were not able to draw further conclusion due to the limited number of samples.

Most Tet^r carriers identified by 16S rRNA gene analysis in our study belonged to the genera reported before[60]. However, for the first time to our knowledge, *tetM* and *tetL* have been found in *Sphingobacteia,* Gram-negative environmental bacteria that rarely cause diseases. Currently *tetX* is the only Tet^r gene reported in *sphingobacteira*[152]. The finding of *tetL* gene in *Brochothrix* is also the first report of tetracycline resistant in this Gram-positive genus.

Enterococci are common bacteria in various environments including gastrointestinal tract of human and other animals, and different foods such as meat, milk, and cheese as reported before[107, 129]. They are also opportunistic pathogens for humans causing increasing numbers of hospital antibiotic resistant infections. Our data showed that AR genes from enterococcal isolates were more likely to be transmitted to other bacteria. Thus the prevalence of antibiotic resistant *Enterocci* in RTC foods represents a high risk of AR gene dissemination from the food chain to humans.

Previous studies demonstrated that newly acquired AR traits usually reduce the fitness of transformants compared to susceptible counterparts in the antibiotic free environment by

imposing extra burden of producing AR related proteins[153]. Therefore, the difference of fitness is supposed to lead to the elimination or reduction of newly acquired resistance. However, the stability analysis of Tet^r determinants in our study showed that Tet^r traits are maintained and expressed in all the progenies of transformants in electroporation experiments. This result suggested that factors other than the reduction in selective pressure, may contribute to the persistency of acquired AR encoding plasmids. A comprehensive study of the genetic analysis of these moveable elements is needed in order to discover the mechanisms of AR persistency and understand the difficulty of reversion of AR simply by the precautious prescription strategy.

Results from this study indicate that the general public can easily be exposed to millions of ART bacteria daily through conventional food intake, and the AR genes from the food isolates can be transmitted to human pathogens and residential bacteria through horizontal gene transfer mechanisms in laboratory settings. However, further studies are needed to illustrate the horizontal gene transmission from the food isolates to pathogens or residential bacteria in vivo, particularly in the host digestive tract ecosystem. Because the AR genes are quite stable in the food isolates even at the absence of the selective pressure, the overall impact of limiting the use of antibiotics on the reduction of ART bacteria in both the environment and hosts might be limited. Further experimental confirmation would be very helpful.

Several strategies can be taken to combat the endless war against AR in the future. First of all, we need to be more prudent with the drugs in use and actively encourage the development of new antibiotics. The appropriate use of antibiotics would reduce the speed

of AR emergence. For example, continued use of the same drugs alone in treating certain diseases should be prevented. The dose of therapeutically useful drug should be standardized. When it comes to food production, prudent use of antibiotics to reduce or control ART bacteria in food is a better choice. Besides, a local, national and global surveillance system of ART bacteria (both pathogens and commensals) in different AR gene reservoirs including food chain is needed to provide information about the prevalence and spread of AR. Further studies about the mechanisms of transmission and maintenance of AR would help scientists develop effective solution to the AR problem.

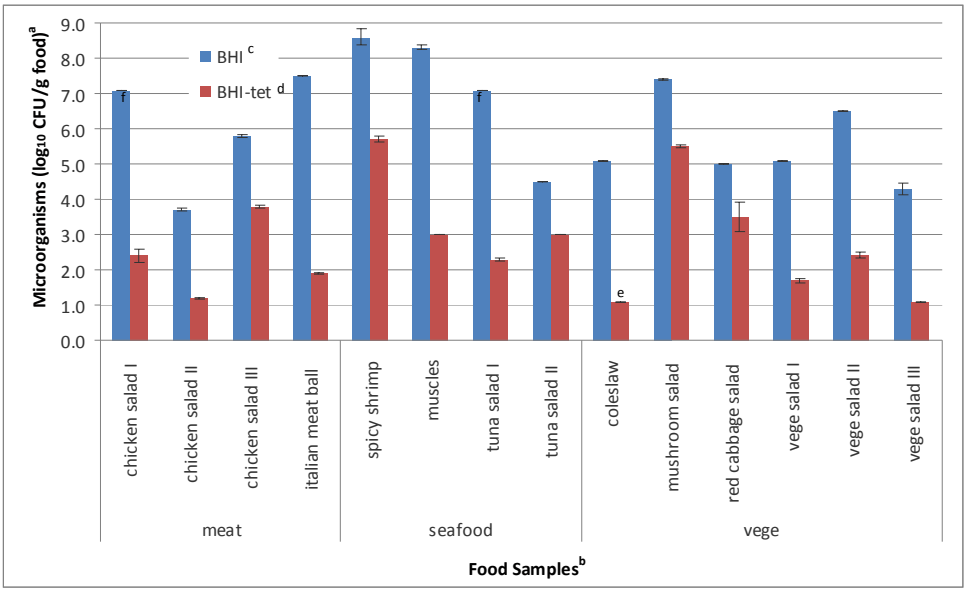

Figure 2.1. Prevalence of ART microbes in food samples (meat, seafood, vegetable)

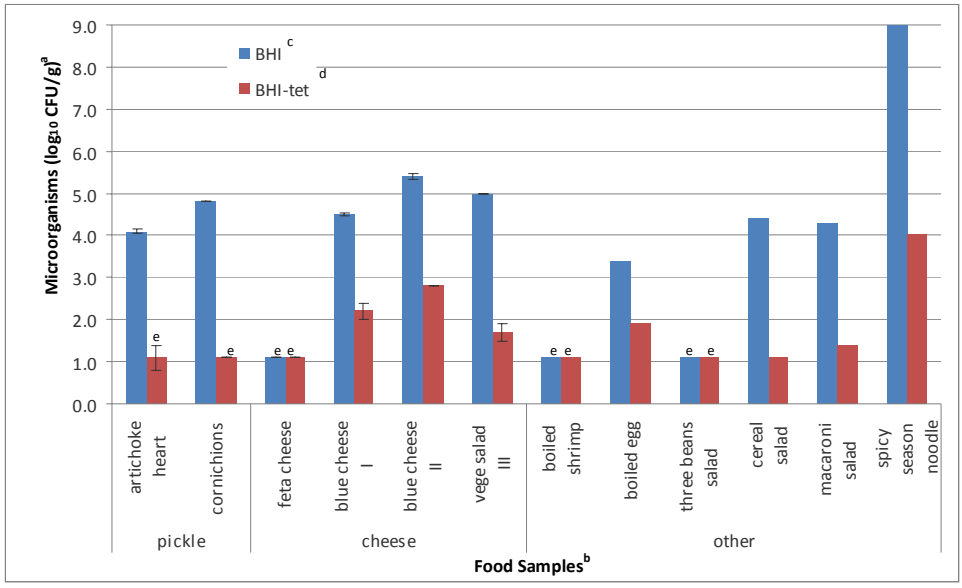

Figure 2.2. Prevalence of ART microbes in food samples (pickle, cheese, other)

a. Colony Forming Units per gram of food samples;

b. Food samples were purchased from salad bars at grocery stores or restaurant in the area of Columbus, OH and were stored in 4°C for less than 48 hours before use. Multiple samples (blue cheese, chicken salad, tuna salad, Italian meat ball, vege salad) were purchased from different stores and were treated as different food samples. The counts of each sample are the average of duplicate plates results obtained from the same sample and standard deviation is shown.

c. Brain and Heart Infusion Agar plates containing 100µg/mL of cycloheximide incubated at 37°C for 48h for total plate counts.

d. Brain and Heart Infusion Agar plates containing 16µg/mL of tetracycline and 100µg/mL of cycloheximide incubated at 37°C for 48h for AR bacteria counts.

e. Tet^r isolates counts were below detection level and reported as < $1.3x10^1$ CFU/g food.

f. Tet^r isolates counts were above detection level and reported as >$1.3x10^7$CFU/g food

No.	Category	Food Item	Total Plate Count[a](CFU[c]/g Food)	Tet-resistant Count[b] (CFU/g Food)
D	Other	boiled shrimp	$<1.3\times10^{1}$	$<1.3\times10^{1}$
N		cereal salad	2.7×10^{4}	1.3×10^{1}
T		spicy season noodle	$1.1.\times10^{9}\pm2.0\times10^{8}$	1.1×10^{4}
aa		macaroni salad	$1.8\times10^{4}\pm7.0\times10^{2}$	$2.7\times10^{1}\pm1.3\times10^{1}$
bb		boiled egg	$2.8\times10^{3}\pm3.0\times10^{2}$	$8.7\times10^{1}\pm4.7\times10^{1}$
dd		three beans salad	$<1.3\times10^{1}$	$<1.3\times10^{1}$
Q	Pickle	cornichions	$6.0\times10^{4}\pm2.0\times10^{4}$	$<1.3\times10^{1}$
cc		artichoke heart	$1.6\times10^{4}\pm2.8\times10^{3}$	$<1.3\times10^{1}$
R	Cheese	blue cheese II	$2.4\times10^{4}\pm1.4\times10^{3}$	$1.2\times10^{3}\pm1.0\times10^{2}$
X		blue cheese I	$4.8\times10^{4}\pm5.0\times10^{3}$	$2.3\times10^{2}\pm3.0\times10^{1}$
H		feta cheese	$<1.3\times10^{1}$	$<1.3\times10^{1}$
ee		vege salad III	$2.6\times10^{5}\pm1.0\times10^{4}$	$1.0\times10^{2}\pm7.0\times10^{0}$
A	Meat	chicken salad I	$>1.3\times10^{7}$	$2.1\times10^{2}\pm9.0\times10^{1}$
P		chicken salad II	$8.7\times10^{3}\pm7.0\times10^{2}$	$3.8\times10^{2}\pm2.0\times10^{1}$
Y		chicken salad III	$9.9\times10^{5}\pm8.0\times10^{4}$	$9.9\times10^{3}\pm8.0\times10^{2}$
GM		italian meat ball	$6.7\times10^{7}\pm3.0\times10^{6}$	$1.7\times10^{2}\pm1.0\times10^{1}$
E	Seafood	spicy shrimp	$2.7\times10^{8}\pm1.3\times10^{8}$	$5.3\times10^{5}\pm1.1\times10^{5}$
G		tuna salad I	$>1.3\times10^{7}$	$2.7\times10^{2}\pm3.0\times10^{1}$
I		muscles	$2.8\times10^{8}\pm4.0\times10^{2}$	$2.6\times10^{3}\pm1.0\times10^{2}$
TU		tuna salad II	$7.1\times10^{4}\pm3.4\times10^{3}$	1.1×10^{3}
K	Vege	coleslaw	1.3×10^{5}	$<1.3\times10^{1}$
M		vege salad I	1.3×10^{5}	$4.7\times10^{1}\pm0.7\times10^{1}$
S		red cabbage salad	9.3×10^{4}	$5.0\times10^{3}\pm3.8\times10^{3}$
V		mushroom salad	$2.5\times10^{7}\pm1.0\times10^{6}$	$3.0\times10^{5}\pm3.0\times10^{4}$
W		vege salad II	$2.9\times10^{6}\pm1.0\times10^{4}$	$2.6\times10^{2}\pm5.0\times10^{1}$
ff		vege salad III	$2.0\times10^{4}\pm7.0\times10^{3}$	1.3×10^{1}

Table 2.2. Prevalence of ART microbes in selected food samples

a. Brain and Heart Infusion Agar plates containing 100µg/mL of cycloheximide incubated at 37°C for 48 hours for total plate counts.
b. Brain and Heart Infusion Agar plates containing 16µg/mL of tetracycline and 100µg/mL of cycloheximide incubated at 37°C for 24 hours for AR bacteria counts.
c. Colony Forming Units per gram of food samples

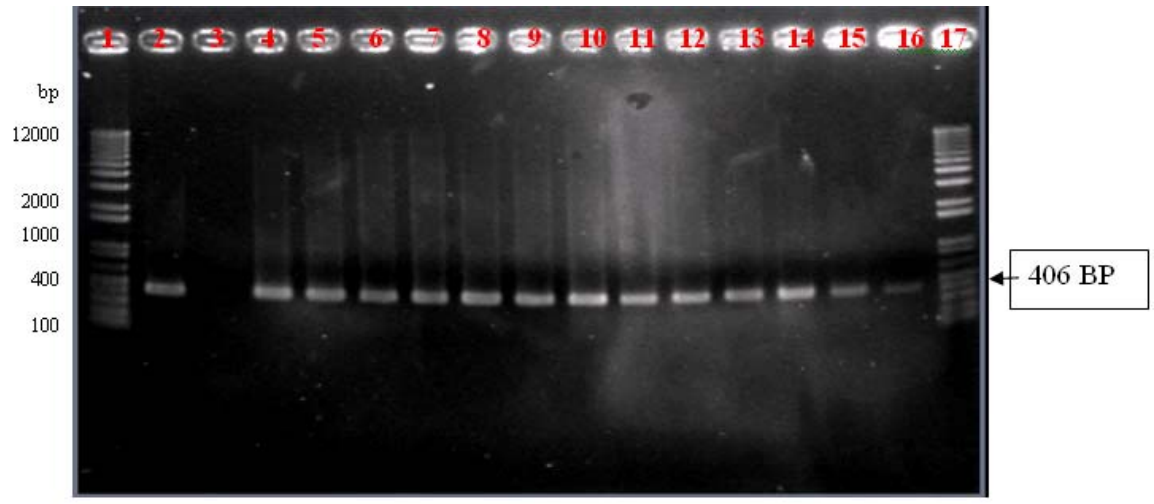

Figure 2.3. Conventional PCR screening for *tetM* gene from selected food samples.
1. 1kb Plus DNA ladder 2. Positive control; 3. Negetive control; 4. A3-11; 5. B2-1; 6. C4-1; 7. EE-3; 8. I2-1; 9. XR-11; 10. R1-14; 11. S1-1; 12. T1-50; 13. RV-42; 14. V2-1; 15. W-31; 16. GM-30; 17. 1kb Plus DNA ladder

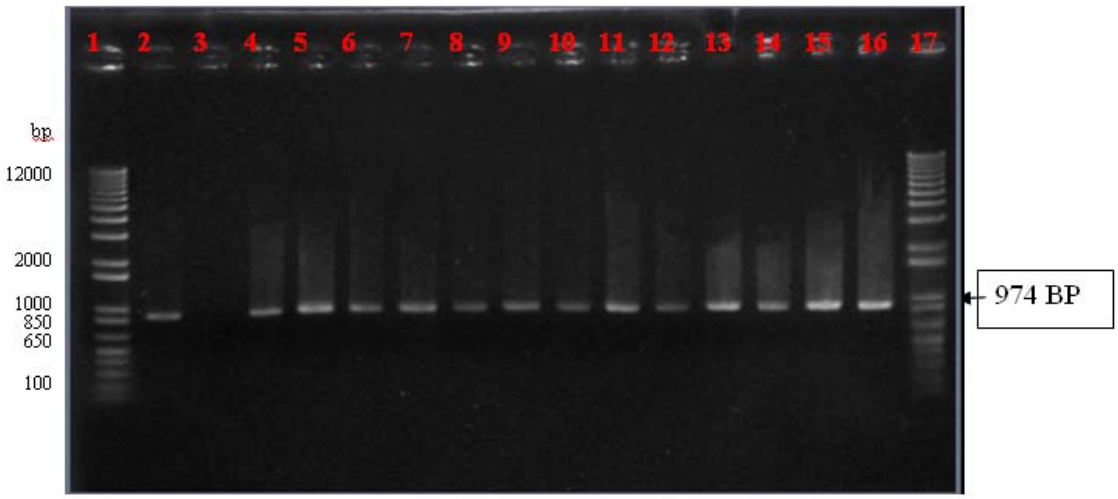

Figure 2.4. Conventional PCR screening for *tetL* gene from selected food samples.
1. 1kb Plus DNA ladder 2. Positive control; 3. Negetive control; 4. A2-11; 5. B2-10; 6. EE-3; 7. I2-1; 8. XR-11; 9. R2-21; 10. S1-1; 11. T1-2; 12. RV-42; 13. V2-1; 14. W-2; 15. GM-6; 16. Y1-32; 17. 1kb Plus DNA ladder

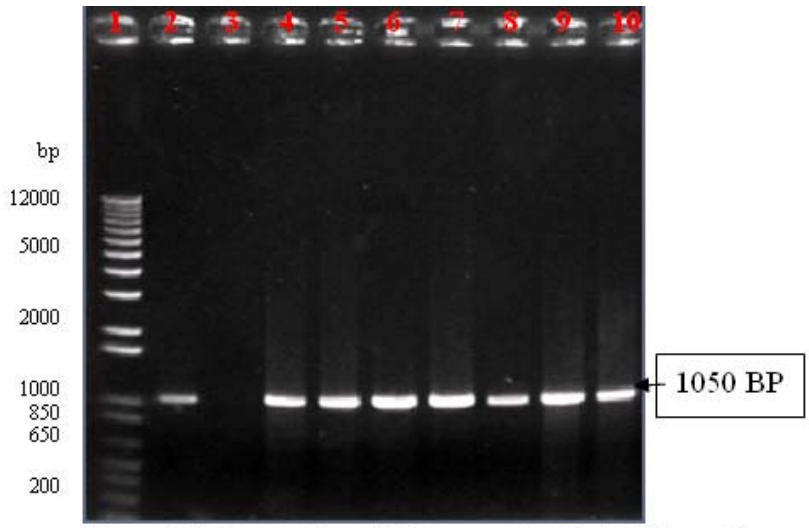

Figure 2.5. Conventional PCR screening for *tetS* gene from selected food samples.
1. 1kb Plus DNA ladder 2. Positive control; 3. Negetive control; 4. B2-9; 5. XR-15; 6.R1-45; 7. T1-34; 8. V2-7; 9. W-6; 10. GM-30

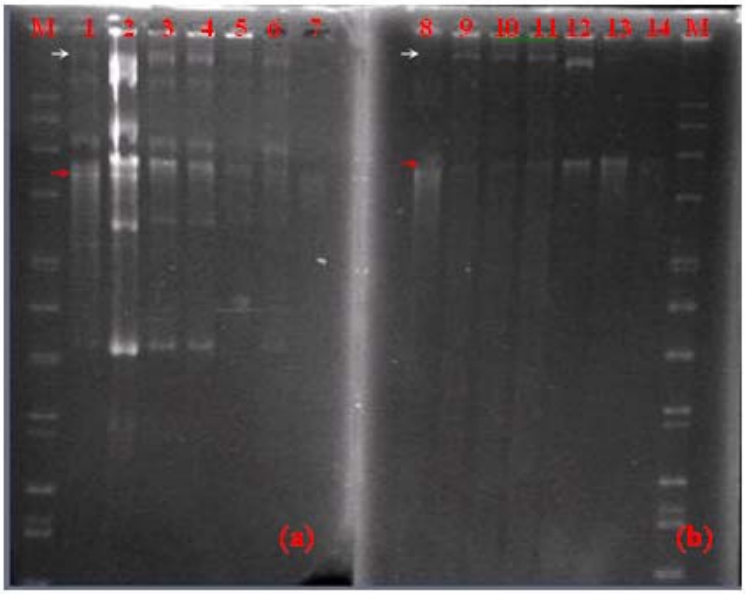

Figure 2.6. Analysis of plasmids profiles of A2-47, EE-3before and after Stability analysis by (a) agarose gel electrophoresis) (b) southern hybridization analysis with *tetM* probe;
A2-47 before stability test; 2-5. A2-47R-1- A2-47R-4, progeny of A2-47 that retain tet^r; 6. A2-47L-1, progeny of A2-47 that lost tet^r; 8 EE-3 before stability test; 9-12. EE-3L-1-EE-3L-4, progeny of EE-3 that retain tet^r; 7, 14. Tet^s, plasmid free *Enterococcus faecalis* OGR1F indicating chromosome position; M. supercoiled DNA Marker (Invitrogen); red arrows indicate the chromosome DNA and white arrows indicate tet^r plasmids

No	Sample	No. isolates screened	Tet^r genes	Strain Identification (No. of isolates within the same category)
A	chicken salad I	60	2 *tetM*, 2 *tetL*	*Lactococcus lactis. tetM* (1)
EE	vege salad III	50	1 *tetM*, 1 *tetL*, 1 *tetS*, 1 *tetM*/*L*/*K*, 1 *tetL*/*K*, 1 *tetM*/*K*,2 *tetM*/*L*	*Staphylococcus epidermidis. tetK*(1), *Enterococcus* sp. *tetM*(1), *Enterococcus* sp. *tetM*/*L*(1), *Ecoli/Shigella* sp. *ML*(1)
GM	Italian meat ball	40	3 *tetL*, 1 *tetM*/*L*	*Enteroccus* sp. *tetM*/*L*(1)
P	chicken salad III	5	1 *tetM*	*Enterococcus faecium. tetM*(1)
I	muscles	70	15 *tetM*, 5 *tetL*, 1 *tetS*, 3 *tetM*/*L*	*Enterococcus* sp. *tetM*(1), *Enterococcus* sp. *tetM*/*L*(1)
R	blue cheese II	120	8 *tetM*, 2 *tetL*, 2 *tetS*, 1 *tetM*/*L*, 1 *tet L*/*S*	*Enterococcus faecium. tetM*(3), *Enterococcus faecium. tetM*/*L*(1), *Lactobacillus* sp./*Enterococcus* sp. *tetL*/*S*(1)
S	red cabbage salad	100	5 *tetM*, 2 *tetM*/L	*Stenotrophomonas* sp./*Pseudomonas* sp. *tetM*(1)
T	spicy season noodle	120	1 *tetM*, 4 *tetL*, 1 *tetL*/*S*, 2 *tetM*/*L*	*Enterococcus* sp. *tetL*/*S*(1)
V	mushroom salad	70	7 *tetM*, 2 *tetL*, 1 *tetK*, 5 *tetM*/*L*	*Enterococcus* sp. *tetM*(3), *Enterococcus* sp. *tetM*/*L*(1), *Sphingobacterium* sp. *tetM* (1) *Sphingobacterium* sp. *tetM*/*L* (1)
W	vege salad II	40	5 *tetM*, 1 *tetL*, 1 *tetS*, 1 *tetL*/*S*	*Enterococcus* sp. *tetL*/*S*(1), *Enterococcus* sp. *tetM*(1) *Enterococcus* sp. *tetS*(1)
X	blue cheese I	25	4 *tetM*, 3 *tetL*, 1 *tetS*, 3 *tetM*/*L*, 1 *tetM*/*L*/*S*, 1 *tetL*/*S*	*Enterococcus faecalis. tetM*(1), *Enterococcus faecalis. tetL*(1)
Y	chicken salad III	40	6 *tetM*, 1 *tetL*, 1 *tetS*	*Carnobacterium* sp. *tetM*(1), *Carnobacterium* sp. *tetS*(1), *Brochothrix* sp. *tetL*(1),
	Total	**740**	**114 (15.4%)**	

Table.2.3. Screening and identification of ART bacteria in selected food samples

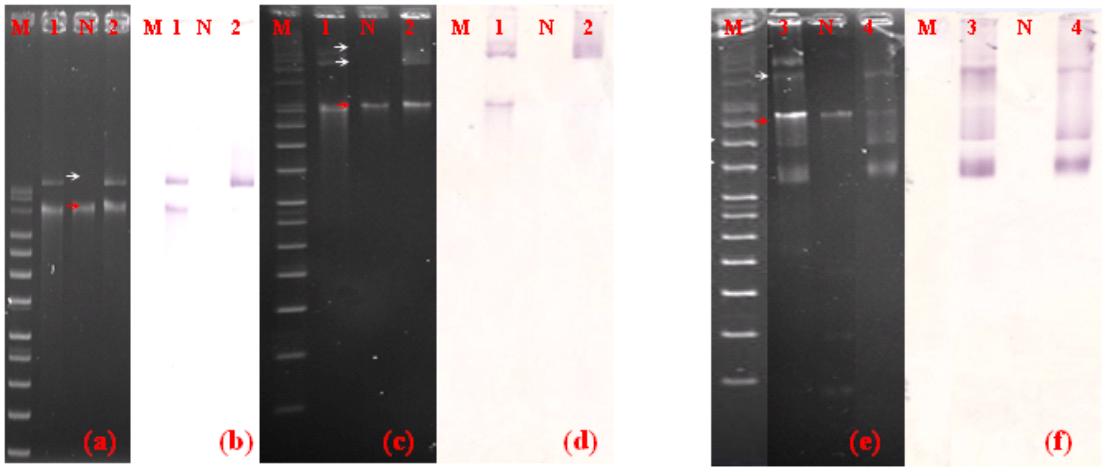

Figure 2.7. Analysis of plasmids profiles of EE-11, W-6 before and after electroporation by (a)(b) agarose gel electrophoresis and Southern hybridization analysis with *tetM* probe; (c)(d) agarose gel electrophoresis and Southern hybridization analysis with *tetL* probe; (e)(f) agarose gel electrophoresis and Southern hybridization analysis with *tetS* probe.
M, supercoiled DNA Marker (Invitrogen); 1. EE-11 before transformation; 2. SREE-11 after transformation; 3.W-6 before transformation; 4. SRW-6 after transformation; N. Recipient *Enterococcus faecalis* OGR1F; red arrows indicate the chromosome DNA and white arrows indicate Tet^r plasmids

Isolates	Source	MIC μg/mL	Tet ^{r} gene	Loci (Tet ^{r} gene)[b]	Stability [c]	Transfer
Lactococcus **A2-47**	Chicken salad I	128	*tetM*	p[b], c [b](*tetM*)	1/100	
E.coli/shigella **EE-3**	Vege salad III	64	*tetM,tetL*	p, c (*tetM*)/ p, c(*tet L*)	1/50	
Enterococcus **EE-11**	Vege salad III	128	*tetM,tetL,tetK*	p, c(*tetM*) /p,c (*tetL*)/ ND(*tetK*) [f]	0/100	N [d],E [e]
Enterococcus **GM-30**	Italian meat ball	256	*tetM,tetL*	p, c (*tetM*) / p, c(*tetL*)	ND	
Enterococcus **P4**	Chicken salad III	128	*tetM*	p, c(*tetM*)	0/100	
Enterococcus **I2-11**	Muscles	128	*tetM*	p, c(*tetM*)	0/100	
Enterococcus **I4-6**	Muscles	128	*tetM,tetL*	p, c (*tetM*) / p, c(*tetL*)	0/100	
Enterococcus **XR-26**	Blue cheese II	16	*tetM*	c(*tetM*)	0/100	
Lactobacillus **XR-37**	Blue cheese II	32	*tetL,tetS*	p, c(*tetL*) / p, c(*tetS*)	0/100	N
Stenotrophomonas **S1-1**	Red cabbage salad	128	*tetM,tetL*	c(*tetM*) /c(*tetL*)	0/100	
Enterococcus **RV-34**	Mushroom salad	128	*tetM*	p, c(*tetM*)	0/100	
Enterococcus **V2-7**	Mushroom salad	128	*tetM*	p, c(*tetM*)	0/100	
Enterococcus **W-6**	Vege salad II	128	*tetS*	p(*tetS*)	0/100	E
Enterococcus **W-20**	Vege salad II	64	*tetL,tetS*	p,c(*tetL*) /c(*tetS*)	ND	
Carnobacterium **Y1-16**	Chicken salad III	64	*tetS*	p,c(*tetS*)	ND	

Table 2.4. Strains used for plasmid stability test, tetracycline MIC[a] and transformation of Tet^{r} gene in the study

a. Minimum inhibition concentration of tetracycline
b. Location of tetracycline genes. P stands for plasmid and C stands for chromosome. Multiple genes were separated with slash.
c. Results of stability analysis were displayed as number of progenies that lost Tet^{r} trait/ total number of progenies tested for tet^{r} trait
d. tet^{r} trait successfully transferred into *Streptococcus mutans* through natural transformation
e. tet^{r} trait successfully transferred into *Enterococcus facalis* through electroporation
f. Not determined

Donor (Genus name)	Tet^r gene (donor/transformant)	No.transformant per recipient cell	tetracycline MIC μg/mL (donor/transformant)
Natural transformation			
Enterococcus sp. EE-11	*tetM,tetL/tetM*	2.2 x10^{-8}	128/64
Lactobacillus sp. XR-37	*tetL,tetS/tetS*	7.2x10^{-9}	32/32
Electroporation			
Enterococcus sp. EE-11	*tetM,tetL/tetM,tetL*	1.8 x10^{-8}	128/128
Enterococcus sp. W-6	*tetS/tetS*	2.7x10^{-8}	64/64

Table 2.5. Natural transformation and electroporation of Tet^r genes

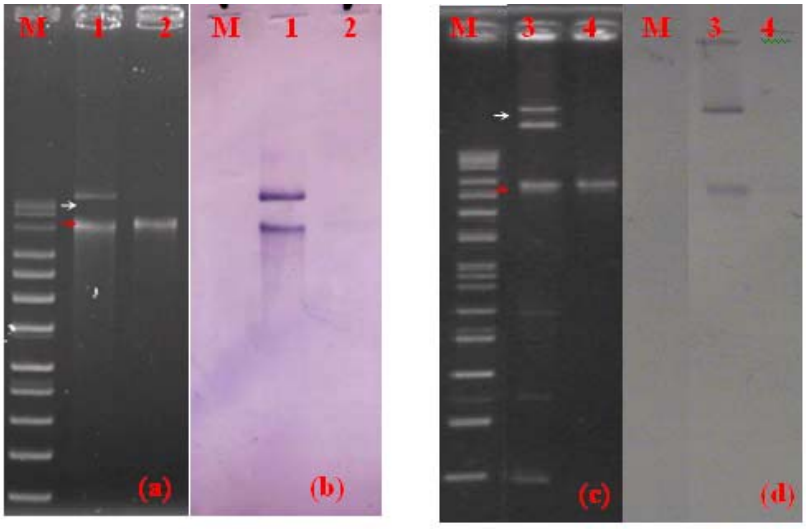

Figure 2.8. Analysis of plasmids profiles of EE-11 and XR-37 before and after natural transformation by (a)(b) agarose gel electrophoresis and southern hybridization analysis with *tetM* probe; (c)(d) agarose gel electrophoresis and Southern hybridization analysis with *tetS* probe

M, supercoiled DNA Marker (Invitrogen); 1. EE-11 before transformation; 2. SMEE-11 after transformation; 3.XR-37 before transformation; 4. SMXR-37 after transformation; red arrows indicate the chromosome DNA and white arrow indicate Tet^r plasmids

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