Establishment and Development of Antibiotic Resistant Bacteria in Host Gastrointestinal Tract—Food, Drug, or Are We Born with It?

Dissertation

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Abstract

The rapid emergence of antibiotic resistance (AR) is a major public health concern. Recent findings on the prevalence of foodborne antibiotic resistant (ART) commensal bacteria in ready-to-consume food products suggested that the food chain likely serves as a major avenue disseminating ART bacteria to the human hosts through daily food intake. To properly assess the impact of antibiotics and the food chain on AR development in hosts and to develop effective mitigation, this study examined 1) the baseline of AR in human gastrointestinal (GI) tract system using multiple infant subjects without antibiotic exposure, and 2) the impact of foodborne ART bacteria and antibiotic delivery methods on AR ecology in GI tract using a murine model. In addition, a small scale study was conducted to examine the prevalence of AR in representative aquaculture products and production environment to assess the potential impact of aquaculture practices on the development of ART bacteria, and the susceptibility of ART bacteria to conventional cooking methods. These studies contributed to a comprehensive understanding of AR development and dissemination, and are essential for the development of effective mitigation strategies.

In the second chapter of the dissertation, the study examined gut microbiota of 6 infant subjects from newborn to 1 year of age, fed on breast milk and/or infant formula during early stage of development and without prior exposure to antibiotics. Predominant
bacterial populations resistant to several antibiotics and multiple resistance genes were found in the infant GI tracts within the first week of age. Several ART population transitions were also observed in the absence of antibiotic exposure and dietary changes. Representative AR gene pools including $tet(M)$, $ermB$, $sul2$ and $bla_{TEM}$ were detected in infant subjects. *Enterococcus* spp., *Staphylococcus* spp., *Klebsiella* spp., *Streptococcus* spp. and *Escherichia coli/Shigella* spp. were among the identified AR gene carriers. ART bacteria were not detected from infant formula and infant foods examined, but small numbers of skin-associated ART bacteria were found in certain breast milk samples. The data suggested that the early development of AR in human gut microbiota is independent of infants’ exposure to antibiotics, but is likely impacted by exposure to maternal and environmental microbes during and after delivery, and that ART population is significantly amplified within the host even in the absence of the antibiotic selective pressure.

The third chapter investigated the prevalence of ART bacteria and AR gene pool in non-food animals using 7 pets and 13 zoo animals. It was found that ART bacteria and AR gene pool were prevalent in many pets and zoo animals, some of which were never exposed to growth promoter or antibiotic treatment. Identified resistance gene carriers included *Lactobacillus* spp., *Streptococcus* spp. and *Escherichia coli*. The finding suggested that non-food animals might also be an important component in AR circulation. Most importantly, antibiotic application is not the essential contributing factor leading to prevalence of ART bacteria in the hosts.
The fourth chapter of the study evaluated the capability of foodborne ART bacteria in colonizing host GI tract and the impact of antibiotics and delivery methods in shaping host GI ecology. Two identified strains, *Enterococcus faecium* A21 and *Streptococcus* sp. A85, isolated from cooked seafood product, were used to feed mouse previously colonized with human originated *Enterococcus faecalis* G37. It was found that foodborne ART bacteria colonized and amplified in mouse GI tract in up to $10^9$ CFU/g in the presence of human associated microbiota, and persisted for up to 2 months after withdrawn of the ART bacteria influx and in the absence of the corresponding antibiotics. Horizontal gene transfer events were not detected under the experimental conditions, in the presence and absence of the corresponding antibiotics. The impact of two antibiotic delivery methods, including intravenous (IV) injection and oral feeding, on host GI ecology was also evaluated in a pilot study. Tetracycline hydrochloride was introduced to mice colonized with environmental microbiota in equal dosage via tail vein injection or gavage feeding once a day for 5 days. The *tetL* and *tetM* gene pools were significantly amplified one day after initial exposure to tetracycline hydrochloride at 50 mg/kg body weight/day by both delivery routes, and persisted throughout subsequent exposure. Though predominant resistant microflora changed in a similar manner in both delivery routes, oral exposure led to *tet* gene pools 10 to 100 times larger than IV injection route in host GI tract. When different antibiotic dosages were applied, the difference in AR gene pool development by the two delivery modes was much more significant.

The last chapter of the study surveyed the prevalence of AR in representative fish, shrimp and environmental samples from aquaculture production, and raw seafood products from
local markets for a preliminary understanding of potential critical control points for AR mitigation in aquaculture production. Further, several household cooking methods were evaluated regarding the efficacy of reducing ART bacteria from raw shrimp samples. It was found that ART bacteria and AR gene pools were prevalent in most samples analyzed. Household cooking methods, including boiling, stir frying, steam and barbecue, were effective as AR mitigation practice.

Data from the infant and non-food animal studies illustrated the critical role of hosts in amplified circulation of ART bacteria even without antibiotics, suggesting manure and feces waste treatment is a critical control point in AR mitigation. The findings on the impact of the mode of drug delivery on ART bacteria development potentially revealed a critically important cause for the rapid emergence of antibiotic resistance. Follow-up studies may lead to major breakthrough in AR mitigation approach and impact future clinical therapy and drug development. The aquaculture study illustrated the prevalence of ART bacteria in aquaculture products and production environment, and set up the foundation for future studies to reveal the impact of processing practice on ART bacteria associated with seafood products. The illustration of the effectiveness of household food processing on reducing ART bacteria in food products suggested the feasibility of interruption of AR transmission to humans through post-harvest processing, and the importance of proper knowledge dissemination in improving food safety and public health.
Dedication

Dedicated to my family
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Fields of Study

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Objectives of the Study

The rapid emergence of antibiotic resistance in pathogenic bacteria is a major public health concern. Recent findings on the prevalence of foodborne ART commensal bacteria in the food chain suggested that human are exposed to foodborne AR through food intake on daily basis. A question of significance to mitigation yet to be addressed is the exact impact of antibiotics and the food chain on AR development in hosts. Therefore, the objectives of the study are: 1) to illustrate the baseline of AR in human gastrointestinal (GI) tract system using multiple infant subjects without antibiotic as well as conventional food exposure; 2) to investigate the prevalence of AR in non-food animals; 3) to examine the impact of foodborne ART bacteria and antibiotic delivery methods on AR ecology in GI tract using a murine model; 4) to examine the prevalence of AR in representative aquaculture products and production environment to assess the potential impact of aquaculture practices on the development of ART bacteria, and the susceptibility of ART bacteria to conventional cooking methods. These studies contributed to a comprehensive understanding of AR development and dissemination, and are essential for the development of effective mitigation strategies.
Chapter One

Literature review

Antibiotics are widely used in human medicine, agricultural production and food processing, and have been essential for ensuring human and animal health as well as the safety of the food supply. Unfortunately, the resistance of microbes to commonly used antibiotics is on the rise globally. Particularly, the spectrum of antibiotic resistant (ART) pathogens is a worldwide concern due to the public awareness of the increased difficulty in treating infections caused by ART bacteria. Hospital acquired infections by ART pathogens and opportunistic pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) have been widely reported (48, 60). In 2003, over a quarter of nosocomial enterococcal infections were resistant to vancomycin, and almost 60% of hospital-acquired *S. aureus* infections were caused by MRSA. This represents a 12% and 11% increase compared to the previous five year averages, respectively (14). Antibiotic resistance (AR) also has economic implications: in the mid-1990s, an estimated $4 billion in human antibiotic sales targeted nosocomial infections caused by ART bacteria (35). The Centers for Disease Control and Prevention and the medical community are promoting changes in antibiotic prescribing practices and encouraging prudent use of antibiotics to reduce the selective pressure that increases the
prevalence of ART microorganisms. These recommendations include renewed emphasis on preventing the spread of infection and targeting specific antibiotics for specific pathogens to limit the use of broad spectrum antibiotics (13). However, despite the efforts, the rising trend of antibiotic resistance did not change. Emerging evidence further suggests that the development, dissemination and persistence of ART bacteria and their resistance-encoding genes are much more complicated than previously thought.

In recent years, the impact of the food chain, particularly foodborne commensal bacteria and agriculture practices, on AR dissemination has become recognized (15, 16, 69, 82, 84, 87). While pathogens only count for a very small percentage of the microbiota, commensal bacteria are in vast quantity and diversity, and likely play an important role in horizontal gene transmission and served as a major reservoir for AR genes. The potential correlation of resistance to various antimicrobials with food processing practice has yet to be revealed. Accordingly, control strategies likely need to be renovated for targeted and effective mitigation.

1.1 Type of antibiotics

Depending on the mode of action, antibiotics can be categorized into bactericidal and bacteriostatic agents. Bactericidal antibiotics normally target key cell structures and activities, such as the biosynthesis of cell wall (β-lactam antibiotics) and DNA (fluoroquinolones); whereas bacteriostatic antibiotics commonly interfere with protein
synthesis, inhibiting proliferation of bacterial cells (41). The three types of commonly used bactericidal antibiotics include β-lactam antibiotics family, aminoglycosides and quinolones; whereas macrolides/telithromycin, sulfonamides and tetracycline are bacteriostatic antibiotics (68).

1.1.1 β-lactam antibiotics

β-lactam antibiotics are most commonly used in clinical therapy for infections. β-lactam antibiotics refer to antibiotic agents containing a β-lactam in the molecular structure and sometimes, also those β-lactamase inhibitors (although they are not true antibiotics). β-lactam antibiotics are bactericidal, as they interfere with the biosynthesis of the peptidoglycan layer in bacterial cell walls by irreversibly blocking penicillin-binding proteins (PBPs, including carboxypeptidases, endopeptidases and transpeptidases). PBPs are a group of proteins that facilitate crosslinking of newly synthesized peptidoglycan to the existing cell wall structure (24). Once treated with β-lactam antibiotics, susceptible bacterial cells develop a weak cell wall and are eventually subject to cell lysis. Four major classes of antibiotics and their derivatives belong to the β-lactam antibiotics category, including penicillin, cephalosporins, carbapenems and monobactams. Based on their antimicrobial activities and antibacterial spectrum, they can be further divided to different groups.

β-Lactamase inhibitors
Apart from the above four families, β-lactamase inhibitors are also recognized as β-lactam antibiotics. One of the most common resistance (against β-lactam antibiotics) mechanisms in bacteria is that they host genes encoding β-lactamase, which hydrolyzes intracellular β-lactam molecules. To be effective, β-lactamase inhibitors are used together with β-lactam antibiotics to minimize the effects of bacterial resistance. β-lactamase inhibitors are not true antibiotics, but antibiotic enhancers (70).

1.1.2 Aminoglycosides

Aminoglycosides are another type of bactericidal antibiotics. Their key structure includes an aminocyclitol ring in the molecule, with different glycosidic linkages and side chains among members in this family (42). Aminoglycosides affect bacterial cells by displacing cations such as Mg\(^{2+}\) and Ca\(^{2+}\) on the outer bacterial membrane, disrupting normal permeability. Moreover, aminoglycosides can also be bacteriostatic, as they impair growth of bacterial cells by binding to the 30S subunit of the bacterial ribosome, inhibiting protein synthesis. Susceptible bacteria are primarily aerobic Gram negative bacteria, such as *Klebsiella* sp. and *Pseudomonas aeruginosa* whereas most Gram positive bacteria and anaerobes are not susceptible to this antibiotic class (59). Representative members of aminoglycosides include gentamicin, kanamycin, neomycin and streptomycin. These classes of antibiotics are primarily used when treating infections on the surface of the skin and in the respiratory system, and they are normally combined with other types of antibiotics for clinical therapy.
1.1.3 Quinolones

Quinolones are also bactericidal as they inhibit DNA synthesis in bacterial cells by binding to DNA gyrase and DNA topoisomerase, both of which are involved in DNA replication, transcription and repair systems (20, 88). Quinolones are effective against many types of Gram negative bacteria, including *Klebsiella* sp., *Escherichia coli*, *Salmonella* sp. and *Shigella* sp (7). Ciprofloxacin, gemifloxacin, moxifloxacin and trovafloxacin are examples of quinolone antibiotics.

Bacteriostatic antibiotics are different from bactericidal antibiotics as they inhibit the proliferation of a bacterial population rather than killing bacterial cells. Most commonly, intracellular nucleotide or protein synthesis is targeted by these types of antibiotics, and a broad spectrum of bacterial species is affected. For example, sulfonamides are competitive inhibitors for dihydropteroate synthase (50), an enzyme with a key role in folic acid synthesis. The lack of folic acid will eventually lead to the breakdown of nucleotide synthesis and interruption of cell proliferation. Four groups of sulfonamides were defined based on their modes of absorption/excretion, including short- to medium-acting sulfonamides, long-acting sulfonamides, gastrointestinal tract sulfonamides and topical sulfonamides. Common sulfonamides include sulfamethoxazole, sulfadoxine, sulfasalazine and silver sulfadiazine.

1.1.4 Tetracycline
Tetracyclines are a group of bacteriostatic antibiotics inhibiting bacterial proliferation by impairing protein synthesis. This type of antibiotic blocks aminoacyl transfer-RNA by binding to the 30S subunit of bacterial ribosomes (2). Generally speaking, tetracyclines present activity against a broad spectrum of bacteria, including most Gram positive bacteria and Gram negative bacteria, aerobes and anaerobes. Depending on pharmacokinetic characteristics, tetracyclines are divided into three groups: short-acting tetracyclines (oxytetracycline, tetracycline), intermediate-acting tetracyclines (demeclocycline) and long-acting tetracyclines (doxycycline and minocycline).

1.1.5 Macrolides/ketolides

Another type of bacteriostatic antibiotics is the macrolides/ketolides, which are capable of binding to the 50S ribosome subunits, inhibiting transpeptidation and translocation during protein synthesis. Macrolides have a relatively broad antibacterial spectrum, especially against Gram positive microflora. Members of this family include erythromycin, clarithromycin, dirithromycin, telithromycin, etc., and they are often used to treat infections in respiratory systems.

1.2 Mechanism of antibiotic resistance in bacteria

Bacteria can become resistant to specific types/groups of antibiotics in many different ways, which can be summarized as: 1) innate resistance; 2) reducing intracellular
concentration of corresponding antibiotics; 3) inactivating corresponding antibiotics; 4) mutations at binding sites of corresponding antibiotics; and 5) formation of additional metabolic pathways that compensate for the loss of key enzymes or substrates (caused by corresponding antibiotics) (37).

Bacterial cells with innate resistance to a particular antibiotic naturally lack the molecule or pathway targeted by the drug, therefore the drug has no impact on the survival of the microbes. Bacteria can also become resistant to antibiotics by reducing the intracellular concentration of the antibiotic, which can be achieved with a reduction of membrane permeability (decreased import) and adoption of efflux pump (increased export). Generally speaking, Gram negative bacteria have better control over importing molecules, as their outer membranes have a smaller size exclusion limit than the peptidoglycan layer surrounding Gram positive bacteria. Hydrophilic solutes diffuse into Gram negative bacteria primarily through porins on the outer membrane. Bacterial mutants that lack porins may present a broad spectrum of resistance. Decreased permeability to chloramphenicol is regarded a cause of resistance in Haemophilus influenzae and Pseudomonas cepacia (9, 10). In Gram-positive Staphylococcus epidermidis, glycopeptide-resistance may result from overproduction of glycopeptide binding sites within the cell-wall peptidoglycan (77). Bacillus subtilis mutants resistant to the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) have altered membranes with reduced amounts of C16 fatty acids and increased ratios of iso:anteiso
branches. The CCCP-resistant mutants are cross resistant to other inhibitors including 2,4-dinitrophenol, tributylin and neomycin (30).

Energy dependent efflux pumps are another mechanism of reducing intracellular antibiotics in resistant bacteria. The efflux pumps can be specific to single antibiotics, or they can export multiple antibiotics. For example, both multi-drug resistant pumps and tetracycline-specific pumps were discovered in tetracycline resistant bacteria. The coordination of reduced membrane permeability and the presence of efflux pumps likely can contribute to high-level resistance to antibiotics. For example, it was found that active efflux pumps as well as a reduction in the amount of major porins contributed to increased resistance to chloramphenicol in some Enterobacter strains (25).

Antibiotic inactivation can be achieved by degradation or modification. One example of antibiotic inactivation is β-lactamase, which is carried by many bacterial species. β-lactamase is capable of hydrolyzing the β-lactam ring in β-lactam antibiotics, inactivating their antimicrobial activity. There are currently four types (Class A, B, C, D) of β-lactamases recognized and each type possesses its own characteristic nucleotide/amino acid sequence. Some lactamases confer resistance to various β-lactam antibiotics, whereas others are responsible for resistance to a single type of β-lactam antibiotics. It was found that many types of β-lactamases are also resistant to a variety of β-lactamase inhibitors. In other circumstances, antibiotics are not degraded but modified by resistant
bacteria. For example, aminoglycosides can be modified by bacterial phosphotransferase and acetyltransferase, losing their capability to bind the ribosome subunit (80). Chloramphenicol is inactivated by acetylation catalyzed by the chloramphenicol acetyltransferase of resistant organisms (74). Resistant pathogens modify aminoglycosides using methylases, acetyltransferases, nucleotidyltransferases, and phosphotransferases (80).

Another strategy of gaining resistance is to modify (mutate) antibiotic recognition sites on the intracellular structure/enzyme in bacterial cells, so that the corresponding antibiotics cannot recognize them. This mechanism is involved in the development of resistance to many types of antibiotics, such as quinolones (mutations on target sites in DNA gyrase), tetracycline and macrolides (mutations on corresponding ribosome subunits) (74).

Formation of alternative metabolic pathways was found to be responsible for resistance to some antibiotics that target critical metabolic activities in bacterial cells. For example, sulfonamides are effective against bacteria by competing with para-aminobenzoic acid (PABA), a substrate involved in folic acid synthesis, which is critical to nucleotide production. The inhibition of sulfonamides can be lifted by additional production of PABA, which was observed in many sulfonamide resistant bacteria species.
In summary, various mechanisms have been adopted by bacteria, leading to resistance towards certain types of antibiotics. Depending on specific mechanisms, resistance to certain antibiotics can be of low-, medium- or high-level. Bacteria can be resistant to single antibiotics, or become multi-drug resistant via one or multiple resistance mechanisms.

1.3 Acquisition of antibiotic resistance in bacteria

Resistance to antimicrobials can be caused by genetic and structural changes or temporary adjustment of metabolic machinery. AR encoded by genetic changes may be transmitted to progenies by vertical transmission and to other susceptible bacteria by HGT mechanisms. Resistance due to a particular mode of living or stress response is transient and thus is not transferable.

1.3.1 Vertical gene transmission

Both mutated genes (resulting from mutations in genes encoding antibiotic recognition site, alternative metabolic pathways, membrane porin/efflux pump or antibiotic degradation systems, etc.) as well as acquired AR genes can be passed to offspring during bacterial proliferation by vertical transmission. Mutations may be caused by errors in DNA synthesis, chemical changes induced by mutagens, or incorrect repair of damage-induced single strand breaks. Spontaneous mutations occur at varied rates in different
organisms regardless of the presence of antibiotics. The reported frequency of mutation ranges from $10^{-6}$ to $10^{-10}$ per bacterial generation (19). Normally, without the selective pressure from antibiotic usage, the mutants would only account for a very small portion of the total bacteria populations and generally would not be detected. Not only does antibiotic usage select for ART population, emerging data also suggest that constant exposure to inhibitory levels of antibiotics may significantly increase the mutation rate in bacteria and lead to multi-drug resistant offspring (40). It was found that sub-lethal levels of antibiotic treatment led to the increase of reactive oxygen species (ROS), resulting in heterogeneous increases in the minimum inhibitory concentration for a variety of antibiotics, regardless of the type of bactericidal antibiotics used. This radical-based mechanism illustrated additional impact of antibiotic usage on multi-drug resistant bacteria evolution (40).

1.3.2 Horizontal gene transmission

Horizontal gene transfer mediates the spread of resistance-determinants (AR genes) to susceptible bacterial cells, most likely by mobile gene elements, such as plasmids, transposons and integrons (85). The transfer can be conducted via conjugation, transformation (uptake of free DNA) and transduction. Unlike the time consuming mutation accumulation process, resistant units can be disseminated rapidly among organisms, within or across species and genus, via HGT events; therefore HGT mechanisms play a key role in the rapid emergence of AR in microbial ecosystems. It is worth noting that immunity gene(s) in antibiotic producing strains that protect
organisms from the antimicrobial compound (if applicable) can potentially serve as resistance-determinant(s) if acquired by others.

Mobile genetic elements in horizontal gene transmission.

Three important mobile gene elements (plasmid, transposon and integron) are involved in HGT more commonly than others. A plasmid is a type of circular DNA that self replicates itself independently of chromosomal DNA. Plasmids may carry beneficial genetic traits, such as additional metabolic utilities, and can be readily transferred to another host bacterial cell via conjugation (49). Various AR gene-encoding, evolutionary related plasmids were isolated from clinical, environmental, food and human microflora, indicating that plasmids are an important shuttle disseminating AR in nature. A transposon is a type of mobile DNA element that can “cut/copy and paste” itself using a transposase enzyme. Once separated from its original position, transposons can integrate to specific/random sites on genome or plasmid DNA (58). Transposons often contain an AR gene, thus contributing to AR dissemination. An integron is a mobile DNA element with a site-specific DNA recombination system that is capable of recognizing and capturing mobile gene cassettes (31). Depending on the content of the gene cassettes, integrons can be divided into resistance integrons, which mainly encode AR genes and can reside on plasmid and genome DNA, and super-integrons (large genome DNA elements containing an array of genes that encode a variety of adaptive functions, such as virulence and metabolic utilities). Resistance integrons are an important contributor in the
dissemination of AR through the food chain. More than 70 resistance genes have been found in resistance integrons, and a single integron may contain multiple resistance genes. Compared with integrons, insertion sequences are small and compact DNA elements that primarily encode for mobility. Once an insertion sequence contains an AR gene, it becomes a mobile vessel capable of disseminating the resistance genes within and between bacterial populations.

Horizontal gene transfer mechanisms.

Common HGT mechanisms include conjugation, transformation and transduction. Conjugation is the transfer of genetic material from donors to recipients through direct contact between the cells. Susceptible recipient cells may become resistant to corresponding antibiotics by acquiring a resistance-encoding genetic element (plasmid or transposon) from the donor cell. The frequency of conjugal transfer of functional genetic elements is affected by both the genetic compatibility of the donor-and-recipient pair and the cell concentration.

Apart from conjugation, it is also possible to transform susceptible bacterial cells with cell-free DNA segments containing AR genes, leading to acquired resistance in these cells to the corresponding antibiotics. A bacterial cell able to absorb naked DNA and integrate into the genome is called a competent cell, and the process is called transformation. The frequency of transformation is commonly low. However, given the
high concentration of free DNA in certain natural and host environments, the intake of DNA by bacteria via transformation may be a critical route in modifying the microbial ecosystem in such cases.

Transduction is a form of HGT mediated by bacterial phage, during which DNA from the donor cell can be packaged into the phage by mistake and then transmitted to the recipient cells in the subsequent round of the infection and eventually integrated into the host (recipient) genome or plasmid. Besides facilitating AR dissemination, transduction also plays an important role in bacterial evolution. For example, DNA sequence data suggest that a bacterial phage likely was involved in the evolution of *Escherichia coli* O157:H7, during which shiga-toxin encoding genes were initially transmitted from another toxin-producing bacterium to an *E. coli* strain via bacterial phage infection (23). Due to the extensive involvement of transduction in microbial evolution including pathogens, it is extremely important to re-evaluate the long-term safety impacts of phage-based intervention strategies intended to control foodborne pathogens.

### 1.4 Antibiotic resistance in human gastrointestinal tract system

It is estimated that there are more than 500 residential bacterial species (most are currently unculturable), over $10^{14}$ bacterial cells colonizing adult human gastrointestinal (GI) tract system, making it one of the most complex bacterial community in nature (28). The relationship between gut microflora and their host is mutually beneficial symbiosis
rather than a simple commensal, as gut flora contribute to a better energy metabolism, a solid immune system and repression of pathogenic bacteria in gastrointestinal tract system (66, 79).

It is widely accepted that newborn gut system is sterile; however, it is quickly colonized by bacteria originated from the mother and environment within a day or two (6). *Escherichia coli* and *Streptococci* are among initial colonizers (53), which further reduce redox potential in gastrointestinal tract system, making it favorable for subsequent residents, most of which are strict anaerobes. Recent study found that delivery method and feeding source contribute to early development of gut microflora in infant subjects. It was noticed that infant subjects born via caesarean section generally requires longer time (up to 6 months) to fully establish their gastrointestinal microflora; whereas vaginally born infant subjects may only need 1 month (78). Delivery method is regarded as a key determinant of initial contact after baby is born, as vaginally born infant subjects may have more chances contacting microflora of the mother, especially those in feces. Apart from contact (with the mother and environment), infant food also plays an important role in early development of microflora in infant GI tract. It is known that breast fed infant subjects are normally dominated with *Bifidobacteria*, while formula fed infant subjects develop more diverse gut flora which is composed of *Enterobacteriaceae*, *Enterococci*, etc. (17, 22, 33)
Previous studies have detected ART bacteria in oral cavity and fecal samples of healthy human individuals, indicating that human is a part of AR circulation in ecosystem. For example, Ready et al. found ampicillin-, penicillin-, erythromycin-, and tetracycline-resistant bacteria in oral cavities of most children subjects not exposed to antibiotics in the past 3 months, with multiple ART genera and species identified (72).

Profile and prevalence of antibiotic resistance in infant GI tract were rarely studied. Also it is known that ART bacteria exist in feces of infant subjects not exposed to antibiotic or traditional solid food, indicating that the development of ART bacteria in human GI tract can be independent of antibiotic treatment and influx of ART bacteria through food (38). Previous studies in our lab found that tetracycline resistant bacteria start to inoculate infant subjects soon after birth, and Enterococcus sp. turned out to be major tetM gene carriers in infant GI tract.

Human GI tract may be an important avenue involved in AR transmission, given the fact it is home of up to $10^{14}$ residential bacterial cells and recipient of ingested foodborne microflora. It was reported that (vancomycin resistance gene) transmission was detected through natural transformation in mouse GI tract between Enterococcus spp (8).
Our current understanding of AR in food and its association with AR in human is still limited. Data from our lab showed that tetracycline resistant bacteria are prevalent in the food chain as well as in human gut microbiota (38, 87), but the prevalence of other ART bacteria remained to be explored. While a detailed assessment of critical control points in fermented dairy products led to recommendation to the industry for effective AR control (47), the origination and the dissemination of AR in other commodity foods across the production and processing chain are yet to be revealed.

1.5 Antibiotic resistance in the food system

AR challenge is mainly due to two related issues: the emergence of multi-drug resistant pathogenic bacteria and the increased level of resistance in resistant pathogenic bacteria. As numerous studies found that the exposure to antibiotics can lead to the evolution of antibiotic resistant bacteria, it is widely accepted that the clinical environment is the major avenue where antibiotic resistance evolves; and restriction of the use of antibiotics during clinical treatment has been the primary AR mitigation strategy.

In the past decade, the isolation and characterization of AR in foodborne pathogens have raised significant concern of the application of antibiotics in food animal and agriculture production, and the potential involvement of the food chain in the dissemination of AR to the general public. Numerous studies have reported the presence of AR in various
pathogens, including but not limited to *Escherichia coli* O157:H7, *Campylobacter* spp., *Salmonella* spp. and *Listeria monocytogenes*, etc. (36, 71, 75)

On the other hand, the scope of AR studies in the food chain is limited before 2005 as most efforts were focused on pathogens, which account for a very limited bacterial population associated with foods. Although ART foodborne pathogens can be a health risk, only limited types of foodborne illnesses are treated with antibiotics. Therefore the overall health impact of the ART foodborne pathogens is limited. However, emerging evidence since 2005 on the prevalence of AR in commensal bacteria associated with seafood as well as commensal and even beneficial bacteria associated with dairy and many other commodity foods raised a serious concern of the impact of the food chain on AR dissemination. Comparing with pathogens, commensal and beneficial bacteria, such as starter culture and natural flora in food materials predominate in food ecosystem. These microflora are consumed in large amount through food intake by the public on daily basis. For example, Manuzon et al reported commercial cheese products carried as many as $10^8$ copies of resistance genes/gram of cheese (56). A broad spectrum of bacteria in the food system is found to be AR gene carriers across the food chain, ranging from raw food materials (such as raw seafood materials, meat and milk) to ready-to-consume food products (such as ready-to-eat salads, cheese, sushi). Many food commensals, including bacteria commonly used as starter cultures or probiotics were found to carry resistance genes, making them potential donors in the dissemination of AR to the consumers through the food chain. It is worth noting that probiotics, the beneficial
bacteria added to food product to minimize the contamination by spoilage and pathogenic bacteria, and to benefit gut health, are also susceptible to horizontal gene transmission, being the carrier and potential donor of AR genes. Many of identified foodborne AR gene carriers turned out to be acid tolerant facultative anaerobes, such as *Enterococcus* spp., *Streptococcus* spp., *Lactococcus* spp., etc (46, 87), indicating that food associated ART bacteria may be able to survive in human GI tract.

The influx of antibiotic resistant bacteria through human digestive tract system may potentially impact the ecology of human gut microflora in intestinal tract system by: 1) colonization in human digestive tract system and become a member of residential microflora; 2) exchange mobile gene elements with other residential gut flora by conjugation/natural transfer/transfection, transmitting antibiotic resistance in bacterial population. In fact, many food commensal bacteria were found to be able to colonize mammals’ intestinal tract soon after input. The transmission of antibiotic resistance genes is largely affected by 1) whether the resistance genes locate on mobile gene elements, 2) the size of the AR gene pool, 3) the chance of contact between gene donor and recipient, and 4) the genetic compatibility of conjugation/natural transfer/transduction between the donor and recipient. The constant supply of ART bacteria through food intake, partnered with occasional colonization and horizontal gene transmission, inevitably will lead to increased resistance in gut microflora, and enhance AR circulation and evolution in the ecosystems. It is worth noting that certain bacteria, such as ART *Enterococci* and other lactic acid bacteria, are found prevalence in both the food and host ecosystems, and can
readily transmit the AR genes to other human residential and pathogenic bacteria in laboratory settings. The data raised a significant concern on the real impact of certain practices, such as the application of probiotics without proper screening of their potential in horizontal gene transfer, in food animal and aquaculture production as AR mitigation control, on the emergence and evolution of AR in the natural and host environment (86, 87, 91)
Chapter Two

Establishment and Development of Antibiotic Resistant Bacteria in Human Infant Gastrointestinal Tract

2.1 Abstract

The rapid emergence of antibiotic resistance (AR) is a major public health concern. Recent findings on the prevalence of foodborne antibiotic resistant (ART) commensal bacteria in ready-to-consume food products suggested that daily food consumption likely serves as a major avenue for disseminating ART bacteria from the food chain to the human hosts. To properly assess the impact of various factors including the food chain on AR development in hosts, it is important to determine the baseline of ART bacteria in human gastrointestinal (GI) tract. The study examined gut microflora of 6 infant subjects from newborn to 1 year of age, fed on breast milk and/or infant formula during early stage of development and without prior exposure to antibiotics. Predominant bacterial populations resistant to several antibiotics and multiple resistance genes were found in the infant GI tracts within the first week of age. Several ART population transitions were also observed in the absence of antibiotic exposure and dietary changes. Representative AR gene pools including tet(M), ermB, sul2 and blatem were detected in infant subjects.
Enterococcus spp., Staphylococcus spp., Klebsiella spp., Streptococcus spp. and Escherichia coli/Shigella spp. were among the identified AR gene carriers. ART bacteria were not detected from infant formula and infant foods examined, but small numbers of skin-associated ART bacteria were found in certain breast milk samples. The data suggested that the early development of AR in human gut microbiota is independent of infants’ exposure to antibiotics, but is likely impacted by exposure to maternal and environmental microbes during and after delivery, and that ART population is significantly amplified within the host even in the absence of the antibiotic selective pressure.

2.2 Introduction
The rapid emergence of antibiotic resistant (ART) pathogens has been a critical public health concern with major social impact. It is estimated that nosocomial infections are responsible for 99,000 deaths in the US every year (39), and more than 70% of the bacteria that caused such infections are resistant to at least one of the antibiotics most commonly used in clinics (12, 12). Besides, drug resistant bacterial infections take longer and cost more during treatment, adding additional $5 billion to the US healthcare system annually (64, 64). It is well documented that the selective pressure due to unrestricted uses of antibiotics likely contributed to the increased antibiotic resistance (AR) seen today (3, 11). However, recent data from food animal studies have shown that limiting the use of antibiotics in food animal production resulted in modest reduction in the prevalence instead of elimination of resistance in certain bacteria (83, 83). In fact, many
AR genes are found to persist in the absence of the antibiotic selective pressure due to various molecular mechanisms (47, 60, 73, 86), and certain ART bacteria even have niche fitness advantage against the wild type strains ((52). These data presented a much more serious and complicated picture of AR than previously realized, suggesting that once AR has evolved in pathogens, simply lifting the antibiotic selective pressure might not be enough for effective mitigation (47, 86).

It is well established that many AR genes from both pathogens and commensal bacteria are highly homologous and encoded by transferable genetic elements; the frequency of horizontal gene transfer (HGT) events is correlated to the size of the AR gene pool as well as the genetic features and compatibility of the donor and recipient (65, 86). Commensal bacteria represent most of the microbial population in diversified host and natural ecosystems, and many commensals shared similar genetic background with the pathogenic variants. Therefore it is becoming recognized that commensal bacteria likely play a key role in the evolution and dissemination of genetic elements including AR genes in microbial ecosystems (4, 51, 57, 63, 81, 86, 87). While the large AR gene pools in food animals, lagoon water and farm manures (3, 15, 43, 63, 69, 82) might be related to the exposure to growth promoting antibiotics, the high prevalence of ART bacteria in oral and fecal samples of healthy human subjects and wild animals without recent history of antibiotic exposure (5, 26, 29, 44, 67, 72, 76) suggested that there might be major knowledge gaps regarding AR acquisition and circulation pathways involving human and animal hosts. In fact, recent studies revealed a large number and broad spectrum of foodborne ART commensal bacteria with resistance-encoding genes in conventional
retail foods including many ready-to-consume items, suggesting humans are constantly exposed to ART bacteria through daily food intake (21, 47, 87). However it was still unknown to what extent the food chain impacted the ART microbiota in host GI tracts. Therefore, the objective of this study was to reveal the baseline of the ART microbiota in human intestinal tracts during early development in infant subjects, who had not been exposed to therapeutic antibiotics as well as the ART bacteria-rich conventional foods consumed by the general public. The knowledge will contribute to a comprehensive understanding of AR origination, dissemination, maintenance and circulation pathways, which is essential for the development of targeted and effective mitigation.

2.3 Materials and Methods

2.3.1 Subjects and samples

Six healthy infant subjects between newborn and 12 months of age, born between 2008 and 2010, were recruited according to OSU IRB protocol 2006H0083. Infant subjects had no history of therapeutic antibiotic exposure, and were fed exclusively on breast milk and/or infant formula until baby foods were introduced to the diet in the later stage of development. While mothers of the infant subjects were not exposed to therapeutic antibiotic treatment during the last 3 months of pregnancy, it was unknown whether they were treated with antibiotics during delivery. Multiple fecal samples were acquired from two individual subjects to monitor the dynamics of the ART bacteria population during infant development, and a single sample was collected from the rest of the infant subjects for surveillance study. Meanwhile, for comparison, mothers of the two infant subjects involved in the study were also recruited during the same sampling period following the
same IRB protocol. Fecal samples from the infants were stored in refrigerators and delivered to the research lab mostly within 2 h of production, and the fecal solids were removed from the diapers and processed in the laboratory immediately upon receipt. Fecal samples from the lactating mothers were collected in sterile containers, delivered to the researcher and processed in the lab following the same approach. In addition, representative infant formula for both babies and breast milk samples from the two mothers were also collected in sterile tubes and delivered to the researcher for further assessment.

2.3.2 Bacterial culture recovery

The following media agar plates were used in recovering the total bacterial population: Tryptic Soy Agar (TSA), Columbia blood agar base with (CBA) and without (CA) 5% defibrinated sheep blood, De Man, Rogosa and Sharpe Agar (MRS), Reinforced Clostridial Agar (RCA), Enterococcus Agar (ENT), MacConkey Agar (MAC) supplemented with 1% glucose, and Brain Heart Infusion Agar (BHI), to assess the cultivable microbial population. The sheep blood was from Fisher Scientific, Hampton, NH, and all bacterial media were from Becton, Dickinson and Company, Franklin Lakes, NJ. The same type of media containing one of the following antibiotics: 16 μg/ml of tetracycline (Fisher Biotech, Fair Lawn, NJ), 100 μg/ml of erythromycin (Fisher Scientific), 152 μg/ml of sulfamethoxazole (Sigma-Aldrich, St. Louis, MO) and 8 μg/ml of trimethoprim (Sigma-Aldrich), 2 μg/ml of cefotaxime (Sigma-Aldrich), or 8 μg/ml of ceftiofur (Sigma-Aldrich), were used to assess the resistant population. Cycloheximide (Sigma-Aldrich) was added to all agar plates to 100 μg/ml to minimize the growth of
yeasts and molds. One gram of fecal sample was serially diluted in 0.85% NaCl, 0.1% peptone water solution and plated in duplicate on the corresponding bacterial agar plates. The plates were incubated under anaerobic conditions using GasPak™ 150 anaerobic system and GasPak™ EZ anaerobe container system with indicator (Becton, Dickinson and Company) at 37°C for 48 h.

2.3.3 DNA extraction

The procedure described by Li et al (47) was followed to extract DNA from bacterial isolates as amplification templates for conventional PCR. For real-time quantitative PCR and DGGE analyses, the DNA templates from fecal and infant food samples were extracted according to Yu and Morrison (90).

2.3.4 Denaturing gradient gel electrophoresis analysis

Bacterial cells were collected from a minimally diluted medium agar plate with a sterilized tongue depressor and subjected to total DNA extraction as previously described (90). The concentration of total DNA was determined with a NanoDrop spectrophotometer (ND-1000, ThermoFisher Scientific, MA), and DNA samples were diluted to 50 ng/µl as templates for PCR amplification. The PCR primer pairs (16S-357F-GC, 16S-518R) targeting the 16 rRNA V3 region were used to amplify the partial 16S rRNA gene following procedures by Muyzer (62). PCR products were loaded on 8% acrylamide gel with 30% to 65% gradient urea. The electrophoresis was performed at 60°C under 83V for 16 hours, using DCode system for DGGE (Bio-Rad, Hercules, CA). The gel was stained in 0.01% ethidium bromide and visualized using the ChemiDoc
XRS+ system (Bio-Rad). DGGE image was further processed with BioNumerics software (version 5.1, Applied Maths NV, Belgium). Specific DGGE bands were selected for excision and sequence determination. Each band was first washed in 500 µl deionized water and soaked in 20 µl deionized water. Bands were then frozen at -20°C, followed by being thawed at 70°C for 5 minutes. The freeze-thaw cycle was repeated twice, and the supernatant was used to amplify partial 16S rRNA gene, which is subjected to direct sequencing analysis at the high-throughput genomics unit at University of Washington, Seattle.

2.3.5 Resistance gene, AR gene carrier and AR gene pool assessments

The presence of representative AR genes from culture recovered resistant isolates was examined by conventional PCR as described previously (87). Primers used in the screening of resistance genes were listed in Table 1 and synthesized by Sigma-Aldrich. Approximately 10% of the positive PCR products for the AR genes screened were confirmed by DNA sequencing using ABI PRISM 3700 (Applied Biosystems, Foster City, CA) at the Plant Microbe Genomics Facility, The Ohio State University and compared with published AR gene sequences deposited in the NCBI database. Up to 50 recovered ART isolates per resistance phenotype were screened for AR genes per infant subject or time point. Approximately 1/4 to 1/3 confirmed AR gene carriers (isolates carrying the representative AR genes) were selected and identified by partial 16S rRNA gene sequence analysis as described previously (87). Taqman real-time PCR was used in assessing representative AR gene pools in both the fecal or food sample DNA extracts, as well as DNA extracts from plate-recovered cultures following procedures described
previously (56). The *tet(M)* carrier was spiked to meconium instead of cheese matrices in the validation study. The probes used in the assessment were synthesized by Biosearch Technology Inc. (Novato, CA), and the probe sequences and sizes of the amplicons were listed in Table 1. The real-time PCR was performed on a CFX96 system (Bio-Rad).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Size of amplicon</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>tet(M)</em> FP</td>
<td>CGAACAAGAGGAAAGCATAAG</td>
<td>974 bp</td>
<td>(47)</td>
</tr>
<tr>
<td><em>tet(M)</em> RP</td>
<td>CAATACAATAGGAGCAAGC</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td><em>ermB</em> FP</td>
<td>TGGTATTCCAAATGCGTAATG</td>
<td>745 bp</td>
<td>(55)</td>
</tr>
<tr>
<td><em>ermB</em> RP</td>
<td>CTGTGGTATGCGGGGTAAGT</td>
<td></td>
<td>(55)</td>
</tr>
<tr>
<td><em>sul2</em> FP</td>
<td>GCAGGCGCGTAAGCTGA</td>
<td>657 bp</td>
<td>this study</td>
</tr>
<tr>
<td><em>sul2</em> RP</td>
<td>GGCTCGTGTTGCGGGATG</td>
<td></td>
<td>this study</td>
</tr>
<tr>
<td><em>bla</em>TEM FP</td>
<td>CATTTCGGTGTCGCCCTTATTC</td>
<td>800 bp</td>
<td>(18)</td>
</tr>
<tr>
<td><em>bla</em>TEM RP</td>
<td>CGTTGATCAGGTGCCGCTGAC</td>
<td></td>
<td>(18)</td>
</tr>
<tr>
<td><em>tet(M)</em> real FP</td>
<td>GAACATCGTAGACACTCAATTG</td>
<td>169 bp</td>
<td>this study</td>
</tr>
<tr>
<td><em>tet(M)</em> real RP</td>
<td>CAAACAGGTTCCACCGG</td>
<td></td>
<td>this study</td>
</tr>
<tr>
<td><em>tet(M)</em> probe</td>
<td>CGGTGTATTCAAGAATATCGTA</td>
<td></td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>GTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tet(S)</em> FP</td>
<td>GAACGCGGAGAGGTTATT</td>
<td>1050 bp</td>
<td>(48)</td>
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Table 1. List of primers and probes included in infant AR study
Table 1 continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Type</th>
<th>Forward/Reverse</th>
<th>Sequence</th>
<th>Length</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>tet(S)</td>
<td>RP</td>
<td>TACCTCCATTTGGACCTCAC</td>
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<tr>
<td>tet(L)</td>
<td>FP</td>
<td>TTGGATCGATAGTAGCC</td>
<td>908 bp</td>
<td>(48)</td>
<td></td>
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<tr>
<td>tet(L)</td>
<td>RP</td>
<td>GTAACCAGCCAACTAATGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ermB real</td>
<td>FP</td>
<td>GAAAGCCRTGCGTCTGACATC</td>
<td>105 bp</td>
<td>this study</td>
<td></td>
</tr>
<tr>
<td>ermB real</td>
<td>RP</td>
<td>CGAGACTTGAGTGCTGCAAGAGC</td>
<td>this study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ermB probe</td>
<td></td>
<td>ACCTTGGATATTCCACCGAAACTAG</td>
<td></td>
<td></td>
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<tr>
<td>sul2 real</td>
<td>FP</td>
<td>GATATTCGCGGTTTTCCAGA</td>
<td>151 bp</td>
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<tr>
<td>sul2 real</td>
<td>RP</td>
<td>CAAAGAAACCGCCGCAATGT</td>
<td>this study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sul2 probe</td>
<td></td>
<td>ATCATCTGCAAAAACGATCGGTA</td>
<td></td>
<td></td>
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<tr>
<td>TGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaTEM real</td>
<td>FP</td>
<td>CACTATTCTCAGAATGACTTGGT</td>
<td>(43)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaTEM real</td>
<td>RP</td>
<td>TGCTAATTCTCTTACTGTCATG</td>
<td>85 bp</td>
<td>(43)</td>
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<tr>
<td>blaTEM probe</td>
<td></td>
<td>CCAGTCACAGAAAAGCATCTT</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CGG</td>
<td></td>
<td></td>
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<tr>
<td>blaCMY-2 FP</td>
<td>GACAGCCTCTTCTCCACA</td>
<td>1143 bp</td>
<td>(93)</td>
<td></td>
<td></td>
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<tr>
<td>blaCMY-2 RP</td>
<td>TGGAACGAAGGCTACGTA</td>
<td>(93)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>16S-357F-GC</td>
<td>CGCCCCCGCGCGCCGGGGGGGCGGCGG</td>
<td>233 bp</td>
<td>(62)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>GGGGCGGGGCGACGGGGGGGCGCTTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGGGAGGCAGCAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S-518R</td>
<td>ATTACCACGGGCTGCTGG</td>
<td>(62)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
2.3.6 Determination of susceptibility to tetracycline

Twenty-four isolates were randomly selected from identified Enterococcus spp. Tet(M) carriers and subjected to antimicrobial susceptibility test by determining the minimum inhibitory concentration (MIC) to tetracycline in Columbia broth (Becton Dickinson and Company, Franklin Lakes, NJ) in microtiter plates, following procedures as described previously(87).

2.4 Results

2.4.1 Prevalence of Tet$^r$ bacteria and tet(M) gene pool in infant gut microbiota.

All seven media were first used in a pilot study to compare their efficacy in recovering both total bacteria and Tet$^r$ bacteria from 3 subjects, by conventional plating method. Among all the media examined, CBA plates had the highest or next-to-highest numbers of bacterial colonies recovered, as well as the greatest diversity in morphology of recovered bacterial colonies. The identified colonies recovered from the CBA plates including a broad spectrum of bacterial species assigned to the genera: Bacteroides, Bifidobacteriaceae, Brevundimonas, Enterococcus, Granulicatella, Lactococcus, Lactobacillus, Serratia, Staphylococcus, Streptococcus, and Enterobacteriaceae (include Citrobacter, Klebsiella, Enterobacter, Escherichia coli/Shigella). Thus CBA was chosen in the rest of the study to assess cultivable bacterial population from infant fecal samples. In addition, MAC plate was used as a supplement to assess resistance particularly in Gram negative population. In agreement with previous report (29), among the 3 Tet$^r$-encoding genes (tet(M), tetS, tetL) screened, tet(M) was most prevalent in resistant isolates from the subjects assessed.
Previous work by a lab colleague illustrated the number of total and Tet\textsuperscript{r} bacteria recovered on CBA plates, and the size of the *tet(M)* gene pool in fecal samples from 9 infant subjects ranging from 1 month to 12 months of age (Fig 1). The Tet\textsuperscript{r} bacterial populations in all infant subjects within the age range were at or more than $10^6$ CFU/g. The cultured total bacterial counts on the non-selective plates were 10 to 100-fold higher than that on the tetracycline-containing plates, suggesting approximately 1-10% of the cultivable gut microbiota were Tet\textsuperscript{r}. The data suggested that Tet\textsuperscript{r} bacteria were detected in infant gastrointestinal tracts at very early stage and persist during infant development. In agreement, the size of the *tet(M)* gene pool reflected the same trend.
Fig. 1 Assessment of tetracycline resistance in fecal samples from infant subjects. ■ Total bacteria count on CBA plates; ▲ Tet<sup>r</sup> bacteria count on CBA plates; □ copy no. of tet<sup>(M)</sup> gene by real-time PCR. Real-time PCR was performed on two independent DNA extract replicates and the coefficient variations of log copy no. of resistance gene were less than 0.05.

2.4.2 The development of ART bacteria and representative AR gene pools in infant subjects

Since the above study was conducted using individual subjects, each sampled at one single time point, variations among subjects could have significant impact on the results. Thus the development of the Tet<sup>r</sup> bacteria and tet<sup>(M)</sup> gene pool in two twin subjects raised in the same family was followed 9 times within 12 months after birth. Data from our laboratory showed that in both subjects, the total bacteria, Tet<sup>r</sup> bacteria counts and the
size of the \textit{tet}(M) gene pool steadily increased within the first month and reached to \(10^9\) - \(10^{10}\) CFU/g, \(10^7\) CFU/g and \(10^6\) \textit{tet}(M) gene copies per gram of fecal sample, respectively, and the values maintained within approximately one log variation throughout the rest of the year (data not shown). The results again suggested that Tet\(^\text{r}\) microbiota was mostly colonized within the first month post delivery (38).

To reveal the details of AR dynamics, including potential resistance against additional antibiotics besides tetracycline within the early stage of infant development, two individual subjects were further examined from newborn to 6 months of age, particularly during the first month post delivery. Fig. 2 illustrated the ART bacterial population as well as the representative AR gene pools in Baby Q. While no cultivable bacterial isolates were detected in meconium sample recovered during the same day (Day 0) of delivery, Erm\(^\text{r}\) (Fig 2B), Sul\(^\text{r}\), and Ctx\(^\text{r}\) bacteria (data not shown) were detected in infant feces within one day (Day 1) after birth, and Tet\(^\text{r}\) (Fig 2C) bacteria were detected from Day 3. Within the first several days, total bacteria as well as ART bacteria counts increased rapidly in infant feces. By Day 8, fecal bacteria count rose up to \(10^{10}\) CFU/g and \(10^9\) CFU/g on CBA and MAC plates, respectively (Fig 2A). ART bacteria counts reached to \(10^7\) CFU/g (Tet\(^\text{r}\)) and \(10^9\) CFU/g (Erm\(^\text{r}\)) on CBA plates containing antibiotics. The ratio of Tet\(^\text{r}\) bacteria to total bacteria changed dramatically, from 0.0006\% at time of initial detection to 1\% by Day 25 and approximately 10\% by Day 100. Similarly, the percentage of Erm\(^\text{r}\) bacteria increased from approximately 1\% of the population at time of initial detection to more than 90\% on CBA plates containing erythromycin by Day 30 and thereafter. The percentages of phenotypically Ctx\(^\text{r}\), Cft\(^\text{r}\) bacteria on CBA plates
containing antibiotics maintained at approximately 1% of the population during the studied period (4 months).
Fig. 2 Assessment of AR in fecal samples from Baby Q between newborn to 4 months of age. A. ■ total bacterial count on CBA plate, □ total bacterial count on MAC plate; B. ■ Erm\(^r\) bacterial count on CBA plate, □ Erm\(^r\) bacterial count on MAC plate, ■ copy no. of \(ermB\) genes by real-time PCR; C. ■ Tet\(^r\) bacterial count on CBA plate, □ Tet\(^r\) bacterial count on MAC plate, ■ copy no. of \(tet(M)\) genes by real-time PCR. Real-time PCR was performed on two independent DNA extract replicates and the coefficient variations of log copy no. of resistance gene were less than 0.05.
Fig. 2 Assessment of AR in fecal samples from Baby Q between newborn to 4 months of age.
ART bacteria recovered from MAC plates emerged relatively late during infant development, mostly detected after one week (Day 8), except that Tet<sup>r</sup> bacteria were not detected from MAC plates containing tetracycline throughout the entire sampling period. The percentage of ART bacteria recovered on MAC plate remained relatively stable since the initial detection. After Day 15, nearly all bacteria recovered from MAC plates were phenotypically Erm<sup>r</sup>. Since emergence, Ctx<sup>r</sup> and Cft<sup>r</sup> bacteria constituted less than 0.0006% and 0.000007%, respectively, of total population.

The sizes of representative AR gene pools varied during infant development in Baby Q (Fig. 2). The tet<sup>(M)</sup> gene pool was not detected in the meconium sample, but increased rapidly afterwards (Fig. 2C). By Day 8, the size of the tet<sup>(M)</sup> gene pool reached to $10^9$.
copies/g of fecal sample and maintained at a stable level thereafter. This result was consistent with data from the above mentioned one year-long study using the pair of twin infant subjects. The development of the $ermB$ gene pool (Fig 2B) followed a similar pattern but more rapidly as that of $tet(M)$, from barely detectable on Day 1 to above $10^7$ copies/g on Day 3. The $sul2$ gene pool was detected at a very low level sporadically in the first week, and was below detection limit thereafter.

Similar development patterns of ART bacteria counts and AR gene pool were observed in fecal samples from another subject Baby P. Tet$^r$, Erm$^r$ and Sul$^r$ bacteria were detected in feces on Day 1 and increased rapidly in the first week. By Month 3, the AR gene pools found in Baby P’s feces included $tet(M)$ ($10^8$ copies/g), $ermB$ ($10^8$ copies/g), $sul2$ ($10^6$ copies/g) and $bla_{TEM}$ ($10^7$ copies/g). The AR gene pools were persistent thereafter ($10^7$ copies/g for $tet(M)$, $10^6$ copies/g for $ermB$, $10^6$ copies/g for $sul2$, and $10^7$ copies/g for $bla_{TEM}$ by Month 6).

Moreover, to reveal the dynamics of bacterial composition within both total and phenotypic resistant population during the first 4 months of infant development, during which the two subjects were exclusively fed with breast milk supplemented with infant formula, fecal samples from Baby P and Q were further assessed by 16S rRNA gene fragment PCR coupled DGGE analysis. Fig. 3 illustrated the predominant ART bacteria recovered from the CBA plates at selected time points during the development of Baby Q. For most resistance subtypes, predominant ART population established shortly after birth, and persisted in weeks. In Baby Y, major population shifts were observed at several time
points during the 4-month period, specifically between 6\textsuperscript{th} and 8\textsuperscript{th} week, 2\textsuperscript{nd} and 4\textsuperscript{th} month for Tet\textsuperscript{r} bacteria (Fig. 3A), 4\textsuperscript{th} and 8\textsuperscript{th} day, 4\textsuperscript{th} and 5\textsuperscript{th} week, 2\textsuperscript{nd} and 4\textsuperscript{th} month for Erm\textsuperscript{r} bacteria (Fig 3B). The initial main ART population in GI tract included \textit{Staphylococcus} spp. (Tet\textsuperscript{r}, Erm\textsuperscript{r}), \textit{Streptococcus} spp. (Tet\textsuperscript{r}, Erm\textsuperscript{r}), \textit{Veillonella} sp. (Tet\textsuperscript{r}), and \textit{Peptoniphilus} sp. (Tet\textsuperscript{r}). By Month 3, \textit{Enterococcus} sp. (Tet\textsuperscript{r}) and \textit{Enterobacteriaceae} (Erm\textsuperscript{r}) become part of the predominant ART population in infant feces. Similar ART population development trends were also observed in Baby P, although the timing of population transition did not exactly overlap with that in Baby Q.
Fig. 3 Predominant ART bacteria during infant (Baby Q) development by DGGE analysis.
A: Tet$^r$ bacteria on CBA plates, B: Erm$^r$ bacteria on CBA plates. Data were presented in a time-lapse manner (D-day, W-week, M-month). DNA ladder was labeled as “L”. Fig. 3A, *Staphylococcus* spp. (a-e), *Streptococcus* spp. (f), *Veillonella* spp. (g-i), *Peptoniphilus* spp. (j), *Enterococcus* spp. (k). Fig. 3B, *Staphylococcus* spp. (l, n), *Streptococcus* sp. (m), *Klebsiella* sp. (o-q), *Enterobacteriaceae* (r).

2.4.3 Antibiotic resistance determinants and cultivable AR gene carriers in infant GI tracts

Since phenotypic resistance to antibiotics may also be due to intrinsic resistance, we further conducted genetic screening of representative AR genes. In addition to the AR
gene pools examined, Table 2 summarized the prevalence of representative AR genes in recovered ART isolates and the identities of representative AR gene carriers. Among studied AR genes, \textit{tet}(M), \textit{erm}B and \textit{sul}2 were detected in all infant subjects examined. Among 1130 \textit{Tet} isolates recovered from CBA-Tet plates examined in this study (from 14 infant subjects), 58\% contained \textit{tet}(M). Limited \textit{tet}(M) carriers (153 of 651) with diversified colony morphology were identified and the majority was found to be \textit{Enterococcus} spp. (74\%). Other identified \textit{tet}(M) carriers included \textit{Staphylococcus} spp. (11\%), \textit{Streptococcus} spp. (11\%), \textit{Escherichia coli}/\textit{Shigella} spp. (2\%), \textit{Providencia} sp. (1\%), and \textit{Veillonella} sp. (1\%). The \textit{erm}B gene was also found to be prevalent in infant GI tract, with 34\% screened resistant isolates being \textit{erm}B\textsuperscript{+}. Most \textit{erm}B carriers turned out to be \textit{Enterococcus} sp. (47\%) and \textit{Streptococcus} sp. (33\%), and \textit{Klebsiella} sp. as well as \textit{Staphylococcus} sp. were also detected. The \textit{sul}2 gene was detected in 35 \textit{Sul} isolates from a single subject by Month 3 and 7, and all isolates were identified as \textit{E. coli}/\textit{Shigella} spp.
Table 2. The prevalence of AR genes detected and identified carriers in infant fecal samples

<table>
<thead>
<tr>
<th>AR gene</th>
<th>Prevalence in infant subjects</th>
<th>Prevalence in corresponding ART population</th>
<th>AR gene carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>tet(M)</td>
<td>100% (14/14)</td>
<td>58% (651/1130)</td>
<td>Enterococcus sp. (114/153), Escherichia coli/Shigella sp. (3/153), Providencia sp. (1/153), Veillonella sp. (1/153), Streptococcus sp. (17/153), Staphylococcus sp. (17/153)</td>
</tr>
<tr>
<td>ermB</td>
<td>100% (4/4)</td>
<td>34% (86/250)</td>
<td>Enterococcus sp. (14/30), Streptococcus sp. (10/30), Klebsiella sp. (5/30), Staphylococcus sp. (1/30)</td>
</tr>
<tr>
<td>sul2</td>
<td>100% (4/4)</td>
<td>54% (85/157)</td>
<td>Escherichia coli/Shigella sp. (36/36)</td>
</tr>
</tbody>
</table>

The identified AR gene carriers during infant development were compatible with the predominant phenotypic ART population by DGGE. For example, the tet(M) carriers (6
out of 75, collected on Day 4 and 8) from Baby Q during the first week of growth were found as *Staphylococcus* spp. and *Streptococcus* spp., which turned out to be the two predominant groups of Tet\(^r\) bacteria by DGGE. However, the overall prevalence of *tet(M)* in Tet\(^r\) population was relatively low (4%) by then. Starting from Week 2, the percentage of *tet(M)* carriers in Tet\(^r\) isolates on CBA plate significantly increased to 30% (15 *tet(M)*\(^+\) out of 50 screened, Day 9), with all identified *tet(M)* carriers as *Staphylococcus* sp. The percentage and identity of the *tet(M)* carriers maintained till Week 4 (15/50, Day 25). A population switch on *tet(M)* carriers was observed following Week 8: while the percentage of *tet(M)* carrier in Tet\(^r\) isolates remained to be approximately 30%, among 13 *tet(M)* carriers identified, 8 were *Enterococcus* sp. and 5 were *Staphylococcus* sp. At the end of Month 4, 70% of the Tet\(^r\) isolates on CBA plate were *tet(M)* carriers (35/50), and most *tet(M)* (63%) carriers were *E. faecalis*.

The prevalence of cultivable *ermB* carriers in Baby Q were found to reach its maximum at Day 3. Out of 50 isolates screened, 50% were *ermB* carriers. Identified *ermB* carriers include *Streptococcus* sp. (10/11), and *Staphylococcus* sp. (1/11). However, no cultivable *ermB* carriers were identified after one week (0/50, Day 8, Day 51) in Baby Q, while the size of *ermB* gene pool did not change significantly. The results indicated a major *ermB* carrier population transition, but the main carriers were not cultivable on CBA or MAC plates. The phenotypic Erm\(^r\) population on the CBA or MAC plates could be due to the intrinsic or acquired resistance mechanism(s) not examined in this study.

2.4.4 Prevalence of AR gene pools in infant foods and mothers’ gut flora
No cultivable bacteria were detected from both infant formula and infant food samples used by Baby P and Q in the study. But a small amount of ART bacteria and AR genes were found in breast milk samples. For instance, a breast milk sample from Mother P contained $10^6$ copies of tet(M) gene, $10^6$ copies of ermB gene and $10^4$ copies of sul2 gene per ml of sample, with major tet(M) gene carriers as *Streptococcus* spp. (10/12), *Granulicatella* spp. (2/12) and sul2 carriers being *Brevundimonas* sp. (1/1). No AR genes examined were found in the ART bacteria isolated from breast milk of Mother Q.

Fig. 4 compared the AR gene pools in fecal samples from infants at 4 months of age with that from their mothers. While the tet(M), ermB, sul2 gene pools were detected in stool samples of both Mother P and Mother Q, the sul2 gene pool, though was detected at the earlier age, was absent in Baby Q, and the blaTEM gene pool found in Baby P was absent in Mother P. One out of 50 Tet\(^r\) isolates screened from Mother P was tet(M) carrier (*Streptococcus* sp.), while 24% Sul\(^r\) isolates were sul2 carriers (11 *Bacteroides* sp., 1 *Enterococcus* sp.). The Ctx\(^r\) *Citrobacter* sp. containing bla\(_{CMY-2}\) gene was detected from Mother P’s feces, but not in the corresponding infant subject.
Fig. 4 AR gene pools in infant subjects and corresponding mothers. Total DNA were collected when Subject P and Subject Q were 4 months of old. ■ tet(M) gene pool; ☐ ermB gene pool; □ sul2 gene pool; ☣ blaTEM gene pool. Data represent means of two independent replicates and coefficient variations of log copy no. of resistance gene were less than 0.05.

2.5 Conclusion and Discussion

The initial purpose of the study was to use infant subjects, presumably free of ART bacteria before birth, to investigate the impact of foodborne ART bacteria and antibiotic exposure on the gradual establishment of the resistant population in the GI tract during development. The discovery of high levels of ART bacteria within the first few days post delivery in fecal samples from all subjects examined, however, clearly indicated that the initial ART population establishment is independent from antibiotic exposure.
Recent evidence illustrated that infant gut microbiota are impacted by microbiota from the mother during delivery, and the gut flora are established rapidly in babies delivered naturally but much slower in those by caesarean section (27). As illustrated in Fig 4, several AR gene pools were found in fecal samples from both the infants and their mothers, but they did not exactly match. Further, the profiles of AR gene carriers of the fecal samples from infant and their mothers also shared some similarity but were not exactly the same. We suspect that birth delivery exposure impacted the establishment of ART flora including anaerobes, since all infant subjects in this study were delivered naturally, but data from this study did not rule out other sources of microbial inoculation. The finding of ART bacteria in breast milk samples indicated another potential AR exposure route. However no cultivable ART bacteria were detected in breast milk sample collected after the nipple was cleaned with over-the-counter rubbing alcohol (data not shown), suggesting environmental contact (from the mother skin in this case) can eventually translate to oral inoculation in infants. In fact some of the main AR gene carriers identified in infant feces such as Streptococcus sp., Staphylococcus sp. and Enterococcus sp. were commonly associated with the human host and natural environment, although no further effort was attempted in this study to compare the detailed genetic background among the organisms from the infant feces, breast milk and skin samples.

Though AR in human oral and intestinal microbiota had been reported in the past, our knowledge regarding drug resistance in a broad spectrum of commensal bacteria in human GI tract remains to be limited, largely due to the technical, cost and labor
challenges associated with studies involving the complex and diversified commensal population. It is worth noting that antibiotic-containing media screen for phenotypic ART population, which include bacteria intrinsically resistant to the drug (such as lack of target molecule for the antibiotic) and those containing resistance-encoding genes (AR gene carriers). Although commensal AR gene carriers do not cause diseases, the concern is their potential involvement in dissemination AR genes to others including pathogens via HGT events. However, AR gene carriers also vary in their abilities in disseminating resistance genes. For instance, chromosomally encoded AR genes not associated with mobile gene elements (such as transposons) have less chance to be successfully transmitted to and retained in other bacteria via natural HGT mechanisms. Since AR gene pool assessment by qPCR did not provide information regarding the functionality and mobility of the AR genes, characterization of ART isolates is very important for proper risk assessment. In this study, among 32 representative plasmid-containing isolates (out of 166 tet(M)$^+$ isolates) examined, tet(M) gene was found located on either the chromosome or both plasmid and chromosome by southern blot analysis (data not shown). Their affiliation with transposable elements (IS elements, transposons) remains unknown. Among 24 tet(M)$^+$ enterococcal isolates examined, 21 had a MIC higher than 128 µg/ml (128 µg/ml for 14, 256 µg/ml for 5, and 512 µg/ml for 2), which illustrated the functionality of the AR genes in these isolates. Further studies are needed to characterize the mobility of the AR genes in vitro and in vivo from representative intestinal isolates.

Our study revealed the rapid development of the ART bacteria and AR gene pools, as well as several major ART bacteria population transitions in the gut flora during the early
stage of infant development, all prior to antibiotic exposure and major dietary change. The findings have several important implications. First, in addition to the one-time oral/nasal exposure to ART bacteria during birth, only small numbers of ART bacteria were found in breast milk samples. The overall ART bacteria intake in infant subjects was much smaller than those found in feces. On the other hand, Erm\textsuperscript{r} bacteria increased from 1% to 90% and Tet\textsuperscript{r} bacteria from less than 0.0006% to 30% of the infant gut microbiota in approximately 1 and 3 months, respectively, after delivery. These data indicated that ART bacteria likely were significantly and unevenly amplified in the host GI tract, in the absence of corresponding antibiotic selective pressure. Thus hosts likely played a significant role in the amplified circulation of AR genes and ART bacteria in the ecosystems, even in the absence of drug exposure. This is in agreement with findings showing the prevalence of Tet\textsuperscript{r} bacteria in feces from home-bred hamsters without prior exposure to antibiotics (45). In addition, animal wastes and lagoon water are also rich in ART bacteria and AR gene pools (43, 69, 82). Since the release of feces/manure further impacts the environmental ART bacteria and AR gene pool, proper fecal/manure treatment should be a critical control point for targeted AR mitigation (86).

In addition, our data showed that although resistance to Tet and Erm were highly prevalent in all subjects examined, the prevalence of sul2 and bla\textsubscript{CMY2} genes was much lower. It is unclear whether the differences in prevalence are related to humankind’s application history of these antibiotics. But arguably it is possible to decelerate the emergence of AR in pathogens and host ecosystems if targeted intervention strategies can be implemented in time.
Second, results from the study uncovered the dynamics of ART bacteria transition, even though the AR gene pools (*tet(M)* and *ermB*) remained relatively stable during early infant development (Fig 2B, 2C). As mentioned, the risk of HGT is correlated to not only the size of the AR gene pool but also the genetic feature of the AR gene carriers (65, 86). Results from this study identified various genus/species in each ART subpopulation, thus enabled further assessment of AR gene flow via both vertical and horizontal gene transfer events. Although with limited cultivation conditions, data from the study clearly revealed that identified AR gene carriers belonged to several groups of commensals, mostly Gram-positive bacteria. However, the identified carriers varied among the specific AR genes, even within the same subject. *E. coli/Shigella* sp. was only identified to be the main *sul2* gene carrier in one subject. No Tet<sup>f</sup> bacteria were identified on MAC plates throughout the testing period in Baby Y. Data as such provided scientific justification for the proper selection of indicators, particularly among the large pool of commensal bacteria, for monitoring the AR status in targeted microbial ecosystems. Despite of all the limitations, the culture-based methods proved to be invaluable in revealing the ART population dynamics and AR dissemination details, which are essential for risk assessment and targeted mitigation.

Due to the extensive labor and cost, this study only focused on limited numbers of cultivation conditions, AR and subjects. Furthermore, a significant portion of the gut microbiota was not cultivable in practice. Therefore the detected sizes of the representative AR gene pools and the cultivable AR gene carriers did not always match. Overall, the data presented here were an underestimation of the extensiveness of the AR
development in infant GI tract. Rather, it broadly described the early establishment of ART bacteria in human gut and the significant role of hosts in the AR circulation. Additional studies are needed to confirm the impact of birth delivery on ART bacteria development in infant subjects, and to assess the contribution of foodborne ART bacteria in further shaping the ART population in gut microbiota.
Chapter Three

Prevalence of Antibiotic Resistance in Non-Food Animals

3.1 Abstract

The prevalence of ART bacteria in the food chain is a raising public concern. Drug resistant flora, especially commensal bacteria, may serve as an important avenue disseminating AR to human. Numerous studies had identified farm food animals as a significant source of ART bacteria, with the bedding, manure and lagoon water rich in these bacteria. The aim of this study was to investigate the prevalence of ART bacteria and AR gene pool in non-food animals. Seven pets and 13 zoo animals were recruited from Columbus, OH in 2011. It was found that ART bacteria and AR gene pool were prevalent in many pets and zoo animals, some of which were never exposed to growth promoter or antibiotic treatment. Identified resistance gene carriers included Lactobacillus spp., Streptococcus spp. and Escherichia coli. The finding suggested that non-food animals might also be an important component in AR circulation. Most importantly, antibiotic application is not the essential contributing factor leading to prevalence of ART bacteria in the hosts.
3.2 Materials and Methods

3.2.1 Fecal sample collection and sample enumeration

Fecal samples from 7 pets, including 3 cats, 3 dogs, and 1 hamster were collected in sterile containers from 4 families in Columbus, Ohio in 2011. Meanwhile, 13 zoo animals, including 3 ponies, 4 lions, 2 tigers, 2 cougars and 2 elephants from Columbus Zoo were included in this study. The following media agar plates were used in recovering the total bacterial population: Brain Heart Infusion (BHI), Columbia blood agar base supplemented with 5% sheep blood, and MacConkey Agar (MAC) supplemented with 1% glucose. One of the following antibiotics was added to the media to recover resistant population: 16 μg/ml of tetracycline, 100 μg/ml of erythromycin, 152 μg/ml of sulfamethoxazole together with 8 μg/ml of trimethoprim, 2 μg/ml of cefotaxime. Cycloheximide was added to all agar plates in 100 μg/ml to minimize the growth of yeast and mold. One gram of fecal sample was serially diluted in 0.85% NaCl, 0.1% peptone water solution and plated in duplicate on the corresponding bacterial agar plates. The plates were incubated under anaerobic conditions using GasPak™ 150 anaerobic system and GasPak™ EZ anaerobe sachets with indicator at 37°C for 48 h.

3.2.2 Examination of profile of AR gene carriers from non-food animal fecal samples

Acquired ART isolates were seeded (50 isolates per resistance type per pet subject) and screened for AR genes, including tet(S), tet(L), tet(M), ermB, blaCTX, blaTEM, blaSHV, blaOXA, blacMY2, sul1 and sul2. The DNA was extracted according to method described
previously. Representative resistance gene carriers were identified according to their 16S rRNA gene sequences.

3.2.3 Total DNA extraction and examination of AR gene pool in feces

Total DNA was extracted from pet fecal sample and pet food using method described by Morrison and Yu (90). Representative resistance gene pools in total DNA, including \textit{tet(M)}, \textit{ermB}, and \textit{sul2}, were measured by Taqman real-time quantitative PCR.

3.3 Result

3.3.1 Prevalence of AR in pet animals

ART bacteria, though not all resistance subtypes, were found in all pet subjects inspected (Fig. 20). In 3 dogs, Tet$^r$ population ranged from $10^5$ to $10^{10}$ CFU/g (0.3-100% in total population); Erm$^r$ population counted as $10^6$ CFU/g (4-15% in total population); Sul$^r$ population ranged from $10^7$-$10^{10}$ CFU/g (5-38% in total population); Ctx$^r$ population ranged from $10^6$-$10^9$ CFU/g (21-26% in total population). In cats, Tet$^r$ population ranged from $10^5$ to $10^8$ CFU/g (0.001-4% in total population); Erm$^r$ population ranged from 0 to $10^3$ CFU/g (0-0.00005% in total population); Sul$^r$ population was $10^9$ CFU/g (20-39% in total population); Ctx$^r$ population was $10^9$ CFU/g (14-27% in total population). In the hamster, Tet$^r$ population was $10^8$ CFU/g (2% in total population); Erm$^r$ population was
$10^5$ CFU/g (0.003% in total population); Sul$^r$ population was $10^7$ CFU/g (0.1% in total population); Ctx$^r$ population was $10^6$ CFU/g (0.03% in total population).

AR gene pools from total DNA extracted from each pet fecal sample were measured by qPCR (Fig. 5). In dogs, the sizes of tet($M$) gene pool were from below detection limit to $10^{10}$ copies/g, while ermB ($10^6$-$10^7$ copies/g) and sul2 (below detection limit to $10^6$ copies/g) gene pools were also detected. In cats, tet($M$) ($10^5$-$10^9$ copies/g), ermB ($10^6$-$10^9$ copies/g) and sul2 (below detection limit to $10^6$ copies/g) were also detected in certain pet subject. In hamster, tet($M$), ermB and sul2 were found to be below detection limit, $10^8$ copies/g and below detection limit, respectively.
Fig. 5 Prevalence of ART bacteria and AR gene pool in pet feces. A, Tet$^r$ bacteria and \textit{tet}(M) gene pool; B, Erm$^r$ bacteria and \textit{ermB} gene pool; C, Sul$^r$ bacteria and \textit{sul2} gene pool.

☆ Bacteria count or size of gene pool is below detection limit.
Further assessment on the profile of ART bacteria in pet fecal samples found that 2-60% of Tet\(^t\) isolates from dogs recovered from BHI plates were *tet(M)* carriers, 0-50% of Ctx\(^t\) isolates were *bla\text{CMY2}* carriers, whereas *tet(S)*, *tet(L)*, *ermB*, *sul1*, *sul2*, *bla\text{CTX}*,-*bla\text{TEM}*,-*bla\text{SHV}*,-*bla\text{OXA}* carriers were not detected with the current methodology. In cats, the percentage of *tet(M)* carriers in Tet\(^t\) isolates were from 0 to 80%, while no carriers of the rest AR genes tested were recovered. In the hamster, 4% of Sul\(^t\) isolates were found to be *sul2* carriers, while no carriers of the rest AR genes tested were recovered. Identities of AR gene carriers were closely associated with each pet subject, rather than the genus of the animal. For example, all *tet(M)* carriers examined (10 out of 26 carriers) in one dog subject are *Lactobacillus* spp., while *tet(M)* carriers from the second dog turned out to be *Streptococcus* spp. (8 out of 31 carriers), and *Clostridium neonatal* (1 out of 1 carrier) in the last dog subject. *tet(M)* carriers were found to be *Lactobacillus* spp. in one cat subjects, while *tet(M)* carriers were not detected in the other two cat subjects. Similarly,
all $\text{bla}_{\text{CMY2}}$ carriers (5 out of 26 carriers) in one dog subject were found to be *Escherichia coli*, while no carriers were found in the other two dog subjects. $\text{sul2}$ carriers (1 out of 1 carrier) found in the guinea pig were identified as *Enterobacteriaceae*.

### 3.3.2 Prevalence of AR in zoo animals

Fecal samples from 1 elephant, 1 pony, 1 cougar, 1 lion and 1 tiger were enumerated (Fig. 6). Total DNA was extracted from all zoo animals recruited and AR gene pools were analyzed by Taqman real-time PCR (Fig. 7). It is worth noting that fecal samples from carnivores generally contain larger AR gene pools than herbivores.
Fig. 6 Prevalence of ART bacteria and AR gene pool in zoo animals. A, Tet\(^r\) bacteria and tet(M) gene pool; B, Erm\(^r\) bacteria and ermB gene pool.

☆ Bacteria count or size of AR gene pool was below detection limit
3.4 Conclusion and Discussion

Antibiotic resistance in foodborne commensals was recognized as a major public health concern in recent years. Besides sporadic existence of AR in food pathogens, large resistance gene pools were routinely detected in food animals during farm practices, the very beginning of the food chain. The prevalence of AR in food animals was believed to
be correlated with the use of antibiotic-based growth promoters, whose application during farm production started to be restricted in the past decade.

Unlike food animals, non-food animals, primarily pets and zoo animals, were rarely exposed to non-therapeutic antibiotics. Meanwhile, AR in non-food animals was barely studied in the past. Revealing AR prevalence in non-food animals would help address the impact of food animal production practice on AR development. Furthermore, the AR prevalence information about non-food animals are important as they may pose significant impact on AR circulation due to 1) the large numbers of animals involved—-it is estimated that there are 8,200,000 pet cats and 7,200,000 pet dogs in the U. S.; and 2) their closer contact relationship, particularly pet animals to humans than food animals.

In this study, ART bacteria and AR gene pools were detected from most pet and zoo animals examined. Both tet(M) and ermB pools were detected in fecal samples from 4 and 7 pets respectively, indicating that pet waste is a significant source of AR in the environment close to human residence. On the other hand, it is worth noting that pet individuals from certain families were relatively more prevalent with AR compared to other pet counterparts. For example, considerable tet(M), ermB and sul2 pools were detected in dog 1, but the sizes of tet(M) and sul2 gene pools were below detection limit
in dog 2 and dog 3. These findings suggested that living habitat and diet may impact the prevalence of AR in pet animals.

Similarly, the study found that AR was prevalent in zoo animals. In 13 fecal samples of zoo animals examined, 10 contained a considerable tet(M) gene pool and 6 contained a similar ermB gene pool. Though the examined AR gene pools were found in most zoo animals, it is worth noting that AR was generally more prevalent in carnivores in terms of frequency and size of AR gene pools than herbivores. This may be due to the difference in diet between the two groups of zoo animals.

Since most pet and zoo animals were not routinely exposed to any antibiotics, the findings of the prevalence of AR in pet and zoo animals suggested that direct antibiotic exposure was not the major contributing factor in these animals. The identified AR gene carriers were found to be commensal bacteria in animal GI tract, such as *Lactobacillus* sp., *Streptococcus* sp. and *Escherichia coli*. The presence of commensal AR gene carriers in non-food animals illustrated another critical pathway that is very close to human vicinity in the entire AR circulation route.Mitigation of corresponding AR related to pet and zoo animals should be carefully considered.
Chapter Four

The Impact of Foodborne Antibiotic Resistant Bacteria and Antibiotic Exposure on the Antibiotic Resistant Microbial Ecology of Gastrointestinal Tract in Animal Model

4.1 Abstract

The prevalence of foodborne antibiotic resistant (ART) commensal bacteria in ready-to-consume food products suggested that daily food consumption likely serves as a major avenue for disseminating ART bacteria from the food chain to the human hosts. The potency of foodborne ART bacteria in shaping microbial ecology in host gastrointestinal (GI) tract was evaluated using an animal model. Germ-free mice were firstly inoculated with human associated microbiota and then exposed to identified AR gene carriers (Tet<sup>+</sup> *Enterococcus faecium* A21 and *Streptococcus* sp. A85) isolated from seafood products, with water and cheese as delivery vectors. The selected foodborne AR gene carriers successfully colonized and significantly amplified in the host GI tracts after a short exposure (2 days) in the presence of human associated microbiota, and persist in the absence of antibiotic selective pressure for up to 2 months. The findings suggested that many foodborne ART bacteria are capable of surviving the acidic environment in host digestive tract and colonizing host GI system. Thus continuous exposure to such ART bacteria, especially AR gene carriers, will inevitably shape AR ecology in host GI tract.
However, horizontal gene transfer events were not detected in this study in the presence and absence of the antibiotic.

Meanwhile, the impact of different antibiotic delivery methods, including intravenous (IV) injection and oral feeding, on antibiotic resistance ecology in host GI tract was studied. Mice colonized by environment associated microbiota were divided into oral group and IV group. Both groups of mice were administered with tetracycline hydrochloride (50 mg/kg body weight/day) for five days. Since only part of the antibiotic by oral delivery was absorbed by the host, the second round of the experiment used increased dosage for oral group and reduced dosage for IV group. Both tetracycline resistant population and tet gene pools (tet(L), tet(M)) in mouse fecal samples with equal dosage of antibiotic exposure were significantly amplified one day after initial antibiotic administration and maintained thereafter. Despite predominant ART bacteria population in mice fecal samples altered in a similar manner after initial antibiotic exposure, detected tet gene pools in mouse fecal sample from the oral group were 10-100 times larger than that of the IV group, indicating that oral drug administration may have bigger impact on AR ecology in host GI tract than IV injection. The difference was even bigger when increased and reduced dosage was applied to oral administration and IV injection, respectively. The study concluded that at least in the case of tetracycline, oral administration may have bigger impact on the development of ART microbiota in host GI tract than IV injection. The results indicated a possible major contributor for the rapid emergence of ART bacteria, and potential intervention point for effective mitigation.
4.2 Introduction

It is becoming widely recognized in recent years that ART bacteria were prevalent in the food production system. Foodborne ART commensals, including those carrying AR genetic determinants, were isolated from various foods including ready-to-eat products from deli market and restaurant (47, 87). Therefore food chain can be an important avenue disseminating AR to consumers. It was estimated that adult were exposed to foodborne AR genes for at least $10^6$ copies/meal when eating ready-to-eat food (54), indicating that there is constant influx of ART bacteria and AR genes into human GI tract through daily food intake. Not all foodborne ART bacteria can survive the acidic environment in host digestive tract; however, a group of acid-tolerant facultative anaerobes were found from ready-to-consume deli and restaurant foods (47, 87). The identified foodborne ART bacteria include those commonly found in human GI tract, supporting the notion that foodborne ART food commensals can potentially colonize human digestive tract and become part of the gut microbiota. On the other hand, since human host is exposed to various environmental foodborne microbes daily, it is also important to evaluate the potential persistency of certain ART bacteria in host GI tract.

It is worth noting that for many years (until 1970’s in China), penicillin and streptomycin had been the antibiotics for treating infectious diseases. Starting from early 1960s, oral penicillin V was introduced to clinics and widely used due to its improved absorption. During the same time period, penicillin resistant *Pneumococci* became one of the most
widespread resistance problems. Meanwhile, antibiotics used to be delivered by muscle or intravenous (IV) injection, and oral pills were relatively new mode of delivery but rapidly became dominant due to its advantage in expense and convenience. However, little is known about its impact on AR ecology in host GI tract system. With the improved understanding on the contribution of gut microflora on ART bacteria development and circulation, it becomes important to assess the mode of antibiotic delivery on ART microbiota development in the GI tract. In fact, oral antibiotics are often prescribed at higher dosage than that for IV injection, when the difference in absorption among population is taken into account. Oral antibiotics can also directly apply selective pressure in host GI tract, as part of them becomes feces regardless of whether the antibiotics excreted through feces or not. The advantage of IV or muscle injection is that it bypasses absorption stage and antibiotics reach highest serum concentration right after injection. Besides, the actual dosage can be managed to avoid over use of antibiotics.

The aim of the study was to evaluate the capability of common foodborne AR gene carriers (commensal bacteria) to colonize and persist in host GI tract, the impact of antibiotic exposure on potential HGT events in the GI tract, and to compare the impact of two delivery methods, oral and IV injection, on AR ecology in host gut system. Mouse model was used in the study.

4.3 Materials and Methods
4.3.1 Mouse preparation

Animal protocol 2009A0167 (The Ohio State University) was followed throughout the study. Germ-free mice (known to be previously colonized with uncultivable Bacillus sp.) were kept in sterile passive vented cages and grouped as three mice per cage. Two groups were introduced as 1) one cage of control group, 2) two cages of mice fed on Enterococcus faecalis G37 (Em\textsuperscript{T} Tet\textsuperscript{s}) previously isolated from infant feces. E. faecalis strain was used to colonize germ-free mice GI tract before exposed to foodborne ART bacteria. The strain was inoculated into feeding water as 4 X 10\textsuperscript{4} CFU/ml (final concentration) and the feeding water is the only water source to mice in the study. Mice were exposed to such feeding water for two days before the strain was withdrawn from the water source.

Two previously identified tetracycline resistant bacterial strains isolated from cooked shrimp samples were used to feed mice after initial colonization. Streptococcus sp. A85 (tet(S)) and Enterococcus faecium A21 (tet(S), tet(L), tet(M)) containing plasmid encoded tetracycline resistance genes were delivered in two independent pathways: 1) the strains were inoculated into feeding water with 10\textsuperscript{5} CFU/ml and renewed every day; 2) the strains were inoculated into sterilized cheddar cheese with 10\textsuperscript{5} to 10\textsuperscript{6} CFU/g and renewed every day. In both cases, the exposure lasted three continuous days before mice fecal samples were collected and finally analyzed.
After the exposure to foodborne ART bacteria through feeding water and/or cheese, ART bacteria were withdrawn from feeding and mice were fed on regular diet (treated with Gamma irradiation) exclusively. At the meantime, mice fecal samples were collected every other week to examine the presence of previously consumed ART bacteria.

Germ-free mice transferred into vented cages and exposed to environmental contamination for one year were observed to have developed cultivable ART bacteria in GI tract system. These mice were used in the antibiotic administration study.

4.3.2 Fecal bacteria enumeration

Fresh mouse fecal sample was homogenized in saline with a W/V ratio of 1 to 20 to 1 to 40. The liquid became zero dilution for plating. The liquid was then subjected to serial dilution and plated on Columbia Blood Agar (CBA) Base supplemented with 5% sheep blood. All media contained 100 µg/ml cycloheximide. Selective antibiotics included tetracycline (16 µg/ml), erythromycin (100 µg/ml), sulfamethoxazole/trimethoprim (152 µg/ml, 8 µg/ml respectively) and cefotaxime (4 µg/ml). 100 µl of selected dilution was spread on agar medium and the plates were incubated in BD GasPak™ 150 anaerobic system with three BD GasPak™ EZ Anaerobe Sachets at 37°C for 48 h.

4.3.3 Antibiotic administration
Mice in oral group were fed with 0.2 ml 5 mg/ml tetracycline hydrochloride at 50 mg/kg body weight per day. Feeding was performed with 20G animal feeding gavage. Mice in IV group were restrained on TV-150 tail vein restrainer (Braintree Scientific) and injected with BD 0.5 ml insulin syringe. A total of 0.2 ml 5 mg/ml tetracycline hydrochloride was injected into mouse tail vein once a day. Mice from both groups were administered with tetracycline hydrochloride for five days. Another comparison group between oral and IV injection utilized 100 mg/kg body weight per day as oral dosage and 10 mg/kg body weight per day as IV dosage. Mouse fecal sample were collected on day 1, 2, 5, 7 to examine ART bacteria and AR gene pool.

4.3.4 DNA extraction from mouse fecal sample

The procedure described by Li et al (47) was followed to extract DNA from bacterial isolates as amplification templates for conventional PCR. For real-time quantitative PCR and DGGE analyses, the DNA templates from mouse fecal samples were extracted according to Yu and Morrison.

4.3.5 Examination of AR gene pool by real-time quantitative PCR

Various AR gene pools, including tet(S), tet(L), tet(M), ermB, sul1, sul2, blaTEM, were examined using total DNA extract from mouse fecal samples. Real-time quantitative PCR
was performed on a CFX real-time system (Bio-rad). Primers and probes were listed in Table 3.

4.3.6 Denaturing gradient gel electrophoresis analysis on plate DNA

DGGE analysis was performed as described in Chapter 2. All recovered DNA bands were sequenced at Plant Microbe Genomic Facility at The Ohio State University.

4.3.7 Identification of AR gene carriers in mouse fecal sample

The same procedure in Chapter 2 was adopted. All AR gene carriers were identified at Plant Microbe Genomic Facility at The Ohio State University.

4.3.8 Evaluation of the effect of antibiotics in mouse tissue

One cage of mice (three) was fed with tetracycline hydrochloride and the other cage of mice (four) were IV injected with the same amount of antibiotic. Two hours after the exposure, these two cages of mice, together with one mouse in the control group, were sacrificed to collect muscle, cecum and colon. Mouse muscle, cecum and colon were first soaked into liquid nitrogen and then stored in -80°C.

4.4 Results
4.4.1 Establishment of human associated flora and foodborne ART bacteria in host GI tract

After being exposed to Erm\(^r\) *E. faecalis* G37 strain for two days (in water), the strain was detected in mice fecal sample in \(2 \times 10^9\) CFU/g on the third day and \(5 \times 10^{12}\) CFU/g in a week. Similarly, with the presence of previously inoculated *Enterococcus faecalis* G37, foodborne *Enterococcus faecium* A21 and *Streptococcus* sp. A85 were detected in mice feces in \(10^9\) CFU/g and thereafter. These strains were still detected in mice fecal samples two months after the exposure.

On the other hand, though the *E faecium* A21 strain colonized mice GI tract well whether through feeding water or cheese inoculation, the *Streptococcus* sp. A85 strain only colonized mice GI tract well when using cheese as delivery vector, as the strain died out in a couple of hours in water. Therefore food delivery method had direct impact on the efficacy of inoculation.

No transconjugants were detected from mouse fecal sample between foodborne ART bacteria strains and previously colonized *E. faecalis* G37, though both foodborne strains contained identified *tet* genes on mobile gene elements.

4.4.2 Change of ART bacterial population in mouse GI tract upon antibiotic exposure
Figure 8 showed total and resistant bacteria count before and after exposure (by two drug delivery methods) to 50 mg tet/kg body weight. Overall, the application of tetracycline hydrochloride did not cause significant impact on total bacteria count in mouse feces. However, it was observed that Tet\(^r\) population significantly increased after the exposure. It is worth noting that oral delivery caused bigger (10-100 times) increase of Tet\(^r\) population than IV injection. Exposure to tetracycline hydrochloride also increased Erm\(^r\) population, but seemed to have no impact on Sul\(^r\) and Ctx\(^r\) population in mouse GI tract.

Fig. 8 Change of ART bacteria population upon exposure to antibiotics. A, total bacteria population; B, Tet\(^r\) bacteria; C, Erm\(^r\) bacteria; D, Sul\(^r\) bacteria; E, Ctx\(^r\) bacteria.
Figure 8 continued

Bacterial Count (Log_{10} CFU/g) vs. Exposure length (day) for Oral and IV exposure.

---

Continued
4.4.3 Change of AR gene pools in mouse GI tract upon exposure to antibiotics
Among seven AR genes examined, \textit{tet(S)}, \textit{sul1}, \textit{sul2}, \textit{bla}_{\text{TEM}} were not detected in mouse fecal samples, regardless whether the mouse had been treated with tetracycline hydrochloride or not. Change of sizes of \textit{tet(L)}, \textit{tet(M)} and \textit{ermB} were shown in Fig 9. It was observed that the levels of these AR genes dramatically increased in mouse fecal samples after antibiotic exposure. Oral administration caused even greater increase (10-100 times) of \textit{tet(L)} and \textit{tet(M)} gene pool than IV injection throughout the exposure. A much smaller AR gene pools were found in mice group with reduced Tet injection dosage.

Fig. 9 Change of AR gene pools upon antibiotic exposure. A. \textit{tet(L)} gene pool; B, \textit{tet(M)} gene pool; C, \textit{ermB} gene pool. Real-time PCR was performed on two independent DNA extract replicates and the coefficient variations of log copy no. of resistance gene were less than 0.05.
Figure 9 continued

B

C

Exposure length (day)

B

C

exposure length (day)

- Oral 50
- IV 50
- Oral 100
- IV 10

Gene pool (Log_{10} copies/g)
4.4.4 Change of profile of predominant ART bacteria after antibiotic treatment

Predominant ART bacteria in mouse GI tract were examined before and after antibiotic exposure. As Fig. 10 illustrated, major population switch existed right after antibiotic administration. For example, *Streptococcus* sp was found to be predominant in Tet\(^r\) population; however, one day after mouse was exposed to tetracycline hydrochloride, *Enterococcus* sp. started to thrive in mouse GI tract. Similarly, *Enterobacter* sp. was found to be predominant bacteria in Erm\(^r\) population and after antibiotic exposure, *Enterococcus* sp. was detected in predominant population. Though an abrupt change of constitution of predominant ART population was observed right after antibiotic administration, the composition of Tet\(^r\), Erm\(^r\), Sul\(^r\) and Ctx\(^r\) population remained relatively stable throughout the entire antibiotic treatment period in oral and IV groups. There was no difference observed in terms of the effect on the profile of ART bacteria in mouse GI tract between oral delivery and IV injection.
Fig. 10 Profile of predominant bacteria population in mouse fecal sample before and after antibiotic treatment. A, Tet<sup>f</sup> bacteria; B, Erm<sup>f</sup> bacteria; C, Sul<sup>f</sup> bacteria, D, Ctx<sup>f</sup> bacteria. O<sub>0</sub>-O<sub>5</sub>, microbiota in oral group before and after tetracycline hydrochloride exposure; I<sub>0</sub>-I<sub>5</sub>, microbiota in IV group before and after tetracycline hydrochloride exposure; M, DNA ladder. a, Enterococcus sp.; b, Streptococcus sp., c-f, Enterococcus sp; g, Citrobacter sp./Enterobacter sp.; h-I, Enterobacter sp.; j-k, Enterococcus sp.
4.4.5 Profile of AR gene carriers in mouse GI tract before and after antibiotic administration

As tet(L), tet(M) and ermB gene pools were detected in total DNA from mouse fecal samples, Tet$^R$ and Erm$^R$ isolates in fecal samples from mice before antibiotic administration, after 5 days continuous exposure via oral delivery or IV injection were randomly selected and screened for these AR genes (Table 3). It was observed that various Enterococcus species and subspecies served as AR gene carriers in mouse GI tract. Prevalence of AR gene carriers in corresponding ART population changed upon antibiotic exposure. For example, no tet(L) or ermB carriers were detected in fecal samples from mouse before antibiotic exposure; whereas percentage of carriers of these genes reached 40% (oral and IV) and 50% (oral) to 70% (IV) after 5 days continuous exposure to 50 mg Tet/kg body weight. Though the application of antibiotic effectively changed prevalence of AR gene carriers in mouse GI tract, no difference was observed between oral delivery and IV injection in terms of the nature and prevalence of AR gene carriers in host GI system despite similar dosage was applied in both delivery methods (50 mg/kg body weight/day).
Table 3. Profile of predominant AR gene carriers in mouse GI tract and its relevance with antibiotic administration (50 mg/kg body weight/day dosage)

<table>
<thead>
<tr>
<th></th>
<th>tet(L)</th>
<th>tet(M)</th>
<th>ermB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>AR carrier %</td>
<td>0 (0/10)</td>
<td>80% (8/10)</td>
</tr>
<tr>
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<td>Enterococcus</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Oral feeding</strong></td>
<td>AR carrier %</td>
<td>40% (4/10)</td>
<td>40% (4/10)</td>
</tr>
<tr>
<td>AR carrier ID</td>
<td>Enterococcus</td>
<td>Enterococcus</td>
<td>Enterococcus</td>
</tr>
<tr>
<td></td>
<td>spp. (2/2)</td>
<td>spp. (4/4)</td>
<td>spp. (4/4)</td>
</tr>
<tr>
<td><strong>IV injection</strong></td>
<td>AR carrier %</td>
<td>40% (4/10)</td>
<td>50% (5/10)</td>
</tr>
<tr>
<td>AR carrier ID</td>
<td>Enterococcus sp.</td>
<td>Enterococcus sp.</td>
<td>Enterococcus sp.</td>
</tr>
<tr>
<td></td>
<td>(3/3)</td>
<td>(3/3)</td>
<td>(3/3)</td>
</tr>
</tbody>
</table>

4.5 Conclusion and Discussion

In this study, common foodborne ART bacteria were used to colonize host GI tract previously inoculated with human associated flora. It was concluded that foodborne commensals are able to colonize and change the ecology of host GI tract even in the
presence of other colonized host gut microbiota. The study used two common tetracycline resistant foodborne bacterial isolates containing \textit{tet} genes on plasmid and both strains were able to colonize host GI tract. The successful colonization is important, as the influx of external AR gene carriers not only modified host gut, but also potentially served as AR gene donors or recipients, further complicating AR dissemination in host GI system. Though initial load was low, consumed ART bacteria likely significantly amplified in host GI tract in the absence of corresponding antibiotic pressure as found in the study, and produced ART bacteria-rich feces that further feed back into the environmental AR gene pool entering another round of AR gene circulation between the host and environment. The study also found that once colonized in host GI tract, foodborne ART bacteria can be quite stable in the absence of antibiotic selection pressure. The maintenance of foodborne ART bacteria did not necessarily depend on continuous supply of corresponding bacterial cells either.

The inoculated foodborne AR gene carriers, \textit{E. faecium} A21 and \textit{Streptococcus} sp. A85 contain plasmid-encoded mobile \textit{tet} genes that may potentially be transferred via HGT events. However, the study did not detect any cross transformants/transconjugants between foodborne AR gene carriers and previously inoculated \textit{E. faecalis} G37. The genetic incompatibility among limited organisms and experimental conditions could have all contributed to the observed outcome.
During the study, the delivery vector of ART bacteria was observed to have an impact on the colonization of foodborne ART bacteria in host GI system. Certain bacteria strains need special food matrix to survive and be delivered to and even survive the host digestive tract. In another word, the nature of food matrix has an impact on the profile of predominant ART bacteria that can be delivered successfully into host digestive tract.

The study also evaluated the impact of antibiotic delivery method (oral feeding and IV injection) on ecology in host GI tract system. Tetracycline hydrochloride was chosen to represent regular antibiotics as it can be delivered in both pathways. The study found that both delivery routes significantly impact AR ecology in host GI tract. Though sizes of total and resistant population in mouse fecal sample persist after antibiotic administration; the sizes of the AR gene pools, constitution of predominant ART bacteria and prevalence of AR gene carriers changed dramatically. For example, sizes of \textit{tet}(L), \textit{tet}(M), and \textit{erm}B gene pools were below detection limit before mice were exposed to tetracycline hydrochloride; however, up to $10^9$ copies/g of corresponding gene pools were detected in one day after the exposure to 50 mg \textit{tet}/kg body weight. Combining data acquired from DGGE analysis and AR gene carrier identification, it was concluded that the increase of these AR gene pools was largely due to the amplification of various \textit{Enterococcus} spp. In detail, \textit{Streptococcus} sp. and \textit{Enterobacter} spp. were predominant Tet$^\prime$ and Erm$^\prime$ bacteria in mouse GI tract before antibiotic exposure. Once tetracycline hydrochloride was introduced, \textit{Enterococcus} spp. were detected by DGGE analysis. As \textit{Enterococcus} spp. were also identified as primary carriers for \textit{tet}(L), \textit{tet}(M) and \textit{erm}B, we concluded that the
same group of Enterococcus spp. detected by DGGE were a group of AR gene carriers which expanded the corresponding resistance gene pool.

The significant amplification of AR gene carriers upon exposure to antibiotics demonstrated that the application of antibiotics may selectively enrich certain group of ART bacteria, which was present as minority in the absence of antibiotic selection pressure. It was also found that continuous exposure to antibiotic had little impact on the sizes of AR gene pool and population switch in predominant ART bacteria in this study. Partially because mouse model included in this study was acquired by exposing germ-free mice to environmental flora, leaving the diversity of gut flora in such mouse model quite limited. This is in accordance with finding that all detected AR gene carriers were Enterococcus spp., though several other species and subspecies were involved.

The study also found that under the same dosage, oral delivery generated larger AR gene pools in host GI tract than IV injection. It is anticipated that effective concentration of tetracycline hydrochloride in host GI tract was higher when delivered through oral route than tail vein injection, as the antibiotic became part of feces and directly contacted with host digestive tract when delivered through oral route. Most blood tetracycline hydrochloride would be excreted in both urine and feces, and the effective concentration is supposed to be lower in host GI tract. As tetracycline hydrochloride is mostly excreted through feces, which diminished difference between oral feeding and IV injection, one
can predict that the difference between the two delivery routes may be even greater in those antibiotics that excreted primarily through urine. Preliminary results using 10mg Tet/kg body weight via IV injection and 100 mg Tet/body weight via oral feeding supported this notion. Additional studies are currently underway to verify the findings.

In a word, different antibiotic delivery methods may not only present different pharmaceutical effects against targets, but also apply different impacts on AR ecology in host GI tract system. Due to this major impact on host gut flora, oral administration, though were most commonly used in current clinics system, should be carefully evaluated and reconsidered.
Chapter Five

Prevalence of Antibiotic Resistance in Aquaculture Products and Susceptibility to Consumer-end Mitigation Controls

5.1 Abstract

Food animal products are recognized as an important vehicle for antibiotic resistant bacteria and antibiotic resistance (AR)-encoding genes, largely due to the modern food animal production practices. The emerging aquaculture farming likely is susceptible to the similar problems. Recently, ART commensal bacteria were isolated from various foods, including both raw and processed seafood products; and the transmission of the AR determinants from seafood isolates to opportunistic pathogens in vitro was also illustrated. The data suggested the potential impact of the food chain on AR ecology of human intestinal tract. An improved understanding of the profile of ART bacteria associated with seafood and its correlation to production and processing conditions is essential for the development of effective mitigation in the seafood production to consumption chain. In this study, we investigated the prevalence of ART bacteria in several aquaculture products, as well as soil and water samples from representative production environment. Fish and shrimp samples were collected from farms and retail
stores in Ohio, Hawaii and Guangzhou, China. Samples were accessed for the prevalence of resistant bacteria against tetracycline, erythromycin, sulfamethoxazole/trimethoprim, or cefotaxime by conventional culture recovering methods and the representative AR genes by PCR. Representative AR gene carriers were subjected to identification by 16S rDNA sequencing analysis. ART bacteria were found prevalent in products from both countries. In general, tetracycline resistant bacteria counted approximately 1%, and the sulfamethoxazole/trimethoprim resistant bacteria ranged from 0.01% to 1% in total bacterial population. Among identified AR genes, tetL, tetK and sul1 were the most prevalent in samples collected from Hawaii, while tetS, tetL and tetM predominated in samples collected from South China. Identified AR gene carriers included Pseudomonas sp. (tetS, tetL, tetM, tetK, Sul1, Sul2), Carnobacterium sp. (tetS, tetM), Acinetobacter sp. (tetK), and Brochothrix sp. (tetL, tetM, tetK). Our results suggest that ART bacteria are prevalent in aquaculture products, but distinct profiles of dominant AR genes and carriers were found in products, likely reflecting the difference in production environment and practices. The study also included an evaluation of the efficacy of household cooking as mitigation method for AR in seafood. It was found that conventional cooking methods, including stir fry, steam and grill effectively reduced ART bacteria in finished seafood products.

5.2 Introduction
A number of critical steps are involved in turning raw materials to food on the table, which include but not limited to: 1) raw material production in farms; 2) food processing treatment in food plants; 3) storage and transportation to retail stores; 4) customer end storage and consumption. Each step may not only significantly amplify or reduce the presence of ART bacteria in food, but also change predominant ART flora associated with food. An improved understanding of the profile of predominant foodborne ART bacteria in each step in the food chain will not only illustrate the extent of exposure to ART bacteria and AR genes through food consumption, but also essential for targeted mitigation.

It is well documented that ART bacteria are prevalent during food animal production process. A variety of resistance gene carriers have been isolated from food animals (84), food animal products (89, 92), farm environment, and particularly in large quantity in lagoon water and animal waste disposal system (manure and waste water) (15, 82). Numerous studies illustrated the direct connection between antibiotic application and the emergence of ART bacteria (1, 11, 34). To combat the AR challenge, various guidelines and regulations are implemented by regulatory agencies to limit the use of antibiotics in both clinical therapy and food animal production. Probiotics are commonly used as an alternative growth promoter in food animal production, with the intention to reduce AR. But the effectiveness of approach is yet to be evaluated. Food processing in processing plant and household are potentially very important for AR mitigation before final consumption. Limited studies showed that ART bacteria may be equally susceptible to
sanitation treatments compared to antibiotic sensitive strains. However, the effectiveness of mitigation control from the consumer end has not been well understood and communicated. Therefore the objectives of this study were to assess the prevalence of ART bacteria in seafood products and the efficacy of household cooking method on AR mitigation.

5.3 Materials and Methods

The overall flow chart of experimental design in this chapter was shown in Fig. 11. Seafood samples were collected and processed through sample enumeration, total DNA extraction, plate DNA extraction and analyzed by real-time quantitative PCR, 16S rDNA gene V3 region DGGE analysis, traditional PCR and 16S rDNA gene sequencing analysis. The study examined AR gene pools, constitution of ART population, prevalence of AR genes and predominant AR gene carriers.

5.3.1 Seafood sample collection

Raw seafood samples, including freshwater shrimp, tilapia and bluegill were collected from 2 farms and several retail stores in Central Ohio. Wild caught fish, including Akule, Weike and Toau were collected from retail stores in Hawaii. Seafood including freshwater shrimp, tilapia, catfish and whitefish from South China were sampled from 2 farms and several retail markets in Guangzhou, China. Processed seafood samples,
including ready-to-eat shrimp and sushi, were collected in several grocery stores in Columbus, OH.

Figure 11 Flow chart of experimental design
Environmental samples in aquaculture farms were also collected to examine the potential impact of productive operation on the prevalence of AR in raw seafood. Pond water, pond mud, and feed samples were collected according to fresh fish and shrimp examined.

5.3.2 Sample enumeration

Fish were rinsed in sterile 0.1% peptone water briefly. The whole fish was then soaked in equivalent amount of 0.1% peptone water (100 gram of fish sample in 100 ml of diluents) and gently massaged. The wash off liquid then became zero dilution for surface rinse sample. On the other hand, fish intestine was collected and homogenized in 9 times 0.1% peptone water (10 gram of fish intestine in 90 ml of diluents). The fluid was used as zero dilution for fish intestine sample.

Pond mud, shrimp sample (raw and cooked) and seafood in sushi sample were homogenized similar to that of fish intestine directly and the fluid is used as zero dilution for corresponding seafood sample.

100 ml pond water was centrifuged at 4°C in 9000 rpm for 10 min. The pellet was dissolved in 1 ml 0.1% peptone water as zero dilution for pond water sample.

All samples were then subjected to serial dilution and spread on Brain Heart Infusion and MacConkey (supplemented with 1% glucose) agar plates. Media containing one of the following antibiotics: 16 μg/ml of tetracycline (Fisher Biotech, Fair Lawn, NJ), 100
μg/ml of erythromycin (Fisher Scientific), 152 μg/ml of sulfamethoxazole (Sigma-Aldrich, St. Louis, MO) and 8 μg/ml of trimethoprim (Sigma-Aldrich), 2 μg/ml of cefotaxime (Sigma-Aldrich), were used to assess the resistant population. Cycloheximide (Sigma-Aldrich) was added to all agar plates to 100 μg/ml to minimize the growth of yeasts and molds. Plates were incubated at 30°C for 48 h.

5.3.3 Total DNA and plate DNA extraction
Total DNA and plate DNA were extracted according to Yu and Morrison (90). Surface wash and fish intestine were sampled in 200 µl zero dilution and 0.2 g intestine tissue respectively. Pond water and pond mud were sampled in 200 µl zero dilution and 0.2 g mud. Feed was sampled in 0.2 g.

Plate DNA was extracted from plates containing sample zero dilution by a sterilized tongue depressor, followed by procedure according to Yu and Morrison.

5.3.4 Quantification of AR gene pool in seafood samples by real-time PCR
Taqman real-time PCR was used in assessing representative AR gene pools, including \textit{tet(S)}, \textit{tet(L)}, \textit{tet(M)}, \textit{ermB}, \textit{sul1}, \textit{sul2} and \textit{bla}_{\text{TEM}} genes, in DNA extracts of seafood and farm environment sample. Primers (Sigma-Aldrich) and probes (Biosearch Technology Inc., Novato, CA) were listed in Table 4. Real-time PCR program include an initial denature at 94°C, 3 min, followed by 40 cycles of 94°C, 20 sec, 60°C, 20 sec, 72°C 20 sec. The real-time PCR was performed on a CFX96 system (Bio-Rad).
<table>
<thead>
<tr>
<th>Primer and probe</th>
<th>Sequence</th>
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<td>GTATGTTCATCTTTTCTAAG</td>
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<td>tet(S) real RP</td>
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<td>tet(S) probe</td>
<td>CCATGTGTCCAGGAGTATCTAC</td>
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Table 4 List of primers and probes used in seafood AR gene pool quantification
Table 4 continued

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5.3.5 Detection of AR genes by conventional polymerase chain reaction (PCR)

The presence of representative AR genes from culture recovered resistant isolates was examined by conventional PCR. Primers (Sigma-Aldrich) and corresponding PCR conditions used during screening of resistance genes were summarized in Table 5. Approximately 10% of the positive PCR products for the AR genes screened were confirmed by DNA sequencing using ABI PRISMs 3700 (Applied Biosystems, Foster City, CA) at the Plant Microbe Genomics Facility, The Ohio State University and compared with published AR gene sequences deposited in the NCBI database. Up to 50 recovered ART isolates per resistance phenotype were screened for AR genes per sample.
<table>
<thead>
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</tr>
<tr>
<td><em>tet(M)</em> FP</td>
<td>CGAACAAGAGGAAGACAAG</td>
<td>50°C</td>
<td>974 bp</td>
<td>(47)</td>
</tr>
<tr>
<td><em>tet(M)</em> RP</td>
<td>CAATACAAATAGGAGCAAG</td>
<td></td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td><em>ermB</em> FP</td>
<td>TGGTATTCAAATGCCTATG</td>
<td>62°C</td>
<td>745 bp</td>
<td>(55)</td>
</tr>
<tr>
<td><em>ermB</em> RP</td>
<td>CTGTGGTATGGCGGTAAGT</td>
<td></td>
<td></td>
<td>(55)</td>
</tr>
<tr>
<td><em>sul1</em> FP</td>
<td>CGGCGTGCGCTACCTGAACG</td>
<td>50°C</td>
<td>433 bp</td>
<td>(32)</td>
</tr>
<tr>
<td><em>sul1</em> RP</td>
<td>GCCGATCGCGTAAGTTCCG</td>
<td></td>
<td></td>
<td>(32)</td>
</tr>
<tr>
<td><em>sul2</em> FP</td>
<td>GCGGCCGCGTAAGCTGA</td>
<td>60°C</td>
<td>657 bp</td>
<td>(32)</td>
</tr>
<tr>
<td><em>sul2</em> RP</td>
<td>GGCCTCGTGTGTGCGGATG</td>
<td></td>
<td></td>
<td>(32)</td>
</tr>
<tr>
<td><em>bla</em>TEM FP</td>
<td>CATTTCGGTGCGCCCTTATT</td>
<td>60°C</td>
<td>800 bp</td>
<td>(18)</td>
</tr>
</tbody>
</table>

Table 5. List of primers used when screening AR genes in ART bacteria isolates recovered from seafood and farm environment sample
Table 5 continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer 1</th>
<th>Temperature</th>
<th>Annealing Temperature</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>bla&lt;sub&gt;TEM&lt;/sub&gt; RP</td>
<td>CGTTCATCCATAGTTGCCTGAC</td>
<td>(18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bla&lt;sub&gt;CMY-2&lt;/sub&gt; FP</td>
<td>GACAGCCTCTTTCTCCACA</td>
<td>50°C</td>
<td>1143 bp</td>
<td>(93)</td>
</tr>
<tr>
<td>bla&lt;sub&gt;CMY-2&lt;/sub&gt; RP</td>
<td>TGGAAACGAAGGCTACGTA</td>
<td>(93)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bla&lt;sub&gt;CTX&lt;/sub&gt; FP</td>
<td>ATGTGCAGYACCAGTAARGT</td>
<td>50°C</td>
<td>593 bp</td>
<td>(61)</td>
</tr>
<tr>
<td>bla&lt;sub&gt;CTX&lt;/sub&gt; FP</td>
<td>TGGGTRAARTARGTSACCAGA</td>
<td>(61)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.3.6 Identification of predominant ART bacteria and AR gene carriers

Approximately 1/4 to 1/3 confirmed AR gene carriers (isolates carrying the representative AR genes) were selected and identified by partial 16S rRNA gene sequence analysis using ABI PRISMs 3730 (Applied Biosystems, Foster City, CA) in High-throughput Genomics Unit at University of Washington, Seattle, WA.

5.3.7 Evaluation of the efficacy of household cooking as mitigation method for ART bacteria associated with raw seafood materials

Common household cooking method, including boiling, steam and stir frying, were adopted in the study. Raw shrimp samples were acquired from local grocery stores. One hundred g of samples were subject to the following treatments: added into 2000 ml boiling water for 5, 10, 15, and 30 min; stirfried together with 10 ml soy sauce
(Kikkoman) for 5 min; steamed for 10 min. Raw and cooked shrimp were then subjected to sample enumeration.

### 5.4 Results

#### 5.4.1 Prevalence of ART bacteria in raw seafood products from aquaculture farms

Fig. 12 shows the prevalence of ART bacteria, including Tet\(^r\), Erm\(^r\), Sul\(^r\) bacterial population in raw seafood products collected in 5 visits from 2 farms in central Ohio. As illustrated, ART bacteria were prevalent in all raw seafood products collected from aquaculture farms. The data indicated that ART bacteria were prevalent in the very upstream of the seafood chain.

![Bacterial count graph](image)

**Continued**

Fig. 12 Prevalence of ART bacteria in raw seafood products in aquaculture farms. Plate counts were read on BHI and MAC (supplemented with glucose) respectively. A, Tet\(^r\) bacteria. B, Erm\(^r\) bacteria. C, Sul\(^r\) bacteria.

☆ Bacterial count is below detection limit in this study (100 CFU/g or ml).
Figure 12 continued
Tet\textsuperscript{r} bacterial population ranged from below detection limit to 10\textsuperscript{4} CFU/g or ml. Tet\textsuperscript{r} bacteria were not detected in three yellow perch samples on BHI and MAC media; while Erm\textsuperscript{r} (up to 10\textsuperscript{5} CFU/g or ml) and Sul\textsuperscript{r} (up to 10\textsuperscript{7} CFU/g or ml) population were detected in all examined raw seafood samples from farms.

Percentage of ART bacteria in total bacterial population varied in different seafood samples. For example, Tet\textsuperscript{r} bacteria in bluegill 1 count approximately 10\% in total population on both BHI and MAC plates; whereas in bluegill 2 and 3, they only count 0.3\% and 0.02\% in total population on BHI plates, and the presence of Tet\textsuperscript{r} population was found to be below detection limit on MAC plates. Similarly, Erm\textsuperscript{r} bacteria in yellow perch 1 and 2 count 1.3\% and 0.6\% in total population on BHI plates; while they were below detection limit in yellow perch 3.

The plate count data also showed that prevalence of resistance in normal Gram negative bacteria (on MAC plate) is relatively limited compared with bacterial population on BHI plate. It is worth noting that though Sul\textsuperscript{r} population was detected on BHI plate in all samples tested, they were mostly found to be below detection limit on MAC plates (5 out of 9 samples).

5.4.2 Prevalence of AR in seafood products from market

5.4.2.1 AR in raw seafood products from domestic and international markets

ART bacteria were found to be prevalent in raw seafood products in U.S. and overseas markets (China), such as shrimp, tilapia, catfish and crustacean. Fig. 13 showed a
comparison of prevalence of ART bacteria in raw seafood products collected from retail markets in central Ohio and South China.

Fig. 13 Prevalence of ART bacteria in seafood market in U.S. and China. A, Tet\textsuperscript{r} bacteria; B, Erm\textsuperscript{r} bacteria; C, Sul\textsuperscript{r} bacteria; D, Ctx\textsuperscript{r} bacteria.
As a comparison, raw seafood materials were also collected from a seafood market in Honolulu, Hawaii, as a control of environment with less impact from agriculture practices. One shrimp and 3 fish samples were assessed and ART bacteria were also found in these
seafood samples from Hawaii (Fig. 14). ART bacteria were detected from shrimp and fish surface wash liquid, ranging from $10^3$ to $10^5$ CFU/ml for Tet$^+$ bacteria; $10^4$ to $10^6$ CFU/ml for Erm$^+$ bacteria and $10^5$ to $10^7$ CFU/ml for Sul$^+$ bacteria.

Fig. 14 Prevalence of ART bacteria in raw seafood product from Hawaii

The surveillance data acquired in this study implied that ART bacteria were prevalent in raw seafood products from both domestic and international markets, from main land to island.

5.4.2.2 ART bacteria in ready-to-consume seafood products

A collection of processed seafood products from retail stores in Columbus, including tuna and salmon sushi, cocktail shrimp, spicy fried shrimp as well as steamed crawfish were
examined. ART bacteria (Tet$^\text{I}$ and Sul$^\text{I}$) were recovered on BHI agar plates (Fig. 15). The data suggested that besides raw seafood products, ART bacteria were also prevalent in processed seafood, including many deli products.

![Graph showing bacterial count in various seafood products](image)

**Fig. 15 Prevalence of ART bacteria in ready-to-consume seafood products.**

- Quantity of bacteria is below detection limit (100 CFU/g).
- The plate count number was an estimate.

### 5.4.3 Presence of AR gene pools in raw seafood products

Representative AR gene pools were determined by Taqman real-time PCR. Fig 16 showed that tet$(M)$, tet$(S)$, erm$B$, sul$1$ and sul$2$, were present in raw seafood products collected from retail stores/markets in both countries.
Fig. 16 Antibiotic resistance gene pools in market raw seafood products. A, \textit{tet}(M) gene; B, \textit{tet}(S) gene; C, \textit{erm}B gene; D, \textit{sul}1 gene; E, \textit{sul}2 gene.

\textbullet Size of AR gene pool is below detection limit.

* Part of samples were recruited by lab colleagues
Figure 16 continued

C

D

Continued
5.4.4 Profile of ART bacteria in seafood products

Among isolated ART bacteria, AR gene carriers were primary concern for AR dissemination and thus were chosen for further identification. The identities of the four \textit{tet} genes (\textit{tet(S)}, \textit{tet(L)}, \textit{tet(M)}, \textit{tet(K)}) and two \textit{sul} genes (\textit{sul1}, \textit{sul2}) containing isolates were illustrated in Table 6.
<table>
<thead>
<tr>
<th>Seafood origin</th>
<th>ART bacteria</th>
<th>AR gene</th>
<th>Percentage of AR gene carriers</th>
<th>Identity of AR gene carrier (#positive isolates/#identified)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U. S.</td>
<td>Tet^r bacteria</td>
<td>tet(S)</td>
<td>19.5% (39/200)</td>
<td>Pseudomonas sp. (1/2), Carnobacterium sp. (1/2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tet(L)</td>
<td>27% (54/200)</td>
<td>Pseudomonas sp. (7/8), Brochothrix sp. (1/8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tet(M)</td>
<td>16% (32/200)</td>
<td>Carnbacterium sp. (1/3), Brochothrix sp. (1/3), Pseudomonas sp. (1/3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tet(K)</td>
<td>26% (52/200)</td>
<td>Pseudomonas sp. (14/16), Brochothrix sp. (1/16), Acinetobacter sp. (1/16)</td>
</tr>
<tr>
<td>Sul^r bacteria</td>
<td>sul1</td>
<td>25% (50/200)</td>
<td></td>
<td>Pseudomonas sp. (12/12)</td>
</tr>
<tr>
<td></td>
<td>sul2</td>
<td>56% (112/200)</td>
<td></td>
<td>Pseudomonas sp. (4/4)</td>
</tr>
<tr>
<td>China</td>
<td>Tet^r bacteria</td>
<td>tet(S)</td>
<td>31.8% (64/201)</td>
<td>Lactococcus sp. (4/12), Macrococcus/Micrococcus sp. (2/12), Exiguobacterium sp. (1/12), Escherichia coli/Shigella sp. (1/12),</td>
</tr>
</tbody>
</table>

Table 6. Profile of AR gene carriers in seafood product
Seafood products from China had a more complex profile of AR gene carriers than those from the US, and some bacteria belong to those commonly used as probiotics in aquaculture production, such as *Bacillus* sp, *Enterococcus* sp., etc. Meanwhile, AR gene
carriers in seafood products from Hawaii contained primarily natural commensal flora associated with food products.

5.4.5 Prevalence of ART bacteria in environmental samples from aquaculture farms

Pond water, bottom mud as well as fish feed were collected from farms and sampled accordingly. A variety of ART bacteria were present in fish feed (Fig. 17). ART bacterial counts were generally higher in feed possessing higher surface-volume ratio, indicating that environment contamination might be a source of ART bacteria in the feed.

![Graph](image)

**Fig. 17 Total and ART bacteria in fish feed collected from a farm**

Apart from feed, live seafood products were also exposed to ART bacteria in the farm environment (Fig. 18), such as pond mud and pond water. For example, it was found that
resistant bacteria against the same spectrum of antibiotics in fish or shrimp samples were
detected either in pond mud or water samples.

Fig. 18 Prevalence of ART bacteria in raw seafood samples and the production
environment. A, ART bacteria in yellow perches and their production environment. B,
ART bacteria in shrimp sample and their production environment.
5.4.6 Efficacy of household cooking as AR mitigation control for AR in seafood products

Household cooking methods as mitigation of AR were evaluated in the lab. Shrimp sample were boiled and stored in the fridge for up to 48 h (Fig. 19). Boiling was effective in eliminating total microbiota and ART bacteria in shrimp samples, for treatment no more than 15 min.

![Bacterial counts in shrimp samples before and after boiling treatment. Data were acquired from two experimental repeats.](image)

Fig. 19 Bacterial counts in shrimp samples before and after boiling treatment. Data were acquired from two experimental repeats.

☆ Bacterial count is below detection limit (100 CFU/g).

Cooked shrimp were stored in the fridge for up to 48 hours. It was found that total bacteria as well as ART bacteria in untreated raw shrimp samples amplified significantly (approximately 1000 times in 48 h) under fridge temperature; however, a 5 min boiling treatment effectively inhibited such amplification. A more intensive treatment (>10 min)
implied even more damage to bacterial cells, that the number of bacteria associated with shrimp samples diminished in 48 h in the fridge. The findings suggest that proper household cooking not only effectively reduced ART bacteria in the seafood, but also minimized their recovery during storage (Fig. 20)

Fig. 20 The impact of boiling and storage treatments on bacteria associated with shrimp samples. A, raw shrimp samples without any treatments; B, shrimp samples boiled for 5 min; C, shrimp samples boiled for 10 min. Data were acquired from two experimental repeats.

★Bacterial count is below detection limit (100 CFU/g).
Additional household cooking methods were also evaluated, including stir frying, steam and grill. It was observed that stir frying and grill applied similar log reduction on total bacterial population as well as ART bacteria (Fig. 21); while steam applied even greater log reduction (>5 log) on bacteria associated with raw shrimp samples (data not shown).
Fig. 21 Efficacy of household cooking on ART bacteria associated with shrimp

* Bacterial count is below detection limit (100 CFU/g).

5.4.7 Waste disposal during seafood production

Large quantity of seafood waste, such as fish internal organ, was disposed during seafood processing. It was found that fish internal organ was a significant source of ART bacteria (Fig. 22). Once disposed into environment without proper treatment, it will become a potential source disseminating ART bacteria.
Fig. 22 Prevalence of ART bacteria in seafood waste (fish internal organ).

* Bacterial count is below detection limit (100 CFU/g).

* The antibiotic was not tested in the sample.

5.5 Conclusion and Discussion

Antibiotic resistance in seafood production chain was examined in this study for potential critical control point assessment. ART bacteria as well as multiple AR gene pools were found prevalent in raw materials, production environment and final products in samples from different locations, indicating this is not an isolated issue. Microbial assessment data suggested that feed as well as environmental factors might impact the ART bacteria seen in the final products.

Evidence also suggested that aquaculture production practice might play an important role determining the profile of ART bacteria contaminants associated with raw seafood materials. It was observed that AR gene carriers isolated from seafood from China were primarily lactic acid bacteria and Bacillus etc., which were commonly used to as
probiotics in the production; whereas AR gene carriers from raw seafood product in Columbus OH and Hawaii were belonged to genera commonly associated with seafood.

Besides raw seafood materials, ART bacteria were also found in certain ready-to-consume seafood products, indicating that current combination of food processing, storage and handling practices might not be sufficient to minimize the exposure of human to ART bacteria in seafood. In particular, certain processed seafood, such as sushi and sashimi, are of high risk of such exposure.

Since a significant portion of the raw food materials are still processed at home, the study evaluated common household cooking methods targeting AR mitigation. Boiling, stir frying, steam and barbecue were found effective in reducing AR in seafood samples, indicating that proper household cooking is a valid control strategy for AR mitigation in the final products. Finally, seafood waste generated during different food processing stages was found to be a significant source of ART bacteria potentially involved in AR dissemination in environmental AR microbiota.
Summary

For a long time, antibiotic mitigation has solely been focused on curbing the use of antibiotics in clinics, food animal and other agriculture productions. Recent findings on the prevalence of foodborne antibiotic resistant commensal bacteria suggested that the food chain likely serves as a critical part in the entire AR circulation route. In order to reveal the impact of the contributing factors on AR development, the baseline of AR in human GI tract system, the prevalence of AR in non-food animals, the impact of foodborne ART bacteria and antibiotic delivery methods on AR ecology in mouse GI tract, and the prevalence of AR in aquaculture products and production environment were examined in this study.

Data from the infant and non-animal study illustrated the critical role of the hosts in amplified circulation of ART bacteria even without antibiotics, suggesting reducing the AR development in the gut microbiota as well as manure and feces waste treatment are critical control points in AR mitigation. The findings on the impact of the mode of drug delivery on ART bacteria development potentially revealed a critically important cause
for the rapid emergence of antibiotic resistance. The aquaculture study illustrated the prevalence of ART bacteria in aquaculture products and production environment, and set up the foundation for future studies to reveal the impact of processing practice on ART bacteria associated with seafood products. The illustration of the effectiveness of household food processing on reducing ART bacteria in food products suggested the feasibility of interruption of AR transmission to humans through post-harvest processing, and the importance of proper knowledge dissemination in improving food safety and public health. Studies are currently underway to reveal the details of impact factors on ART bacteria development in gut microbiota, as well as in the aquaculture food chain.


13. **Centers for Disease Control and Prevention.** 2004. 12 steps to prevent antimicrobial resistance among long-term care residents. [http://www.cdc.gov/drugresistance/healthcare/ltc/12steps_ltc.htm](http://www.cdc.gov/drugresistance/healthcare/ltc/12steps_ltc.htm).


