ASSESSMENT OF ANTIBIOTIC RESISTANT COMMENSAL BACTERIA IN FOOD

A Thesis

Presented as a Requirement for the Master of Science in the Graduate School of The Ohio State University

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2006

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
13 SEP 2006	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Assessment Of Antibiotic Resistant Commensal Bacteria In Food	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **The Ohio State University**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) **AFIT/CIA**	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**122**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

ABSTRACT

The rapid emergence of antibiotic resistant (ART) pathogens is a major public health concern. Although antibiotic resistance (AR) in foodborne pathogens has been studied extensively, the contribution of foodborne commensals in disseminating the resistance genes has been neglected in the past. Foodborne pathogens only account for a very small portion of microbes associated with food; meanwhile, AR encoding genes can be transferred readily from commensals to pathogens by natural gene transfer mechanisms. Horizontal transmission of genetic material from one organism to another has been established as a major mechanism for the expedited development of resistance. The impacts of antibiotic applications in clinical treatments, veterinary medicine, animal husbandry practices and animal feed on the emergence of antibiotic resistance (AR) have been well-documented. However, knowledge on other major routes in the dissemination of AR is limited. This information is essential to properly evaluate the contribution of food chain in the evolvement of AR pathogen, particularly in susceptible host populations.

The objective of this study is to reveal the significance of the food chain in AR dissemination by investigating the prevalence of ART commensal organisms in a variety of ready-to-eat (RTE) and raw foods, Samples were evaluated for the total microbial counts, as well as resistant population for tetracycline (Tet) and erythromycin (Em). All food items were analyzed within the use-by dates. RTE salad mixes contained ART population greater than 10^3 CFU/g, more than 80% of which were resistant to erythromycin. Block cheeses had resistant counts greater than 10^2 CFU/g. Raw meat products, both raw and cooked shrimps all have resistant population greater than 10^2 CFU/g.

Further studies confirmed the presence of mobile AR genes in the food isolates, by assessing the presence of AR genes in ART commensals and their transmission to human residential bacteria, using *erm*C gene as an example. Nine RTE bagged salads and nine RTE deli-meats from local retail stores were examined for the prevalence of tetracycline –resistant (Tet^r) and erythromycin-resistant (Em^r) bacteria using plate count agar (PCA) with tetracycline (16μg-mL^{-1}) or erythromycin (50μg-mL^{-1}), and without antibiotics. Cycloheximide (100μg-mL^{-1}) was added to all plates to inhibit the growth of molds and yeasts. All nine salad samples contained at least 10^3 CFU of Em^r bacteria per gram of food., but the ART bacteria were only found in RTE deli meats sporadically. A total of 108 Em^r colonies were screened by conventional PCR and nearly 14% possessed the

*erm*C gene. The *erm*C isolates were found to be either *Pseudomonas* spp. or *Staphylococcus* spp. using partial 16S rRNA gene sequence analysis . The *erm*C gene from a salad *Staphylococcus epidermidis* isolate was transferred to *Streptococcus mutans* UA159 by natural transformation. The *erm*C gene was found associated with a plasmid(s) of approximately 2.4 kb to 4.0 kb in both the *S. mutans* transformants and the *Staphylococcus epidermidis* donor strain by Southern blot analysis. The minimum inhibitory concentration (MIC) of Em for the transformants was significantly increased compared to the parental *S. mutans* strain, but comparable to the donor strain. These data suggest that ART bacteria are prevalent in the food chain and might serve as a potentially important avenue transmitting AR genes to human microflora and possibly pathogens.

This work is dedicated to Damara, Zachary and Brian for all their support and understanding.

ACKNOWLEDGMENTS

I would like to thank Dr. Hua H. Wang, my adviser, who served as a true mentor by providing knowledgeable advice on all aspects of academic life and offering the encouragement and intellectual support that made my research possible. I want to thank her for her patience and directions to conduct the research.

I would also like to thank Dr. Ahmed Yousef for thoughtful conversations, including providing feedback and suggestions on my research and for serving as a committee member. I would also like to thank Dr. Randall Harris for serving as a committee member, even though this is outside his usual discipline. People like you create a positive learning environment both inside and outside the classroom.

I am very grateful to Hongliang Luo, Scott Hanna, Michele Manuzon, and Kai Wan, whose help in the laboratory made my education possible.

This education program was funded through the USAF AFIT/CI program. The views expressed in this presentation are those of the author(s) and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the U.S. Government.

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PUBLICATIONS

Wang, H., M. Manuzon, M. Lehman, K. Wan, H. Luo, T. Wittum, A. Yousef, L. Bakaletz. 2006. Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes. FEMS Microbiol. Lett. 254, 226-231.

FIELDS OF STUDY

Major Field: Food Science and Technology

Minor Field: Public Health

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CHAPTER 1

INTRODUCTION

The emergence of resistant pathogens untreatable by antibiotics is a major health concern. Several organizations, including the World Health Organization (WHO), Centers for Disease and Prevention (CDC), and the European Union (EU), have all stressed the need to control the spread of resistance (1,5,18). The potential link between the applications of antibiotics in food animal production and agriculture, and the increased resistance in antibiotic resistance (AR) in agricultural environment and products triggered several government-coordinated actions. In the European Union, steps have been taken not only to track the use of antibiotics in veterinary and human medicine, but to ban the application of certain antimicrobial agents (tylosin, spiramycin, bacitracin, and virginiamycin) as growth promoters in food animal production; chicken and swine farmers, as well as beef producers in Denmark voluntarily stopped using antimicrobial agents as growth promoters since the late 1990's (1). However, effective control strategies are yet to be recommended and implemented in the US. In 1996 the National Antimicrobial Resistance Monitoring System (NARMS) was established as a collaborative effort among the CDC, United States Department of Agriculture (USDA), Food and Drug Administration (FDA), and state and local public health departments. The primary mission of NARMS is to monitor antibiotic resistance (AR) in foodborne enteric pathogens (2). The increases in AR among enteric pathogens, i.e.,

Camplyobacter, *Salmonella*, and *E. coli*, were recognized (3). Furthermore, it was suggested that commensal enteric bacteria of both animals and humans could be used as indicators of potential AR development, and could serve as genetic reservoir, allowing pathogens and opportunistic bacteria to develop AR in an accelerated manner (6,10,15). According to Mellon et al (8), 28 million pounds of antibiotics are used annually in agriculture in the absence of disease. The Union of Concerned Scientists estimated over 31 million pounds of antibiotics are used in animals annually (8), representing 89% of the total annual antibiotic usage in the United States. In 2004, the Animal Health Institutes estimated that 21.7 million pounds of antimicrobials were used in veterinary medicine in the US (4), down from 24.4 million pounds in 1999. A landmark development is FDA banned the use of enrofloxacin in poultry farmers in 2005 (7).

It is demonstrated that AR bacteria occur in a variety of environmental compartments; i.e. water, sewage, and soil. Furthermore it is documented that the AR genes from these bacteria can be transferred to other bacteria within the same compartment and between compartments (5,9,13). Therefore reducing the use of antibiotics may not result in the immediate slowing down of the emergence of ART bacteria. For instance, a recent study showed that the number of ART *E. coli* isolates for ten antimicrobials from dairy cows raised on organic dairies did not vary much from those from conventional dairies (12). Further, ART bacteria are found prevalent in oral microflora of healthy adults and children (16,17), and the gut microbial flora of normal humans have become a potential reservoir for AR genes, likely involved in acquiring and donating AR genes to transient intestinal bacteria (11). The food supply, specifically animal products, has been

implicated in the transfer of AR genes from the farms to the human intestinal tract (11,14). However, despite the isolation and identification of ART pathogens in food animals and retail products, which only represent a very small percentage of the microbial flora associated with foods, the impact of the food chain on AR dissemination is yet to be revealed.

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CHAPTER 2

LITERATURE REVIEW

2.1 History of antibiotics

As the germ theory grew in acceptance in the 19th century, so did the search for antibiotics. In 1887, Rudolf Emmerich demonstrated that animals artificially infected with streptococci were protected from developing cholera. In 1896, a French medical student, Ernest Duchesne, first discovered that the soil mold *Penicillium* was able to inhibit the growth of some bacteria. (37). In 1928, a Brittish physician Alexander Fleming observed the similar phenomenon. One of his bacterial plate cultures was contaminated with the blue-green mold *Penicillium*, and the bacterial colonies in close approximation to the mold showed disrupted growth. Subsequently, Dr. Fleming grew cultures of the mold and was able to demonstrate its ability to kill bacteria (26). Antibiotics were first used in mass by the military in World War II. Their success in reducing mortality due to wound infections didn't go un-noticed. Soon after, the application of antibiotics in therapeutics was expanded to the general population and the production of antibiotics kept growing during the postwar years. Antibiotics quickly

earned the nickname "magic bullets", in part because they were much safer than previous remedies which often involved toxic compounds such as arsenic (46).

2.2 Classes of antibiotics

Antibiotics can be classified in several different ways. These classifications can be based on the mechanism of action (bactericidal versus bacteriostatic), chemical structure, spectrum of activity (broad versus narrow), or route of administration. Describing antibiotics by their common chemical structure is often the most useful way to classify antibiotics because those with similar structure often have similar functionality, mode of action, substrate spectrum as well as toxicities. Penicillins are perhaps the best known of all the antibiotics and are similar in structure to cephalasporins. Both penicillins and cephalasporins contain a beta-lactam structure and are referred to as beta-lactam antibiotics. Beta-lactam antibiotics are considered bactericidal against Gram-positive and Gram-negative bacteria. Beta-lactams inhibit the synthesis of the cell walls via the disruption of metabolic functions. Macrolides, another class of antibiotics, can be differentiated from other antibiotics by their macrocyclic lactone chemical structure and are derived from *Streptomyces* spp. Erythromycin, azithromycin and clarithromycin are all common antibiotics in this class. This class is used most commonly to treat Gram-positive cocci, but can also be used against gram-negative anaerobes. However, reduced susceptibility to erythromycin is commonly observed in *Staphylococcus aureus* due to acquiring resistance genes thus it is recommended not to routinely use erythromycin

when treating *Staphylococcus aureus*. Clarithromycin is commonly used to treat *Helicobacter pylori* infections. Macrolides are primarily bacteriostatic, inhibiting protein synthesis by binding to the 50S subunit of the ribosome (6). Tetracyclines are named because of their four ring structure. Although they are similar to macrolides in that they are derived from *Streptomyces* spp., these bacteriostatic antibiotics differ slightly from macrolides in their mode of action by binding to the 30S subunit of the ribosome. Fluroquinolones are synthetic antibacterial agents. They are considered to be broad-spectrum, bactericidial antibiotics, inhibiting DNA gyrase activity (31)

2.3 Antibiotic applications

Originally used to treat bacterial infections in both animals and humans, the applications of antibiotics have expanded beyond their obvious use against bacteria. For instance, antibiotics are also used extensively as growth promoters in food animal production (53). The animals can gain approximately 5% more body weight when antibiotics are included in their feed (14). In agriculture, antibiotics are sprayed on crops to combat plant pathogens, but are classified as pesticides when used in this manner. Even in human medicine, antibiotics are sometimes used therapeutically, but not to combat bacteria. For example, macrolides have been used in cystic fibrosis patients as an inflammatory mediator suppressor (16). Tetracycline and some tetracycline derivatives are also used to treat the syndrome of inappropriate antidiuretic hormone (SIADH) secretion, and protozoan infections such as malaria (31).

2.4 Antibiotic Resistance

Since its discovery, the scientific community has recognized the development of antibiotic resistance. In 1943, four years after drug companies began mass-producing antibiotics, the first reports of penicillin resistant *Staphylococcus aureus* were documented (26).

Dr. Fleming recognized the potential seriousness of this development and in an interview with *The New York Times*, Fleming warned the public of the implications of resistant bacteria. Through a series of experiments using various doses of antibiotics, Flemming postulated two theories on how resistance developed: bacterial proteins which could degrade the drug(s) or strengthening of the cell wall (24). Unfortunately the threat of resistance was considered unwarranted until the 1970s, when resistant strains of bacteria causing meningitis and gonorrhea proved fatal (7). Because of the similarity in antibiotic use between animals and humans, including but not limiting to tetracyclines, sulfonamides, penicillins, macrolides, fluoroquinolones, cephalosporins, aminoglycosides, chloramphenicols, and streptogramins, a serious concern is that once resistance develops in animals it will soon affect humans (5).

Resistance to any environmental pressure is an adaptive process. It is generally believed that resistance to antibiotics develops from new mutations which proves to be beneficial or through the acquisition of resistant genes (53). On the other hand, if the selective pressure is removed, the organism may lose the necessity to carry the gene over time, but

this will occur at a much slower rate, if at all (24,43). Resistance traits gained due to natural mutation usually are genetically stable; on the other hand, resistance gained through acquisition of resistance determent via horizontal gene transmission, which is rapid and common in resistance development, is affected more by selective pressure. Based on this understanding, the two major areas of antibiotic applications, human and veterinary medicines, have been the focus of regulations in an effort to reduce the selective pressures by minimizing non-therapeutic antibiotic usage. However, a recent CDC study still showed mixed results in the prescription rates of some common antibiotics. During the study period, the antimicrobial prescribing rate at all ambulatory care visits declined for amoxicillin and ampicillin, cephalosporins , and erythromycin; however rates rose for amoxicillin/clavulanate, quinolones, azithromycin and clarithromycin (29). Besides, even if the antibiotics were used appropriately, resistance to antibiotics might still be inevitable.

There has been a steady downward trend in non-therapeutic antibiotic use in animals in the last five years. Yet despite a drop from 24.4 million pounds of antibiotics used by veterinarians in 1999 to 21.7 million pounds in 2004 (12), there continues to be an increase in resistant organisms. Furthermore, bacterial isolates from organic food production, where the use and thus the exposure to antibiotics were limited, still exhibit antibiotic resistance. A recent study showed mixed results in the antibiotic resistance of *E. coli* isolated from organic dairy herds. Of the 17 antibiotics tested for, 10 showed no significant decrease in resistance when comparing organic dairy herds to convention production herds (40). Because of these mixed results, the debate as to the impact of

veterinary use of antibiotics continues on. Some argue that veterinary medicine contributes very little risk to humans when it comes to the development and transmission of antibiotic resistant infections (48). The more popular opinion is that the use of antibiotics, especially in food animals, will lead to the development of antibiotic resistance which in turn can be disseminated through the environment and led to resistant infections in humans (1). Several studies support this idea. *E. coli*, for example, has been shown to develop and disseminate antibiotic resistance in correlation with antibiotic usage in animal feed (18,25,43,52).

2.5 Mechanisms of resistance

Since most of the antibiotics in use have a natural origin, produced either by bacteria or by fungi, development of resistance is a natural process. The extent of resistance can be limited to one drug within a class (8), an entire class such as sulfanomides (31), or multiple drugs (51). Resistance development can be due to natural mutation in the bacterium. In this case the resistance trait is stable and independent from environmental selective pressure, and the trait can be disseminated vertically through multiplication. Resistance can also be due to the acquisition of resistance-encoding genetic elements via horizontal gene transmission. In this case the resistance trait can be disseminated rapidly in the microbial population, but its distribution and maintenance is greatly affected by the selective pressure. Several types of genetic determinants can lead to antibiotic resistance (38). Efflux pumps are energy dependent mechanisms which can be either acquired or located on the chromosome, and are of concern because they can extrude not only antibiotics but other biocides, often lead to multidrug resistance (23).

These pumps have been further classified into two categories, ATP-binding cassette (ABC) and secondary transporters (33). These pumps can be found in a multitude of bacteria important in food fermentation and food safety (35,36). Another common mechanism is enzyme modification. For instance, rRNA methylases can alter the rRNA and thus inhibit antibiotics from attaching to a specific region of rRNA. In the case of macrolide antibiotics there are 30 known genes which encode for rRNA methylases. All of these methylases inhibit antibiotics from binding to the 50S ribosome subunit (38). This is significant because other classes of antibiotics, such as lincosamides and streptogramins, can also be blocked (49). Similar in action to methylases are ribosomal protection proteins (RPP) which block specific binding sites of antibiotics such as tetracyclines (7). A third mechanism includes a variety of inactivating enzymes. These enzymes are specific for specific antibiotics, but are common in Gram-positive and Gram-negative bacteria (28,32). The immunity genes from the antibiotic producing strain could be an import source of resistance as well.

2.6 Gene transfer mechanisms

There are several basic types of resistance: intrinsic, mutation and acquired. Intrinsic resistance refers to the bacterial reduced susceptibility to certain anti-microbial(s) due to a specific property of a given organism, such as the molecular features of the cell wall or membrane, or the lack of a particular enzyme targeted by the antibiotic. For instance, Gram-negative bacteria are less susceptible to erythromycin. Mutation happens naturally at the frequency of somewhere around 10^{-8} to 10^{-10}. Antibiotics can accelerate the domination of resistant bacteria in the population by acting as selective agents and cause

vertical dissemination of the mutated bacteria. Genetically such mutants are stable. But if the mutants have additional auxotroph, once the antibiotics are removed, the mutated organisms may be out-competed by the wild-type organisms. Acquired resistance refers to bacterium exhibiting reduced sensitivity to a given antibiotic due to acquisition of a genetic determinant encoding specific resistance mechanism. Acquired resistance is often due to horizontal transmission of mobile elements, most commonly transposons and plasmids, carrying genes encoding resistance. Transformation, transduction, and conjugation are three common mechanisms of gene transmission. Transformation occurs when DNA or RNA from the environment is taken up and expressed by a bacterium. Transduction involves a bacteriophage mediating the transmission of bacterial DNA from one bacterium to another. Conjugation requires direct cell-to-cell contact, allowing genetic material to be passed from one bacterium to another.

2.7 Food microbiology

The interactions between microorganisms and foods, both beneficial and harmful to humans, can be traced down since recorded history. Spoilage of processed foods was first documented around 6000B.C. (42). Beer making dated back as far as 7000BC (34). Egyptians used milk, cheese and butter as early as 300 B.C. (20). Louis Pasteur demonstrated the souring of milk by microorganisms in 1837. Seventeen years later, he successfully used heat to destroy unwanted microbes in beer and wine. This technique of Pasteurization was then adapted to process milk in 1880 (11).

In most cases, microorganisms are abundant in raw food materials and considerable amount of them can survive various processing conditions. Very few foods are considered sterile, free of any microorganisms. Some of the microbes can cause diseases and are referred to as foodborne pathogens. Despite pathogens only account for a very small percentage in foodborne microbes, they have received the most attention because of their significance in public health. According to the CDC, foodborne pathogens account for over 76 million illnesses, 325,000 hospitalizations and 5000 deaths annually (30). Most of these diseases are self-limiting, and thus the number of illnesses is thought to be grossly underestimated. Of all the known pathogens, 90% of all food related illnesses and deaths are caused by five pathogens, *Salmonella*, *Listeria*, *Toxoplasma*, Norwalk-like viruses, *Campylobacter*, and *E. coli* O157:H7.

Most microorganisms associated with foods do not cause sickness in humans and are referred to as commensals. An important concern of foodborne commensals is that some of them can lead to food spoilage. Despite of all the advances in food processing and preservation, microbial spoilage continues to be a problem today. A study by the USDA Economic Research Service reported that 5,449 million pounds of edible food, which represented approximately 2% of the total edible food supply in the United States, were lost at the retail stage in 1995. Almost half of the losses came from perishable items such as dairy products and fresh fruits and vegetables (22). A number of spoilage bacteria responsible are also considered as opportunistic pathogens in humans, such as *Pseudomonas*, *Enterococcus*, and *Staphylococcus*, in immuno-compromised patients.

One of the beneficial applications of microbes is food fermentation, during which microorganisms can convert food materials such as carbohydrates through metabolic activities into desirable end products such as lactic acid, ethanol, and other flavor compounds. The rapid growth of fermentation cultures can inhibit the outgrowth of other spoilage and pathogenic microbes through various means such as producing antagonistic compounds, decreasing the pH, changing the redox potential, and minimizing the availability of essential nutrients, etc. Therefore a primary function of fermentation is preserving perishable raw food materials. As a consequence, many fermentation products distinctive from the original raw materials, such as cheese, bread, and beer, were added to our list of favorite foods. Although fermentation is usually desired, in the wrong food category a beneficial fermentative bacterium could be considered as a spoilage bacterium. For example, *Pediococcus cerevisiae* is considered desirable in the final stage of sauerkraut fermentation, but in beer *P. cerevisiae* is a spoilage organism. The most common fermentative bacteria are lactic acid bacteria (LAB). This group consists of 12 genera of Gram-positive bacteria (20) and has been extensively studied (39).

2.8 Antibiotic resistant bacteria and food safety

A number of antibiotics, such as tetracyclines and macrolides are commonly used in both animals and humans, and bacteria resistant to these antibiotics have been found in humans, animals and the food supply consisting of pathogens, fermentation bacteria and commensal bacteria (3,27,44,45,47). While there are several reasons antibiotic resistant bacteria pose a particular problem to food safety, the CDC emphasizes the following three major concerns (4).

First, the emergence of ART foodborne pathogens diminishes the efficiency of therapeutic treatment of the corresponding foodborne diseases. In the United States alone, several million people are infected with foodborne pathogens annually. Antibiotics are sometimes needed to successfully treat these diseases and can be life-saving especially in infants, immuno-compromised, pregnant and elderly individuals. The most tracked and studied foodborne pathogens are those associated with animals, such as *E. coli*, *Salmonella*, *Campylobacter*, and *Listeria*, in both the United States and worldwide (3,21,41,50). However, various resistance genes have been identified in many of these pathogenic bacteria. Unfortunately, the research emphasis has so far been limited to examine the prevalence of ART pathogens in food animals and its potential transfer to humans, and the ecological complexity of antibiotic resistance has not been fully explored (41). A few studies have shown that human sewage instead of animal waste might be the source of resistant bacteria into the environment (17,19). Obviously, just illustrating the prevalence of antibiotic resistant microbes in the food supply is not enough. Appropriate regulation and monitoring can only be accomplished when the origins and entire ecology of antibiotic resistance is understood (9,21).

Second, the normal microflora of animals and humans can be disrupted when exposed to sublethal doses of antibiotics, which can increase the susceptibility of the subjects to disease causing agents. Normally healthy people can tolerant the infection of a small amount of pathogenic bacteria without getting the disease because the normal intestinal microflora of a healthy individual can out-compete the pathogens. When antibiotics are given the subsequent disruption of normal flora can make healthy individuals more

susceptible to foodborne pathogens, such as *Salmonella*, by reducing the number of bacteria needed to cause disease, and leading to a more severe disease because the resistant bacteria has a selective advantage over the normal microflora (5). It is also important to note that the intestinal track of both humans and animals has been speculated to be the location of gene transfer for *Listeria* (10) thus further promoting the pathology of the disease.

Related to this is the third major concern of antibiotic resistance relating to food safety. Although pathogens are of major public health concern the number of commensal bacteria far outnumber that of pathogens. Thus, if antibiotic resistance develops in commensal bacteria this genetic material could be transferred to pathogens. While the CDC states this phenomenon would be difficult to quantify, several studies have demonstrated this phenomenon. Genes encoding for resistance against tetracycline, erythromycin and vancomycin have been detected in several commensal lactic acid bacteria from fermented meats and dairy products (27,47). Several countries in the European community have tracked resistant zoonotic species using non-pathogenic *E. coli* as an indicator organism (3,15). DeFrancesco et al (2004) demonstrated that commensal *E. coli* had higher rates of resistance than pathogenic multi-drug resistant Salmonella from the same herds, suggesting that commensal bacteria could be used to survey the prevalence of antibiotic resistant bacteria. Another study demonstrated similarities in virulence factors between human isolates and food isolates examining *Enterococcus* isolates, concluding that food commensals may be an overlooked aspect of antibiotic resistance (13). Boehme et al (2004) examined antibiotic resistant enterococci

in agricultural foodstuffs and found that vegetable foods, although not as well studied as animal products, could serve as a common gene pool for resistant genes.

Therefore, the overall impact of the food chain in AR transmission has not been fully understood, and studies on the prevalence of AR in the foodborne microbes, particularly in the dominant commensal bacteria, and the potential transmission to host ecosystems, need to be evaluated.

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CHAPTER 3

OBJECTIVE

The objective of this study was to assess antibiotic resistance in various foods. This would be accomplished by 1) determining the prevalence of ART bacteria targeting two commonly used broad-spectrum antibiotics, erythromycin and tetracycline, using both conventional microbiology as well as molecular biology techniques; 2) conducting a more focused study to determine the genetic characteristics of the ARTs based on information from the prevalence study as well as to demonstrate the transfer of the genetic material from food commensal bacteria to human residential bacterium in laboratory settings. While several studies have been done to assess antibiotic resistance in both foodborne pathogens and clinical pathogens, only a few have attempted to demonstrate the role of commensal bacteria from food in disseminating antibiotic resistance to humans. This study is in an attempt to fill this knowledge gap and to help better understand the ecology involved in the development of antibiotic resistance.

CHAPTER 4

PREVALENCE OF ART BACTERIA IN SELECTED RETAIL FOOD PRODUCTS

4.1 Summary

The rapid emergence of antibiotic-resistant (ART) pathogens is a major threat to public health. While the surfacing of ART food-borne pathogens is alarming, the magnitude of the antibiotic resistance (AR) gene pool in food-borne commensal microbes is yet to be revealed. Incidence of ART commensals in selected retail food products was examined in this study. The presence of 10^2–10^7 CFU of ART bacteria per gram of foods in many samples, particularly in ready-to-eat, 'healthy' food items, indicates that ART bacteria are abundant in the food chain. AR-encoding genes were detected in ART isolates, suggesting that food could be an important avenue for ART bacteria evolution and dissemination.

4.2 Introduction

Resistant pathogens to various antibiotics are emerging rapidly. Surfacing of these resistant pathogens, untreatable by antibiotics, constitutes a real threat to public health. To effectively combat this problem, a comprehensive understanding of the major pathways in antibiotic resistance (AR) gene dissemination, as well as the key mechanisms in the evolution of antibiotic-resistant (ART) bacteria is essential. Horizontal gene transfer among pathogens in the hospital environment has been recognized as an important avenue for the rapid spread of AR genes among pathogens. It is also believed that horizontal transfer of AR genes between commensal and pathogenic microorganisms in ecosystems are much more likely events than direct AR gene dissemination from one pathogen to another (1). The presence of AR gene reservoirs in commensal microbes in various environmental and host ecosystems (6,9,12,13,14,15, 18,19), and the illustration of commensals as facilitators for human microbiota (11) suggest the importance of commensals in mediating the dissemination of AR genes. The isolation of AR genes in food-borne pathogens from retail products exemplified the potential contribution of the food chain in transmitting ART pathogens to humans (2,7,11,20). These studies on commensal bacteria, however, are limited and primarily focused on the opportunistic pathogen enterococci (4,8).

A standard laboratory enrichment procedure (http://www.fda.gov/cvm/ Documents/AppendicesA-6.pdf) is often used to detect the presence of the ART bacteria, masking the real magnitude of the AR problem associated with the food chain. To

examine the AR risks associated with the food chain, this study aimed at revealing the distribution spectrum and magnitude of ART commensal bacteria and AR gene pool in retail foods, by targeting total food microbiota instead of a particular group of microorganisms or pathogens. Therefore, food samples were analyzed without any laboratory enrichment procedures. Prevalence of microbial resistance to tetracycline (Tet) and erythromycin (Em), still commonly used in animal production and human therapy, was investigated (3,17). The presence of representative resistance gene markers in selected food isolates was examined and main AR gene(s) and hosts were identified. Minimum inhibitory concentrations (MIC) were used to determine the susceptibility levels to further distinguish between intrinsic and extrinsic resistance.

4.3 Materials and methods

Food sample preparation and enumeration of total and ART populations.

Seventy-one various food samples, were purchased from five local grocery stores and analyzed within the products sell-by dates. Among the 11 RTE bagged or boxed salad samples, representing different mixtures of greens and other vegetables RTE, two were from two different fast-food chain restaurants. Five grams of each sample were aseptically removed from the product packaging and placed in disposable plastic bags containing 10mL of sterile 0.1% peptone water. Bagged samples were hand massaged for 10 min. Homogenized samples or rinsing liquids were serially diluted and plated on nonselective plate count agar (PCA, Becton Dickinson and Company, Sparks, MD) for nonselective total microbial counting, and on PCA plates containing 16 μg-mL^{-1} of Tet or

50 μg-mL^{-1} of Em (Fisher Biotech, Fair Lawn, NJ). Plates were incubated aerobically at 20^{o}C and/or 37^{o}C for up to 48 h for assessing Tet- and Em-resistant population of non-fermented food products. Homogenized fermented food samples were serially diluted and plated on Difco Lactobacilli MRS Agar (MRS, Becton Dickinson and Company) incubated anaerobically at 30^{o}C for 48 hours. The levels of antibiotics used in selective agar plates were based federal standards (http://www.fda.gov/cvm/Documents/ AppendicesA-6.pdf). The ART bacteria counts for each of the duplicated samples were converted to CFU/g and the means and standard deviations for both the total plate count on PCA and Em^{r} count on Em plate were calculated. The peptone water rinse from an unused sandwich bag was included as the negative control for each examination.

Antibiotic resistance gene detection and host isolates identification.

Conventional PCR was conducted to detect AR genes in the ART isolates. Bacterial cells from single colonies were re-suspended in 300 μL sterile dH_2O containing 100 mg of 1:1 mixture of 0.5 mm diameter and 0.1 mm diameter glass beads (Biospec Products Inc., Bartlesville, OK). The sample mixtures were homogenized using the Mini-Bead-Beater-8 (Biospec Products Inc.) for 3 min. at maximum speed. The resulting cell extracts were placed in a boiling water bath for 10–15 min and 5 μL of the supernatant were used as PCR templates. The primer pair *erm*C FP 5'-GCTAATATTGTTTAAATCGTCAAT-3' and *erm*C RP 5'-TCAAAACATAATATAGATAAA- 3' were used to amplify the 640 bp *erm*C fragment (Chung et al.,1999). PCR was conducted using reagents and conditions as described previously (Luo et al., 2004). PCR products with expected sizes were purified using the QIAquicks kit (Qiagen, Valencia, CA) following manufacturer's instruction.

ART isolates containing resistance genes were identified by PCR amplification of the 16S rRNA gene fragment and sequence analysis following procedures as described previously (5). DNA sequences of the 16S rRNA gene and the *erm*C gene fragments were determined using a DNA analyzer (ABI PRISMs 3700, Applied Biosystems, Foster City, CA) at the Plant Genome Sequence Facility, The Ohio State University. The DNA sequences were compared with published Em resistance gene sequences deposited in the NCBI database.

Minimum inhibition concentration (MIC) profiles of ART isolates.

The MIC profiles of selected ART isolates were determined using a commercial kit (Sensititres 18–24 h MIC and Breakpoint Susceptibility Plates; TREK Diagnostic Systems, Cleveland, OH) following the manufacturer's instructions, with modifications. Tryptic soy broth (TSB) broth instead of the standard Mueller–Hinton broth was used to culture fastidious organisms. The MIC panels were incubated at either 30 or 37^0C for 24–48 h. Additional 96- well microtiter plates with wells containing up to 256μg-mL^{-1} of Tet and Em were used to determine the MIC of Em^r or Tet^r isolates which exhibited positive growth in wells containing 16 μg-mL^{-1} of Tet or 8 μg-mL^{-1} of Em, the maximum concentrations on the Sensititres plates. The MICs were reported as the minimum concentration of the antibiotic that inhibited visible growth, as indicated by a lack of turbidity of broth or deposition of cells at the bottom of the wells. Control strains used in the study include *Staphylococcus aureus* ATCC 292136 (American Type Culture Collection (ATCC), Manassas, VA), *Pseudomonas aeruginosa* ATCC 27853 (ATCC),

and *E. coli* DH5α .

(http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=13773).

4.4 Results

Prevalence of ART bacteria in food samples.

ART microbes were detected in majority of the retail foods examined.

All of the raw meat samples contained culturable ARTs, of which 88.9% (8/9) had both Em^r and Tet^r organisms (Fig 4.1.). All twelve raw seafood samples contained ARTs, 91.7% (11/12) of these samples had both Em^r and Tet^r organisms (Fig 4.2.). Raw vegetables including carrots and mushrooms were also sampled. ART bacteria were cultured from all the samples. All of the produce samples yielded higher counts on the Em plates than the Tet plates.

Ready-to-eat (RTE) samples included yogurts, cheeses, deli meats, and bagged salads.

No detectable ART microbes were found in any of the four yogurt samples (Fig 4.3.).

Seventeen cheese samples were analyzed, all were made with pasteurized milk except sample XVII. Three different styles of block cheeses, cheddar, mozzarella, and colby, and one processed sliced cheese (sample IV), were examined in the study. Twelve samples contained ART organisms, of which 91.7% (11/12) had both Em^r and Tet^r microbes. Plate counts from the Tet plates were consistently greater than those from the

Em plates (Fig 4.4.). Samples IV, VII, XIII, XIV and XVII had no growth on either the Em or Tet plates, despite total plate counts ranging from 4.0 X 10^1 to 5.6 X10^8 CFU/g cheese. Ten pre-packaged deli meats were analyzed. Half of the samples had no detectable growth of microbes, and only one sample had growth on both the Em and Tet plates (Fig 4.5.).

The counts for the RTE salad samples did not differ much between the two incubation temperatures. Total plate counts from the 20^{o}C and 37^{o}C incubation temperatures ranged from 8.4 X 10^4 to 10.3 X 10^9 CFU/g, and 5.2 X10^4 to 2.5 X 10^9 CFU/g, respectively. Em^r bacteria were detected in every salad sample, and the numbers of Em^r bacteria were consistently greater than those of the Tet^r ones (Figs. 4.6 and 4.7).

Detection of AR genes and ART isolates identification.

To confirm that most of the ART organisms detected by growth on the Em agar plates were resistant bacteria because of the possession of AR determinants, PCR was conducted to detect the presence of selected AR genes in these organisms. Grouping single isolates and screening for individual genes produced more useful results than screening for multiple AR genes using multiplexed PCR (Appendix B). PCR products using *erm*C primers yielded a specific band with proper size in produce and meat sample analyses, and the sequences of the PCR amplicons were determined and compared with published *ermC* gene sequences deposited in the GenBank database. The *erm*C gene was found in the isolate CX-I EM (Fig. 4.8A) from packaged sliced chicken lunchmeat, and the strain was identified as *Pseudomonas* sp. ART bacteria were isolated sporadically in

lunchmeat, which is probably because of occasional contamination after food processing. Other members in our group examined the presence of other representative Em^r and Tet^r genes in dairy and raw meat samples. Their data showed that although the *erm*B-primers were problematic in screening for the target gene in produce samples (Fig. 4.8B), the primers functioned well in identifying the Em^r bacteria in cheeses, perhaps due to the difference in food matrices or different bacteria. They have found that among the Em^r isolates from cheese, more than 50% contained the *erm*B gene, and the carrier organisms identified so far include *Staphylococcus* sp. (five out of 28) and *S. thermophilus* (23 out of 28). Related results were summarized in our recent publication (Appendix A).

Minimum inhibition concentration analysis.

Minimum inhibition concentration (MIC) analysis showed that the *erm*C^+ *Staphylococcus* sp. CX-I EM from packaged sliced chicken lunchmeat was resistant to both Tet ($\leq$64 μg-mL^{-1}) and Em (>256 μg-mL^{-1}) as compared to the control strain *S. aureus* ATCC 292136 (Tet <2 μg-mL^{-1}, Em $\leq$1 μg-mL^{-1}). The MIC analysis of the *Pseudomonas* sp. Pork Tet 24 from ground raw pork was resistant to Tet (>32 μg mL^{-1}) and susceptible to Em (<0.5 μg mL^{-1}). Control strains of *P. aeruginosa* ATCC 27853 and *E. coli* DH5Aα were both susceptible to Tet at less than 4 μg mL^{-1}. The MICs of other isolates were run by other members of the lab and can be found in Appendix A.

4.5 Discussion

It is worth noting that the study was conducted using limited incubation conditions, and the antibiotic concentrations used to screen for resistant organisms might not be optimal for all bacteria. Therefore the numbers reported here only represent a portion of the total ART bacterial load in these foods. Despite the fact that this current study only screened for a limited number of resistance markers, it illustrated the prevalence of ART commensals and AR genes in retail foods.

Many ART bacteria-containing RTE products are consumed without further cooking or processing. Consequently, human are routinely inoculated with ART bacteria through daily food intake, including opportunistic pathogens and commensals such as *Pseudomonas* sp., *Streptococcus* sp. and *Staphylococcus* sp. The detection of high numbers (i.e. up to 10^8 CFU per serving of food) in several products is suggestive of the potential role food could have in transmitting ART bacteria. This finding is in agreement with a previous report showing that consuming sterile foods can significantly decrease the presence of ART bacteria in the gastrointestinal system (10). While further research is needed to establish the direct correlation between the ART microbes from foods and the ART population in the host ecosystems, it is evident that a constant supply of ART bacteria, partnered with occasional colonization and horizontal gene transfer, are at least partially responsible for the increased AR profiles seen in human. Such an intrinsic AR gene pool could have significant impact on pathogen resistance in susceptible population, particularly those receiving antibiotic treatment. Oral cavity could be an important area

where many initial interactions between food microbes and human microbiota, including horizontal gene transfer events such as conjugation and transformation, took place during the retention of food residues in the oral cavity. Furthermore, some acid resistant bacteria, such as *Helicobacter*, can survive the acidity of the stomach and are known to be antibiotic resistant. The AR genes from commensal food bacteria may complicate such a situation and aid in the development of such resistance. In fact, the *tet*S/M (Appendix A) and *erm*B genes were found to be abundant in bacteria isolated from foods, which is in agreement with the prevalence of these Tet- and Em-resistance genes in human oral microflora (18).

Interestingly, no ART bacteria were detected on neither of the selective plates containing antibiotics from yogurt, another fermented dairy food with high total plate counts. This suggests that the variabilities in starter strain selection or processing conditions may have a role in the emergence of ART bacteria in the fermented dairy products. This may point to the future direction of using altered processing conditions to control the spread of ART. Also, the produce, which normally is not associated with antibiotic exposure, contained some of the highest levels of ARTs. This is especially concerning because of these foods are often consumed without further processing by the consumers. However, the ART bacteria in produce may reflect the AR profiles of the associated environments, such as water, soil, fertilizers, and processing environments. Therefore agricultural food items such as produce may further be used as an indicator for the AR status of the related ecological environments.

Future streamlining of a surveillance methodology is needed and should include molecular techniques such as real-time PCR to better evaluate the total resistance gene pools in foods. In doing so we could better estimate the true risk by including AR genes form fastidious organisms as well as non-viable cells.

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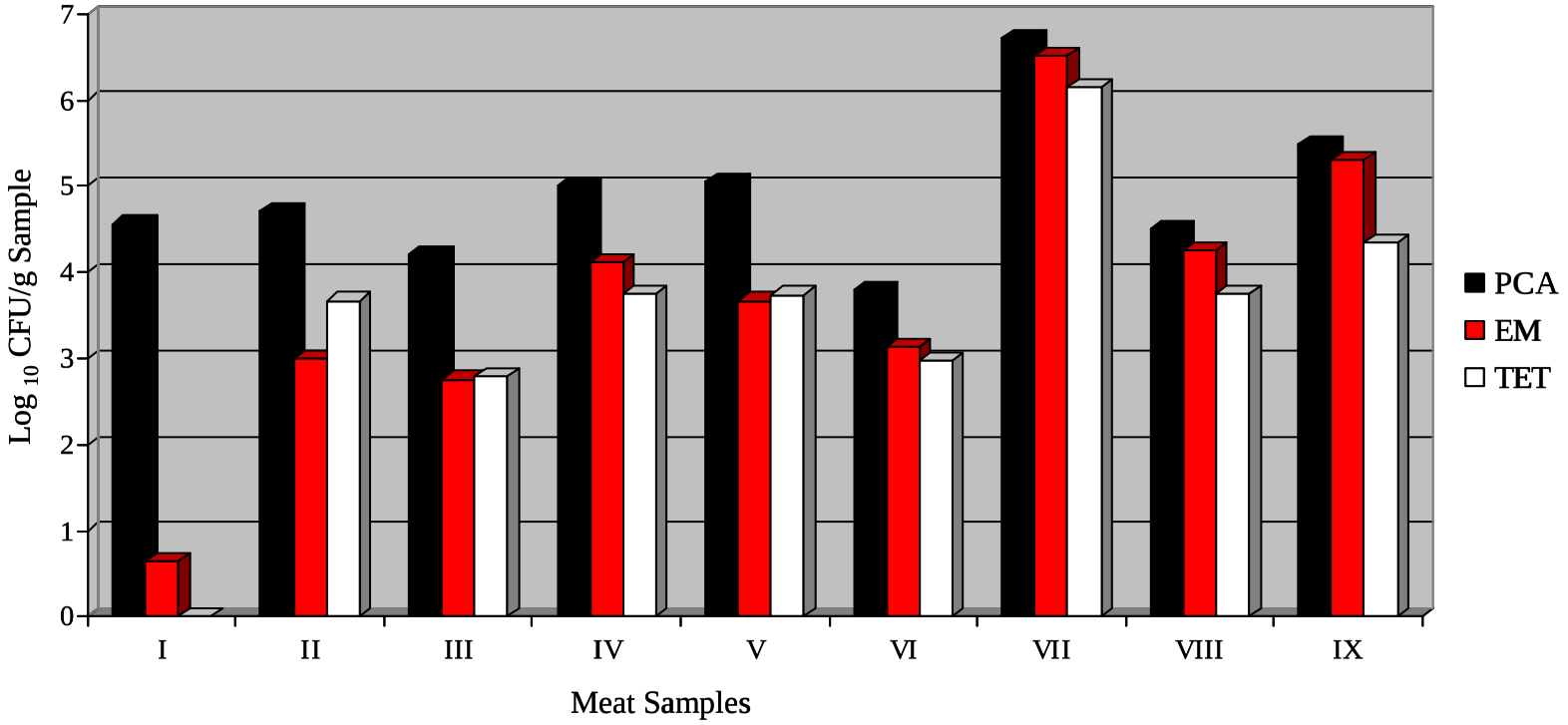

Figure 4.1. Prevalence of ART microbes in raw meat samples: I. Ground Beef; II. Ground Turkey; III. Ground Beef; IV. Breakfast sausage patties V. Ground Pork; VI. Ground Beef; VII. Ground Beef; VIII. Ground Beef; IX. Pork Chop.

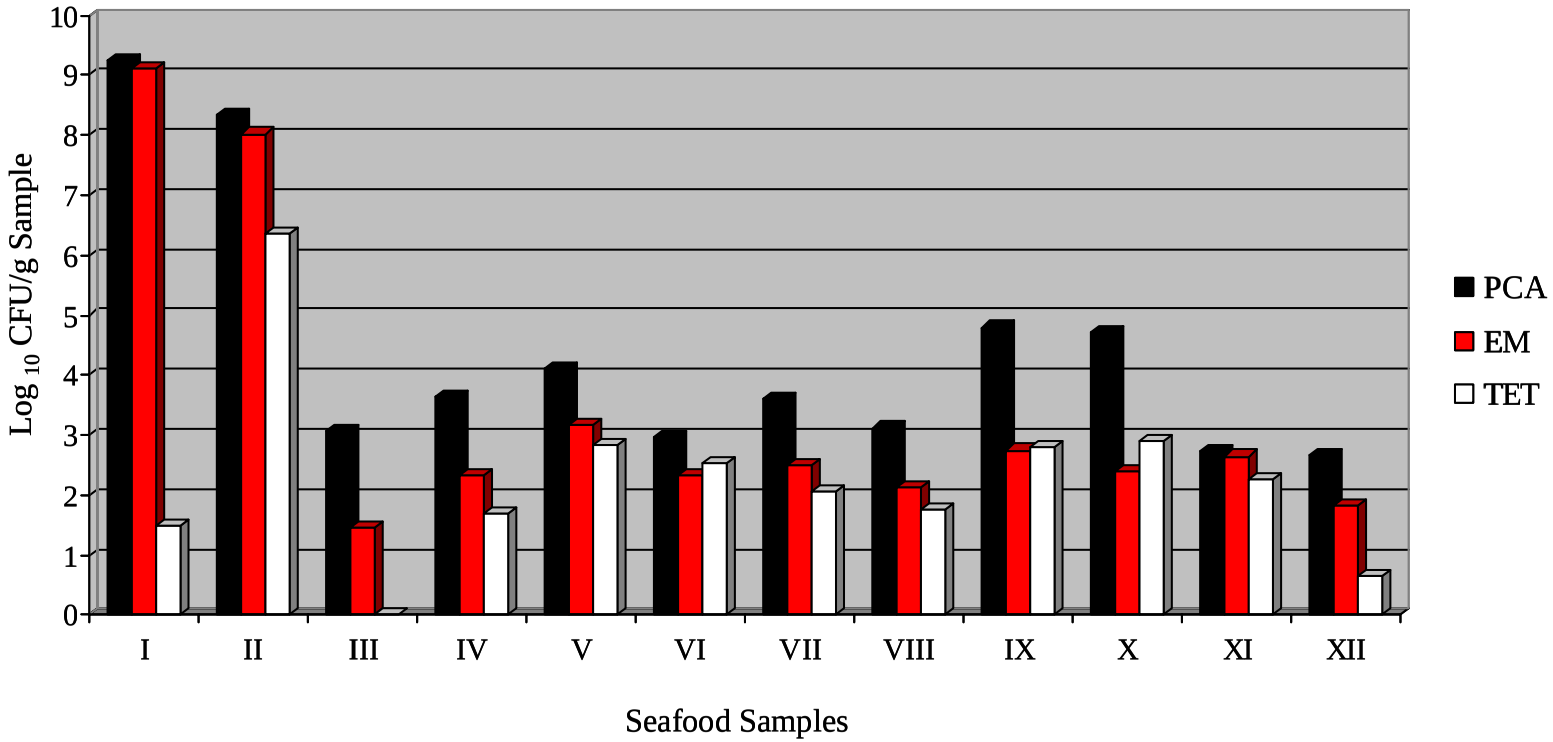

Figure 4.2. Prevalence of ART microbes in seafood samples grown at selected incubation temperatures. I. Cooked shrimp, 37ºC; II. Raw catfish nuggets, 37ºC; III. Whole fresh shrimp, head and thorax only, 20ºC; IV. Fresh whole shrimp, tail only, 20ºC; V. Frozen raw shrimp, 20ºC; VI. Frozen raw shrimp, 37ºC; VII. Frozen cooked shrimp, 20ºC; VIII. Frozen cooked shrimp, 37ºC; IX. Fresh raw shrimp, 20ºC; X. Fresh raw shrimp, 37ºC; XI. Frozen cooked shrimp, 20ºC; XII. Frozen cooked shrimp, 37ºC.

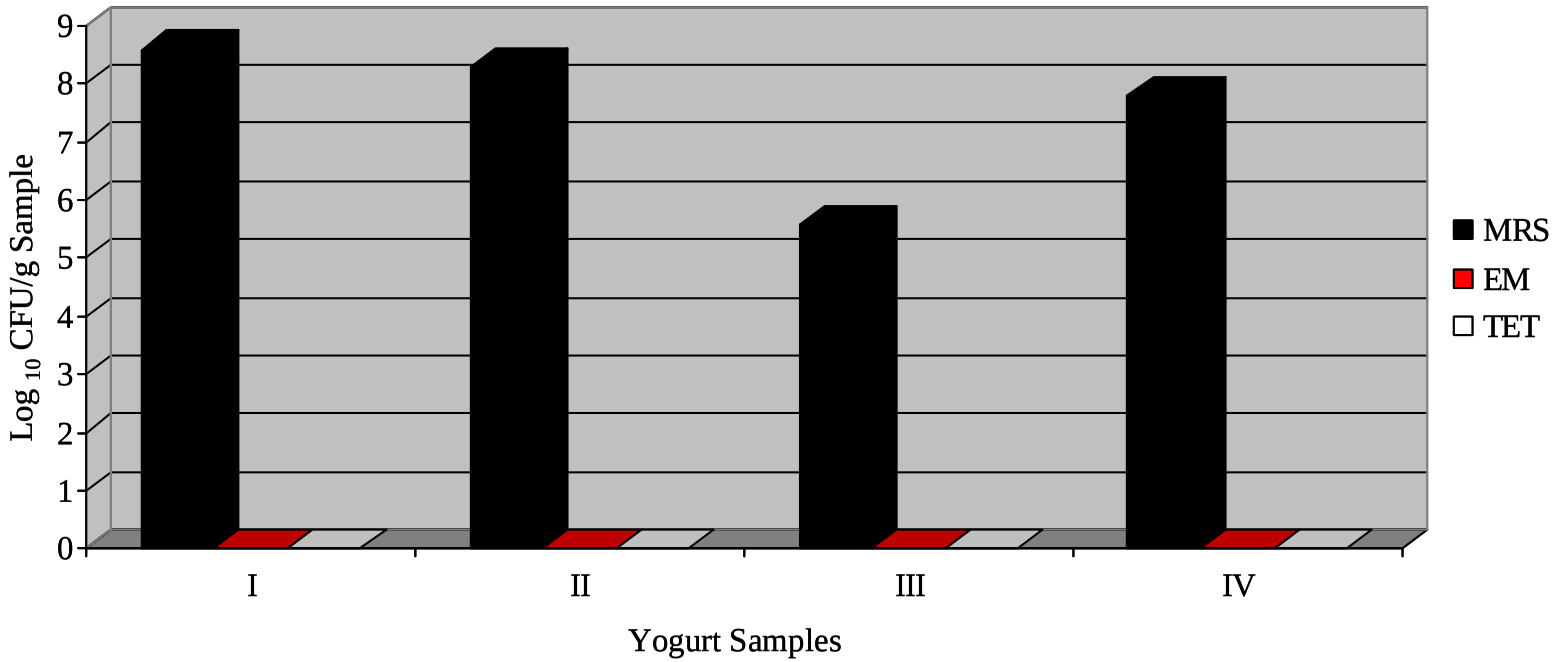

Figure 4.3. Prevalence of ART microbes in yogurt. Samples: I. Brand A low-fat; II. Brand B low-fat; III. Brand A full-fat; IV. Brand C low-fat.

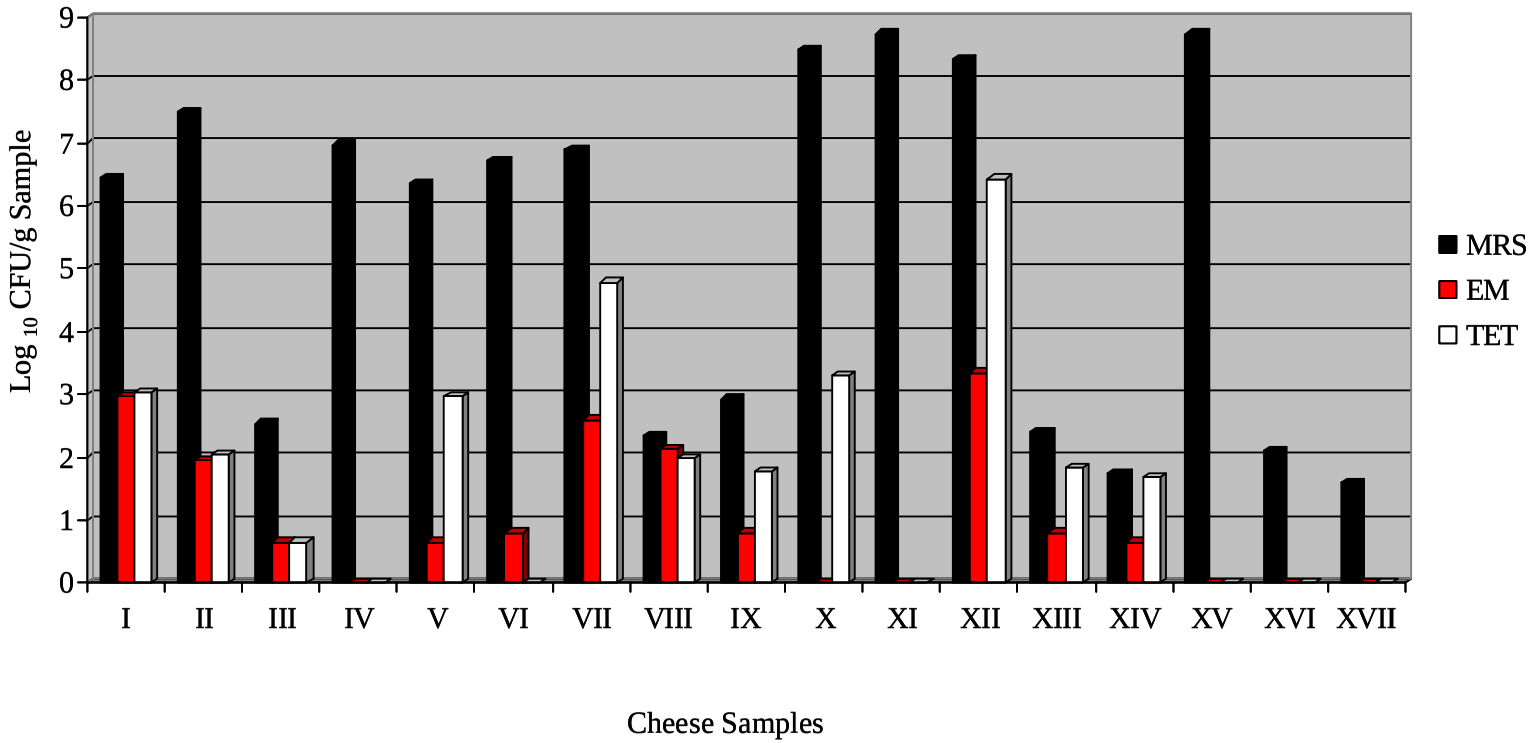

Figure 4.4. Prevalence of ART microbes in cheese. Samples: I. Cheddar A; II. Cheddar B; III. Mild cheddar A; IV. Mild cheddar B; V. Mild cheddar C; VI. Mild cheddar D; VII. Medium cheddar A; VIII. Medium cheddar B; IX. Sharp cheddar; X. Extra-sharp cheddar; XI. Organic cheddar; XII. Colby A; XIII. Colby B; XIV. Mozzarella A; XV. Mozzarella B; XVI. Mozzarella B; XVII. Sliced American.

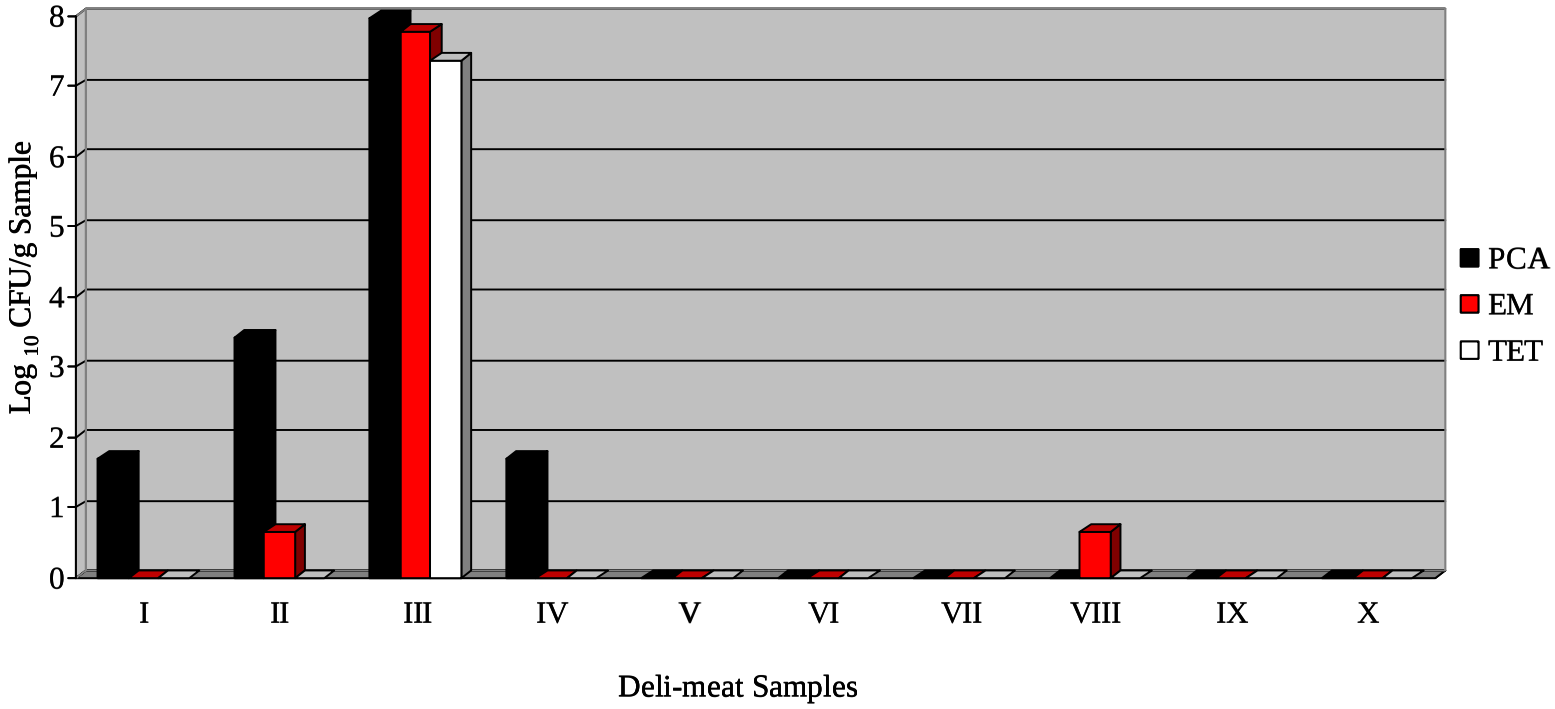

Figure 4.5. Prevalence of ART microbes in deli meats. Samples: I. Brand A. Ham; II. Brand B. Ham; III. Brand C. Ham; IV. Brand B. Beef; V. Brand A. Turkey; VI. Brand B. Turkey; VII. Brand C. Turkey; VIII. Brand B. Chicken; IX. Brand B. Chicken; X. Brand C. Chicken.

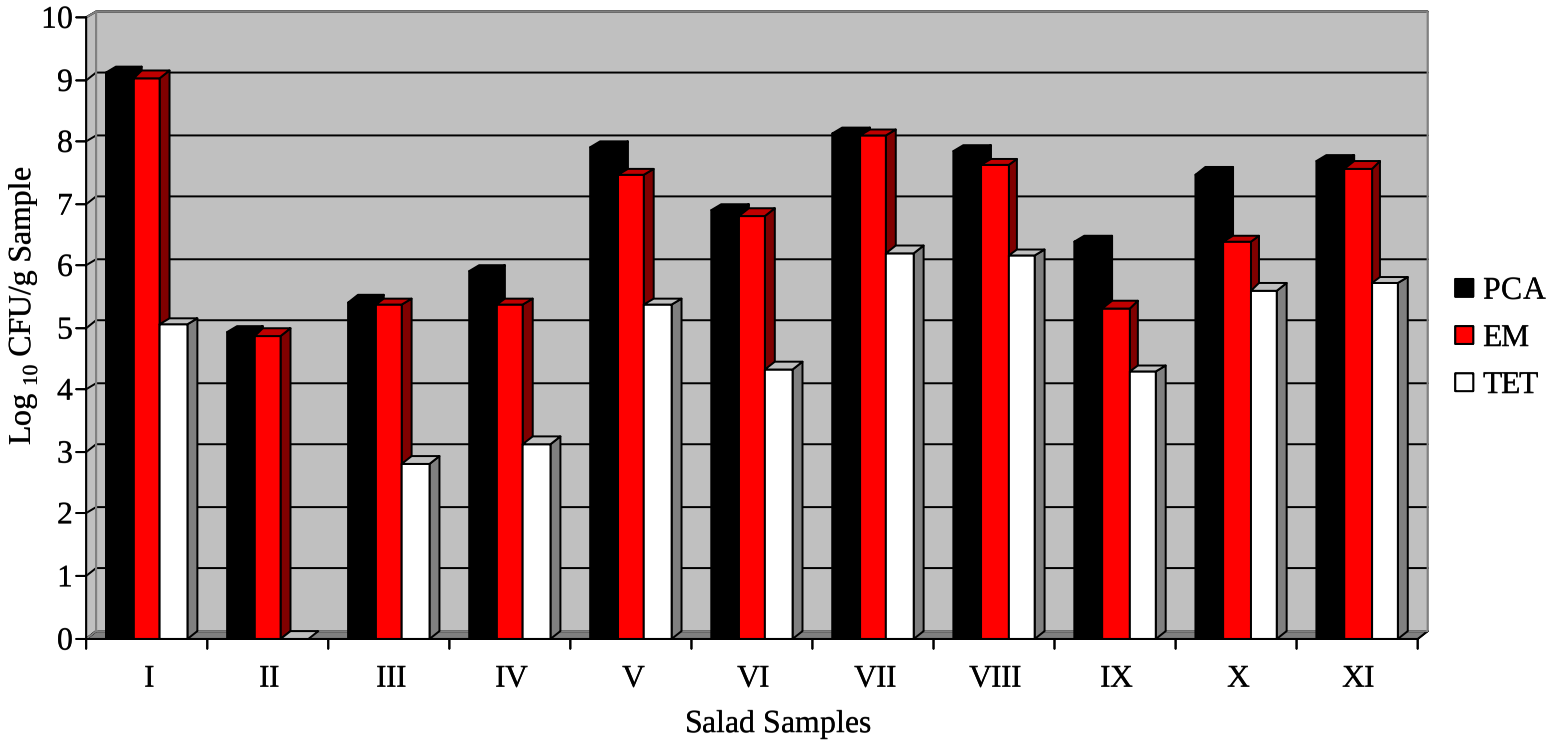

Figure 4.6. Prevalence of ART microbes in salad. Samples were incubated aerobically at 20°C for 48 hours. Samples: I. Brand A lettuce mix; II. Brand B Iceberg mix; III. Iceberg head; IV. Brand C salad mix; V. Brand A mix; VI. Brand E tossed green; VII. Brand F; VIII. Brand B iceberg mix; IX. Brand F; X. Brand G field mix; XI. Brand A iceberg mix.

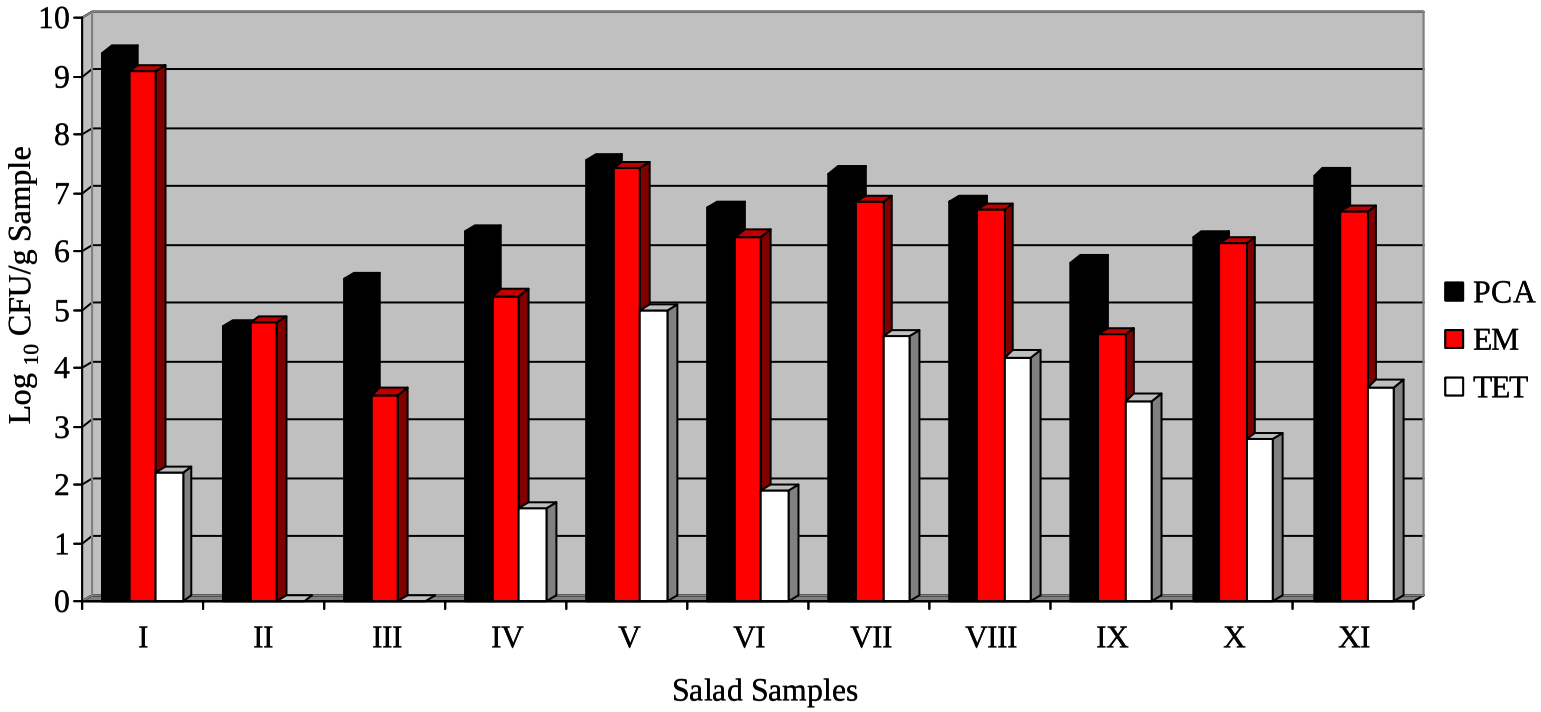

Figure 4.7. Prevalence of ART in salad. Samples were incubated aerobically at 37°C for 48 hours. Samples: I. Brand A lettuce mix; II. Brand B Iceberg mix; III. Iceberg head; IV. Brand C salad mix; V. Brand A mix; VI. Brand E tossed green; VII. Brand F; VIII. Brand B iceberg mix; IX. Brand F; X. Brand G field mix; XI. Brand A iceberg mix.

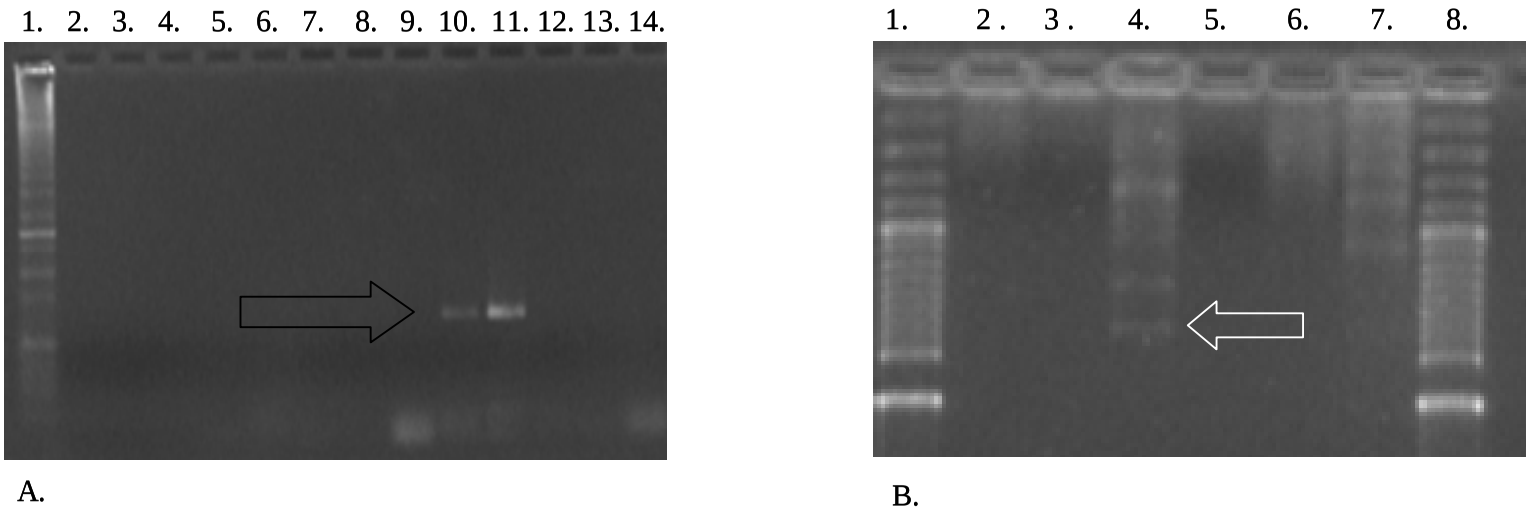

Figure 4.8. A. PCR screening of the *erm*C gene from food isolates. Lanes 2-5; salad isolates: lanes 6-9; cheese isolates: lane 10; CX I isolated from Em plate: lane 11; CX I isolated from Tet plate: lanes 12-14 shrimp isolates. The arrow indicates a positive band (640bp). B. PCR screening of the *erm*B (389 bp) gene from food isolates. Lanes 2-3; salad isolates: lanes 4-5; cheese isolates: lane 6 beef isolate: lane 7; salad isolate. The arrow indicates a possible positive band (380 bp).

CHAPTER 5

ANTIBIOTIC RESISTANCE IN READY-TO-EAT SALADS

RUNNING TITLE: Antibiotic resistance in RTE salad

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Key Words: antibiotic resistance, ready-to-eat, salad, *ermC*

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5.1 Summary

The rapid emergence of antibiotic resistant pathogens is a major public health concern and the contribution of the food chain in the dissemination of antibiotic resistance is yet to be evaluated. In this study, the prevalence of resistant commensal organisms in ready-to-eat salads was examined and the potential impact on the resistance profiles of human microflora was discussed. At least 10^3 CFU of erythromycin-resistant bacteria per gram of food were isolated from all nine ready-to-eat bagged salads purchased from local retail stores. A total of 108 Em^r colonies were screened by conventional PCR and nearly 14% possessed the *ermC* gene. Identified *ermC* carriers included *Pseudomonas* spp., *Staphylococcus epidermidis* and *Staphylococcus* spp., by partial 16S rRNA gene sequence analysis. The *ermC* gene from a salad isolate *Staphy. epidermidis* S102-35 was transferred to a human oral residential bacterium *Streptococcus mutans* UA159 by natural transformation in the laboratory setting. The *ermC* gene was found associated with a plasmid of approximately 2.4 kb in both the *S. mutans* transformants and the donor strain *Staphy. epidermidis* S102-35 by Southern blotting analysis. The *S. mutans* transformants acquired resistance to erythromycin, comparable to the donor staphylococcal strain but more than 100 times higher than the recipient UA159. Thus the resistance gene from the foodborne bacterium is transmissible to and functional in human residential bacterium. Our data suggest that a large antibiotic resistance gene pool already exists in the food chain, which may serve as a potentially important avenue in resistance gene dissemination.

5.2 Introduction

The rapid emergence of antibiotic resistant (ART) pathogens is a main threat to public health. Horizontal gene transfer among pathogens in the hospital environment has been recognized as the major avenue for the rapid spread of antibiotic resistance (AR) genes among pathogens. The potential contributions of other pathways in the dissemination of AR are also under investigation. In the past couple of decades, many AR genes have been identified in foodborne pathogens isolated from retail foods, particularly meat products (4, 21, 25), and it is well-documented that the increased use of antibiotics in veterinary and agricultural practices could have contributed to the AR in foodborne pathogens (22). Recently, a much larger AR gene pool in commensal microbes was found, not only in various environmental and host ecosystems (3, 10, 15, 18, 20), but in many ready-to-eat (RTE) food items such as shrimp, block cheeses and even sporadically in RTE lunch meats (7, 24). Commensal bacteria may not just serve as reservoirs for AR genes, but also as enhancers for the dissemination of genetic determinants beneficial to microbial survival in biotic and abiotic environments (13). Because the amount of bacteria introduced into human through routine food intake is quite large, it is important to identify the prevalence of ART bacteria in various foods comprising a typical diet, which would urge implementing proper processing conditions at both the industry and consumer levels to interrupt this AR gene transmission pathway.

So far, AR studies on RTE vegetable products other than sprouts are very limited (2, 8, 9, 17). These food items are important components in the western diet and are often

consumed on daily basis without further processing. Thus the AR status in these items directly reflects the magnitude of resistant bacteria introduced into the human microbiota via food intake. The *erm*C gene is one of the common erythromycin (Em) resistance determinants found in human staphylococcal and oral commensal isolates (16, 19), but its status in the food chain has not been examined much. Therefore, the AR profiles, particularly the prevalence of the *erm*C gene in RTE bagged salads were investigated in this study.

5.3 Materials and methods

Food sample preparation and enumeration of total and ART populations.

Nine RTE bagged salads (3 types of products, included 5 different brands) were randomly purchased from 4 different local and chain grocery stores and analyzed within the products' sell-by dates using procedures previously established in our laboratory (24), with minor modification. Basically, five grams of each sample were aseptically removed from the product package and placed in disposable plastic bag containing 10 ml of sterile 0.1% peptone water. Bagged samples were hand-messaged for 5 min and the rinsing liquids were serially diluted and plated on nonselective plate count agar (PCA, Becton Dickinson and Company, Sparks, MD) for counting total microbes, on PCA plates containing 16 μg/ml of tetracycline (Tet, Fisher Biotech, Fair Lawn, NJ) for Tet^r microbes, and on PCA containing 100 μg/ml of Em (Fisher Biotech) for Em^r microbes. All plates were incubated aerobically at 37^0C for 48 hours. All agar plates contained 100 μg/ml cyclohexamide (Fisher Biotech) to inhibit the growth of molds and yeasts.

Reported data were means of duplicated results with the standard deviations less than 10% of the mean values.

Antibiotic resistance gene detection and host isolates identification.

Conventional PCR was used to detect the presence of AR genes in ART isolates following procedures described previously (Wang et al., 2006). The primers *erm*C FP 5'-GCTAATATTGTTTAAATCGTCAAT-3' and *erm*C RP 5'-TCAAAACATAATATAGA TAAA-3' were used to amplify the 640 bp *erm*C fragment (5). PCR products with expected sizes were purified using the QIAquick® kit (Qiagen Inc., Valencia, CA) following manufacturer's instruction and subjected to DNA sequence analysis using a DNA analyzer (ABI PRISMs 3700, Applied Biosystems, Foster City, CA) at the Plant Genome Sequence Facility, The Ohio State University. The DNA sequences were compared with published *erm*C gene sequences deposited in the GenBank database.

ART isolates containing the *erm*C gene were identified by PCR amplification of the partial 16S rRNA gene fragment and sequence analysis following procedures as described previously (6).

DNA extraction.

Total DNA of representative ART isolates used for Southern blotting analysis and natural gene transformation was isolated following procedures described by Yu and Morrison (26) with modification. Basically, each bacterial isolate was inoculated into 25 ml of tryptic soy broth (TSB, Becton Dickinson and Company) containing 50 µg/ml Em and

incubated aerobically in a C24 incubator shaker at 125 r.p.m (New Brunswick Scientific Co. Inc., Edison, NJ), 35^0C for 24 hr. The cells were harvested by centrifugation and the cell pellet was resuspended in 1 ml lysis buffer, and transferred into 2 ml screw-cap tubes along with 0.4 g sterile glass beads (0.3g of 0.1 mm and 0.1g of 0.5 mm, Biospec Products Inc., Bartlesville, Okla). The sample was homogenized for 3 min on the Mini-Beadbeater™ (BioSpec Products), centrifuged for 5 min, and the supernatant was transferred to a microcentrifuge tube. The extraction was repeated one more time by adding 300 µl of fresh lysis buffer to the lysis tube followed by homogenization and centrifugation. The supernatant aliquots from two extracts were combined (total of 1.3 ml) and the total DNA was precipitated with 3M ammonium acetate and 1 vol. of isopropanol at -20^0C for 18 hr. The pellet was dried and resuspended in TE buffer. RNA and protein contaminants were removed by incubating with 20 µl RNase (1mg/ml) at 37^oC for 15 min, and 15 µl protienaseK at 70^oC for 10 min. DNA was purified using the DNeasy® tissue kit (Qiagen Inc.).

Southern blotting analysis.

The purified 640 bp *erm*C PCR amplicon was DIG-labeled using the DIG DNA labeling and detection kit (Roche Diagnostics Co., Indianapolis, IN) as the hybridization probe. Hybridization and color detection were conducted following procedures described by the manufacturer (Genius System User's Guide, version3), and the hybridization buffer contained standard buffer plus 50% formamide.

Natural gene transformation.

DNA extract from salad isolate *Staphy. epidermidis* S 102-35 was used to transform *S. mutans* UA159 following procedures described previously (12). Em^r transformants were selected on BHI agar containing 5µg/ml Em and incubated anaerobically for 48 hours at 37^0C. Transformation efficiency was calculated based on the ratio of Em^r transformants to the total number of viable cells.

Minimum inhibition concentration (MIC) profiles of ART isolates.

MIC profiles of the *erm*C^+ salad isolates, *S. mutans* UA 159, and *S. mutans* UA 159 transformants (ZB35-A and ZB35-B) were determined using commercial kit (Sensititres 18–24 h MIC and Breakpoint Susceptibility Plates, TREK Diagnostic Systems, Cleveland, OH) with modification, following procedures described previously (24).

5.4 Results

Prevalence of ART in RTE salad samples.

Nine RTE salad samples were examined for the presence of ART bacteria. Total bacterial counts for the salad samples ranged from $1.2X10^5$ to $4.3X10^7$ CFU per gram of food. The Em^r bacteria ranged from $1.1X10^3$ CFU to $4.3X10^6$ CFU per gram of food, corresponding to less than 1% to more than 84% of the total bacterial population. The Tet^r bacteria ranged from non-detectable to $5.3X10^3$ CFU per gram of food. In all cases, the Em^r bacterial counts for the salad samples were much higher than the Tet^r counts (Fig. 1).

Detection of *erm*C gene and ART isolates identification.

A total of 108 isolates from the 9 salad samples (12 isolates from each) were examined for the presence of the *erm*C gene by conventional PCR (Fig. 5.2). About 14 % (15 isolates) contained the resistance gene. The overall prevalence for each salad sample ranged from non-detectable to around 42% (Table 1).

The *erm*C positive isolates identified by 16S rRNA gene sequence analysis included *Staphylococcus epidermidis*, *Staphylococcus* spp., and *Pseudomonas* spp. (Table 1).

Southern blotting analysis.

Southern hybridization was conducted to analyze the location of the *ermC* gene from six of the staphylococcal isolates and two of the pseudomonas isolates, from different salad samples. The *erm*C gene was found to be associated with two plasmid bands corresponding to 2.4 kb and 3.9 kb (Fig. 5.3) for all three staphylococcal isolates from salads, in agreement with those found in staphylococcal clinical isolates (16), and with the genomic DNA for the *Pseudomonas* spp. strains. The 3.9 kb band in staphylococcal strains most likely represents the open or relaxed circular form of the 2.4 kb plasmid, although the presence of a second plasmid carrying the *erm*C gene is still possible.

Natural Transformation.

DNA extracted from salad isolate *Staphy. epidermidis* S102-35 was successfully used to transform the human oral residential bacterium *Streptococcus mutans* UA-159. Transformation efficiency ranged from $1.8X10^{-8}$ to $2.3X10^{-8}$ transformants per recipient

cell with saturated DNA. The presence of the *ermC* gene in the transformants was first confirmed by conventional PCR, followed by Southern blotting analysis with the *ermC* probe hybridized to two bands in the *S. mutans* transformant ZB35-A, with the sizes identical to those found in the donor strain *Staphy. epidermidis* S102-35 (data not shown).

Minimum Inhibitory Concentration (MIC).

The MIC profiles of the *ermC*$^{+}$ salad isolates and the *S. mutans* transformants were examined and all *ermC*$^{+}$ salad isolates exhibited reduced sensitivity to Em up to the maximum concentration tested (256 μg/ml). The natural transformation recipient strain *S. mutans* UA159 was sensitive to Em (MIC less than 2 μg/ml). However, both of the *S. mutans* transformants, ZB35-A and ZB35-B, exhibited reduced sensitivity to Em with the level similar to the *ermC* gene donor strain *Staphy. epidermidis* S102-35. Both MIC and Southern hybridization data suggest that increased resistance to Em and antibiotics with overlapping mode of actions (such as clarithromycin and clindamycin) can be disseminated from the salad isolate to the human residential bacterium *S. mutans* UA159 through horizontal transmission of the resistance-encoding element, at least in the laboratory settings. It is worth noting that several Em^{r} isolates exhibited reduced sensitivity to other types of antibiotics, for instance up to 256 μg/ml Tet, indicating the possible possession of multi-drug resistance determinant(s) in these ART bacteria. Characterization of additional resistance determinants in these isolates is currently underway.

5.5 Discussion and Conclusion

In this study, the prevalence of AR in RTE salads and the potential transmission of the AR gene from food isolate to human residential bacterium were investigated. While samples reported in this study were limited to those purchased from retail stores, several boxed salads purchased randomly from fast food chain restaurants were also examined in our laboratory, and the AR assessment results were comparable with the findings from retail foods (data not shown). The results suggest that this popular "healthy" food item could be an important vehicle transmitting ART bacteria to human on daily basis. The AR status in RTE produce could be affected by microflora in soil and water, which are prevalent in ART bacteria. So far, the size of the food samples analyzed was relatively small and only the profiles of a few AR genes were examined by genetic means in this study and our previous work. But the 100% prevalence of the ART bacteria in sampled salads from multiple brands and sources suggests that this is not a rare occurrence, but rather a widespread phenomenon. Nevertheless, a nationwide large scale prevalence study for ART bacteria and more resistance genes may be necessary in the future for a thorough assessment of the AR risks in the food chain.

It is worth noting that limited cultural conditions and genetic screening are applied in the study, thus the data only reflect part of the AR profiles in these foods. In addition, a noticeable difference between AR profiles from the salad (this study) and cheese samples (24) is that the numbers of Em^r bacteria are much higher than those of Tet^r ones in all salad samples, while the trend is opposite in cheeses. Because neither antibiotic is used in vegetable production or cheese fermentation, the difference could be due to the

survival advantage by other genes associated with the mobile element(s) encoding the corresponding AR genes, or the difference in antibiotic residues (serving as the selective pressure) found in these environments.

While the prevalence studies on foodborne ART bacteria, particularly pathogens and opportunistic pathogens in the past decade suggested the potential involvement of the food chain in transmitting such resistant organisms to human, there are still knowledge gaps to be closed in assessing the impact of the food chain on public health, especially on the emergence of AR in host microflora through horizontal gene transmission. The recent demonstrations of the large AR gene pools in many popular RTE items and the identification of the potential AR gene donors with diversified genetic background (7, 24) confirmed the constant inoculation of ART bacteria to human microflora through food intake. Thus horizontal transmission of AR genes from foodborne bacteria to human pathogens becomes a likely event. The prevalence of ART bacteria in oral microflora even from healthy children and adults without antibiotic treatments makes the food chain a logical source for the AR genes (23). The facts that applications of antibiotics in food animals affected the AR profiles of microbiotia in farmers, consuming sterile foods reduced the numbers of ART bacteria in gut microflora, and the AR patterns of clinical isolates from persons with *Salmonella* infections showed more resistance to antibiotics used in agriculture than to those used for the treatment of *Salmonella* infections in humans (1, 11, 14) also support the notion that food likely played an important role affecting the AR status of host microbiota, besides the clinical route. Horizontal transfer of the AR genes among food commensals, oral commensals and pathogens by

conjugation and transformation in the laboratory settings illustrated the functionality and mobility of the resistance genes (19, 24). However, the direct connection between AR in foods and in human microflora is yet to be established by well-designed human studies.

Revealing potential AR transmission pathway(s) is important for scientific understanding. But the ultimate goal of this line of applied research is to develop counter strategies to effectively interrupt AR gene dissemination to humans. Our preliminary results showed that simple processing modification such as applying brief heat treatment and good manufacturing practices can significantly reduce the amount of ART bacteria in targeted foods, suggesting focusing our effort on interrupting the AR gene transmission through the food chain is likely practical and affordable.

5.6 Acknowledgements

This project was sponsored by OSU start-up fund and OARDC project OHOA1265 for H. H. Wang. M. Lehman is supported by US Air Force Institute of Technology.

The views expressed in this article are those of the author(s) and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the U.S. Government.

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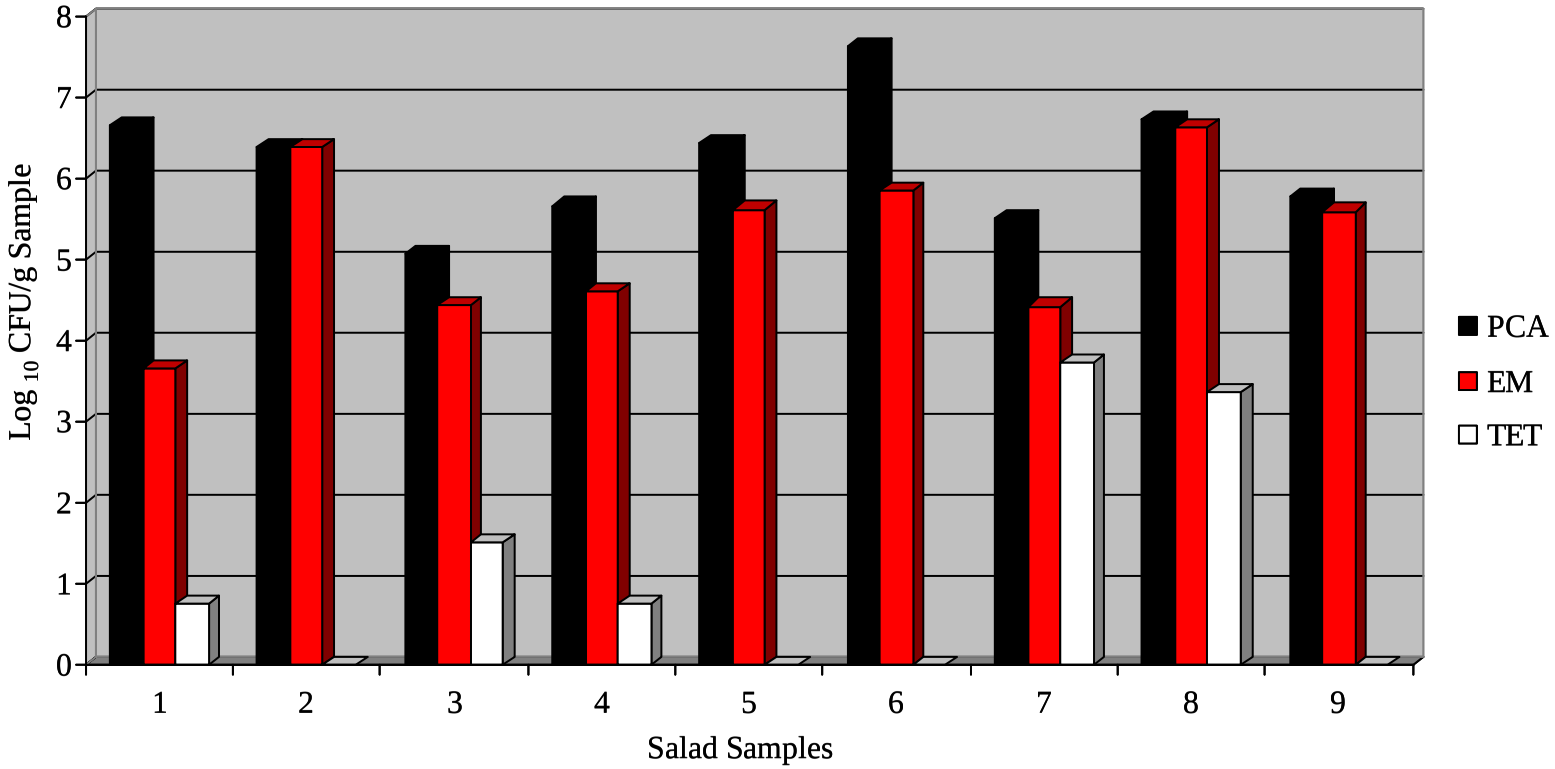

Figure 5.1. Prevalence of antibiotic-resistant microbes in salads. Samples; 1. Brand AI; 2. Brand AII; 3. Brand AIII; 4. Brand BI; 5. Brand BII; 6. Brand CI; 7. Brand DI; 8. Brand EI; 9. Brand EII.

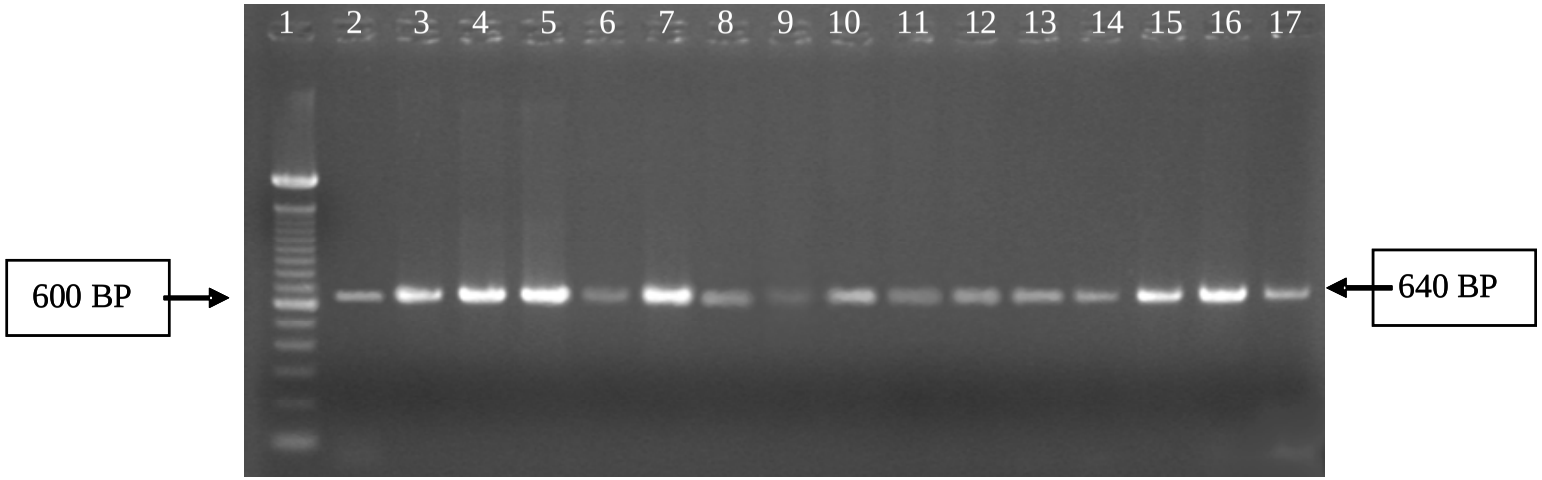

Figure 5.2. Conventional PCR screening for *erm*C gene from selected salad samples. 1. 100 bp ladder; 2. S101-29; 3. S101-30; 4. S102-29; 5. S102-35; 6. S105-7; 7. S107-2; 8. S107-3; 9. S107-5; 10. S107-7; 11. S107-8; 12. S108-4; 13. S108-5; 14. S108-7; 15. S108-12; 16. ZB35-A; 17. Positive control

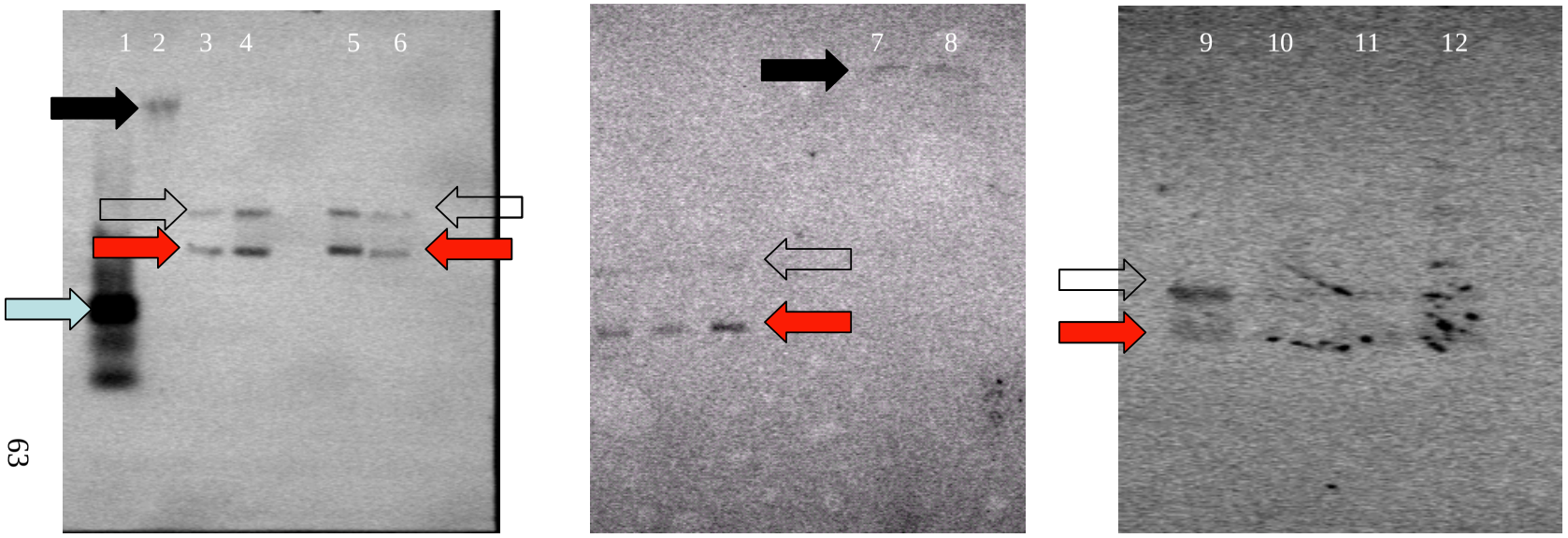

Figure 5.3. Results of Southern hybridization. Black arrows indicate genomic DNA; Red arrows indicate supercoiled plasmid (2.4 kb); Open arrows indicate open circular plasmid (3.9 kb). Lanes: 1. ermC PCR product (blue arrow 640 bp); 2. S105-7; 3. S101-29; 4. S101-30; 5. S102-35; 6. CX-I; 7. S105-7; 8. S102-29; 9. S107-7; 10. S108-4; 11. S108-5; 12. ZB35-A.

Sample	No. of *erm*C gene carriers/ No. isolates screened	Strain Identification (number of isolates within the category)	*erm*C gene location
Salad #1	2/12	*Staphylococcus* sp. (1) *Staphylococcus epidermidis* (1)	Plasmid Plasmid
Salad #2	2/12	*Staphylococcus epidermidis* (1) *Pseudomonas* sp.(1)	Plasmid Genomic
Salad #3	0/12		
Salad #4	0/12		
Salad #5	1/12	*Pseudomonas* sp. (1)	Genomic
Salad #6	0/12		
Salad #7	5/12	*Staphylococcus epidermidis* (4) *Pseudomonas* sp. (1)	Plasmid ND
Salad #8	4/12	*Staphylococcus epidermidis* (1) *Staphylococcus* sp. (1) *Pseudomonas* sp. (2)	Plasmid Plasmid ND
Salad #9	1/12	ND	
Overall	15/108		

ND=Not determined

Table 5.1. The prevalence of *erm*C^{+} bacteria in RTE salads.

Antibiotic	Control			Salad Isolate									Transformant	
	P. aeruginosa ATCC 27853	*S. mutans* UA 159	*S. aureus* ATCC 292136	29@	57@	30*	35*	73*	77*	85*	72#	84#	ZB35-A	ZB35-B
Erythromycin	>256	<0.25	1	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Clarithromycin	>8	<1	<1	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8
Tetracycline	8	<2	<2	2	<2	>256	32	128	<2	128	128	>256	<2	<2
Clindamycin	>4	<0.5	<0.5	>4	>4	>4	>4	<0.5	>4	0.5	2	0.5	>4	>4
Vancomycin	>32	<1	1	>32	>32	2	<1	2	>32	2	2	2	<1	<1
Cefazolin	>16	<2	<2	>16	>16	<2	<2	<2	>16	<2	<2	<2	<2	<2
Ampicillin	>16	<0.12	1	>16	>16	0.25	<0.1	<0.1	>16	.05	0.5	0.5	<0.12	<0.12
Gentamicin	2	4	2	<2	<2	<2	<2	<2	<2	<2	<2	<2	2	2
Penicillin	>8	<0.06	>8	>8	>8	0.5	<0.06	0.06	>8	0.5	0.25	0.25	<0.06	<0.06
Rifampin	>4	<0.5	<1	2	4	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
TMS	>4/76	<0.5/9.5	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76	<0.5/9.5	<0.5/9.5
Oxacillin	>8	<0.25	0.25	2	>8	<0.2	0.5	0.5	<0.2	>8	>8	>8	<0.25	<0.25

**S. epidermidis*
@Pseudomonas spp.
 # Staphylococcus spp.

Table 5.2. Minimum inhibitory concentrations from controls, Em^r salad isolates and transformants.

CHAPTER 6

CONCLUSION AND FUTURE DEVELOPMENT

Despite the limitations in experimental conditions and the number of AR genes screened, data from this study clearly illustrate that the commensal bacteria in the food chain could serve as a significant source of AR genes introduced into human. The total plate counts yielded anticipated results for total microbial counts of all food items examined; however the prevalence and magnitude of the ART bacteria in almost all of the food commodities examined, except yogurt, was a major surprise. In addition, results from examination of a few organic foods tested were mixed. These data indicate that merely limiting the use of antibiotics likely is not enough to slow down the future development of antibiotic resistance, at least in the near future. Additional strategies need to be in place to interrupt the gene transmission pathways. And a practical and economical way is through modifying food processing at both industrial and consumer levels to minimize the transmission of live ART bacteria to human through the food chain. In addition, screening for resistance profiles of all the environmental microflora possibly involved in food production seems inefficient. Developing a proactive surveillance system to monitor changes in the commensal bacteria of agricultural products such as foods seems much more realistic especially because food serves as an intermediate between the

environment and humans regardless of where you live. In order to do this a more streamline approach would need to be developed. Replacing the conventional PCR techniques described here with a multiplex real time PCR or microarrays would seem to be the next logical step. This would be beneficial because genetic materials from fastidious and non-culturable organisms could also be assessed.

The plamid(s) yielding the *erm*C gene should be sequenced to determine if it is a previously recognized plasmid or a different one. Also, the isolates demonstrating possible multi-drug resistant (MDR) patterns, based on MIC results, should be further evaluated to determine if their resistance is from multiple plasmids or perhaps a single MDR plasmid. Resistance patterns from hospital isolates need to be more thoroughly evaluated to determine if there is a commonality between pathogens, healthcare-acquired infections (HCAI) and these commensal bacteria. This is especially concerning since organisms such as MRSA and *Pseudomonas arugeniosa* are of the same Genera as those ART isolated from the foods sampled in this study.

While pathogen monitoring is important commensal and opportunistic bacteria should not be overlooked. Recent studies have shown that better healthcare in this country and most developed countries is leading towards an older population as well was a population growing in the number of immuno-compromised individuals. These are the people most at risk for acquiring an antibiotic resistant infection. The lack of monitoring of antibiotic usage in this country, coupled with, at best, a reactive surveillance system creates an environment void of any safety net for those individuals at greatest risk. We need to take

a more proactive approach, including monitoring of the emergence of ART bacteria. A better understanding of the genomics of bacteria needs to be developed, rather than waiting for selective pressure in pathogens to create “superbugs” especially when found in organisms closely related so gene transfer would be more likely.

APPENDIX A

FOOD COMMENSAL MICROBES AS A POTENTIALLY IMPORTANT AVENUE IN TRANSMITTING ANTIBIOTIC RESISTANCE GENES

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RUNNING HEAD: Food commensals in AR transmission

Key Words: Foods; commensals; antibiotic resistance; horizontal gene transfer; *Streptococcus thermophilus*

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A.1 Summary

The rapid emergence of antibiotic resistant (ART) pathogens is a major threat to public health. While the surfacing of ART foodborne pathogens is alarming, the magnitude of the antibiotic resistance (AR) gene pool in foodborne commensal microbes is yet to be revealed. Incidence of ART commensals in selected retail food products was examined in this study. The presence of 10^2 to 10^7 CFU of ART bacteria per gram of foods in many samples, particularly in ready-to-eat, "healthy" food items, indicates that the ART bacteria are abundant in the food chain. AR-encoding genes were detected in ART isolates, and *Streptococcus thermophilus* was found to be a major host for AR genes in cheese microbiota. *Lactococcus lactis* and *Leuconostoc* sp. isolates were also found carrying AR genes. The data indicate that food could be an important avenue for ART bacteria evolution and dissemination. AR-encoding plasmids from several foodborne commensals were transmitted to *S. mutans* via natural gene transformation under laboratory conditions, suggesting the possible transfer of AR genes from food commensals to human residential bacteria via horizontal gene transfer.

A.2 Introduction

Resistant pathogens to various antibiotics are emerging rapidly. Surfacing of these resistant pathogens, untreatable by antibiotics, constitutes a real threat to public health. To effectively combat this problem, a comprehensive understanding of the major pathways in antibiotic resistance (AR) gene dissemination as well as the key mechanisms in the evolution of antibiotic resistant (ART) bacteria is essential.

Horizontal gene transfer among pathogens in the hospital environment has been recognized as an important avenue for the rapid spread of AR genes among pathogens. It is believed also that horizontal transmissions of AR genes between commensal and pathogenic microorganisms in ecosystems are much more likely events than direct AR gene dissemination from one pathogen to another (1). The presence of AR gene reservoirs in commensal microbes in various environmental and host ecosystems (2-8), the illustration of commensals as facilitators for AR gene dissemination (9), and the correlation of antibiotics usage in animals with increased AR in human microbiota (10, 11) suggest the importance of commensals in mediating the dissemination of AR genes. The isolation of AR genes in foodborne pathogens from retail products exemplified the potential contribution of the food chain in transmitting ART pathogens to humans (12-14). The studies on commensal bacteria, however, are limited and primarily focused on

the opportunistic pathogen enterococci (15, 16). A standard laboratory enrichment procedure (http://www.fda.gov/cvm/Documents/AppendicesA-6.pdf) is often used to detect the presence of the ART bacteria, masking the real magnitude of the AR problem associated with the food chain.

To examine the AR risks associated with the food chain, this study aimed at revealing the distribution spectrum and magnitude of ART commensal bacteria and AR gene pool in retail foods, by targeting total food microbiota instead of a particular group of microorganisms or pathogens. Therefore, food samples were analyzed without any laboratory enrichment procedures. Microbial resistance to tetracycline (Tet) and erythromycin (Em), which are still used in animal production and human therapy, was investigated (17, 18)**.** The presence of several AR markers including *erm*B, *erm*C, *tet*S/M and *tet*A, encoding ribosomal modification and Tet efflux mechanisms, in selected food isolates was examined and main AR gene hosts were identified.

A.3 Materials and Methods

Food sample preparation and enumeration of total and ART populations.

Food samples (Fig. 1) were purchased from local grocery stores and analyzed within the products sell-by dates. Fresh raw milk was obtained from the dairy pilot plant at OSU and analyzed the same day as shipped. Five grams of each sample were aseptically removed from the product packaging and placed in disposable plastic bags containing 10 ml of

sterile 0.1% peptone water. Bagged samples were hand-massaged for 10 minutes. Homogenized samples or rinsing liquids were serially diluted and plated on non-selective plate count agar (PCA, Becton Dickinson and Company, Sparks, MD) for non-selective total microbial counting, and on PCA plates containing 16 μg ml^{-1} of Tet or 8 μg ml^{-1} of Em (Fisher Biotech, Fair Lawn, NJ). Plates were incubated at conditions as indicated (Fig.1) for up to 48 h for assessing Tet and Em resistant population. The levels of antibiotics used in selective agar plates were based on that used to screen for ART enterococci (http://www.fda.gov/cvm/Documents/AppendicesA-6.pdf). Serially diluted samples were also plated on Difco Lactobacilli MRS Agar (MRS, Becton Dickinson and Company) and *Pseudomonas* isolation agar (PIA, EMD Chemicals Inc., Gibbstown, NJ) plates with proper antibiotics to recover ART lactic acid bacteria and *Pseudomonas* species, respectively. The cell numbers reported (CFU g^{-1} of food, Fig. 1A and 1B) were the mean values from duplicates.

AR gene detection and host isolates identification.

Conventional PCR was conducted to detect AR genes in the ART isolates. Bacterial cells from single colonies were re-suspended in 300 μl sterile dH_2O containing 100 μg of 1:1 mixture of 0.5 μm diameter and 0.1 μm diameter glass beads (Biospec Products, Inc, Bartlesville, OK). The sample mixtures were homogenized using the Mini-Bead-Beater-8 (Biospec Products, Inc, Bartlesville, OK) for 2 min at maximum speed. The resulting cell extracts were placed in a boiling water bath for 10-15 min and 5 μl of the supernatant were used as PCR templates. The PCR primers *tet*A-FP 5'GCTACATCCTGCTTGCC TTC3' and *tet*A-RP 5'CATAGATCGCCGTGAAGAGG3' were used to amplify the 220

bp *tet*A fragment (19), *tet*S-FP 5'CATAGACAAGCCGTTGACC3' and *tet*S-RP 5'AT GTTTTTGGAACGCCAGAG3' for the 667 bp *tet*S/M fragment (19), *erm*B-FP 5'GGAA CAGGTAAAGGGC3' and *erm*B-RP 5'GGTTTAGGATGAAAGC3' for the 389 bp *erm*B fragment (this study), and *erm*C FP 5'GCTAATATTGTTTAAATCGTCAAT3' and *erm*C RP 5'TCAAAACATAATATAGATAAA3' for the 640 bp *erm*C fragment (20). PCR was conducted using reagents and conditions as described previously (21). PCR products with expected sizes were purified using the QIAquick® kit (Qiagen, Valencia, CA) following manufacturer's instruction. DNA sequences of the 16S rRNA, *erm*C, *tet*A gene fragments and approximately 50% of the *erm*B and *tet*S/M gene fragments were determined using a DNA analyzer (ABI PRISM® 3700, Applied Biosystems, Foster City, CA) at the Plant Genome Sequence Facility, The Ohio State University. The DNA sequences were compared with published Tet or Em resistance gene sequences deposited in the NCBI database. ART isolates containing the resistance genes were identified by PCR amplification of the 16S rRNA gene fragment and sequence analysis following procedures as described previously (22).

The minimum inhibition concentration (MIC) profiles of ART isolates.

The MIC profiles of selected ART isolates were determined using a commercial kit (Sensititre ® 18-24 Hour MIC and Breakpoint Susceptibility Plates; TREK Diagnostic Systems, Cleveland, OH) following the manufacturer's instructions, with modifications. MRS or brain heart infusion (BHI) broth instead of the standard Mueller-Hinton broth

was used to culture fastidious organisms. The MIC panels were incubated at either 30^0C or 37^0C for 24-48 h. Additional 96-well microtiter plates with wells containing up to 256 μg ml^{-1} of Tet and Em were used to determine the MIC of Em^r or Tet^r isolates which exhibited positive growth in wells containing 16 μg ml^{-1} of Tet or 8 μg ml^{-1} of Em on the Sensititre® plates. The MICs were reported as the minimum concentration of the antibiotic that inhibited visible growth, as indicated by increased turbidity or by deposition of cells at the bottom of the wells. Control strains used in the study include *Staphylococcus aureus* ATCC 29213 (American Type Culture Collection (ATCC), Manassas, VA), *Pseudomonas aeruginosa* ATCC27853 (ATCC), *L. lactis* ML3 (22), and *S. thermophilus* LMD-9 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj&cmd =Retrieve&dopt=Overview&list_uids=13773).

Plasmid isolation and natural gene transformation.

Lactococcus sp. CZ-T4 (Tet^r) and CZ-T8 (Tet^r) were isolated from commercial cheddar cheese, while strain RMK-T14 (Tet^r) was obtained from raw milk (this study). The multi-drug resistant *L. lactis* K214 was isolated from soft cheese made from raw milk (24). The strains were grown in MRS broth or M17 broth with 0.5% glucose, supplemented with 5 μg ml^{-1} Tet, and the mixture were incubated at 30°C for 24 h. Plasmids were isolated from these strains following the method of Anderson and McKay (25) and were used in the natural transformation experiments, following procedures as described by Li et al (26). For the selection of Tet^r transformants, BHI plates were supplemented with 5 μg ml^{-}

[1] Tet. Plates were incubated in a 5% CO_2 incubator at 37°C for 48 h. Transformation efficiency was calculated based on the ratio of Tet^r transformants to the total number of viable cells.

A.4 Results

Prevalence of ART bacteria in food samples.

ART microbes were detected in majority of the retail foods examined, from raw food materials such as meat and shrimp to ready-to-eat items such as cheeses and salad. No detectable ART microbes were found in processed cheese (heat treated during manufacture) and yogurt samples, with representative data illustrated in Fig. 1. Twenty out of the 23 cheese samples analyzed contained Tet^r and/or Em^r microbes ranging from 10^2 to 10^7 CFU g^{-1} of food, which are equivalent to 10^3 to 10^8 CFU ART microbes per slice of cheese (about 20 g). In general, the number of Tet^r microbes was greater in cheeses than that of Em^r bacteria. It is worth of noting that the study was conducted using limited incubation conditions, and the antibiotic concentrations used to screen for resistant organisms might not be optimal for all bacteria. Therefore the numbers reported here only represent a portion of the total ART bacterial load in these foods.

Detection of AR genes and ART isolates identification.

To confirm that most of the ART organisms detected by growth on the selective agar plates were resistant bacteria due to the possession of AR determinants, conventional PCR was conducted to detect the presence of selected AR genes in these organisms and

the results were summarized in Table 1. Among the Tet^r isolates recovered from cheese, about 10% contained the *tet*S/M gene. Seven out of 11 *tet*S/M^+ cheese isolates identified were *S. thermophilus*; 2 *tet*S/M^+ isolates were found to be *L. lactis*. Two additional cheese isolates CZ-T4 and CZ-T8 had 97% 16S rRNA gene sequence identity to unidentified *Lactococcus* sp., and particularly had 93-94% identity to *L. garvieae* and *L. lactis*, similar with that of the raw milk ART isolate RMK-T14, suggesting this might be a common organism from milk. Therefore, it is possible that the *Lactococcus* sp. *tet*S/M^+ isolates from cheese were originated from milk (pasteurized but not sterile) or dairy processing environment during cheese fermentation. A *tet*S/M^+ isolate from raw milk was identified as *Leuconostoc* sp. In addition, the *tet*A gene was found in two cheese isolates CZ-T3, CZ-T7 and several isolates from raw pork meat. These isolates were all identified as *Pseudomonas* sp.

Among the Em^r isolates from cheese, more than 50% contained the *erm*B gene, and the carrier organisms identified so far include *Staphylococcus* sp. (5 out of 28) and *S. thermophilus* (23 out of 28). Both *tet*S/M and *erm*C genes were found in the isolate CX-I EM from packaged sliced chicken lunchmeat, suggesting a multi-drug resistance phenotype of the strain. CX-I EM was identified as *Pseudomonas* sp. ART bacteria were isolated sporadically in lunchmeat (data not shown), which is probably due to occasional contamination during the processing of the meat.

MIC analysis.

MIC tests of selected cheese isolates showed that *Lactococcus* sp. CZ-T4 and RMK-T14 (*tet*S/M$^+$) were resistant to at least 128 μg ml^{-1} Tet, and *S. thermophilus* E4 (*erm*B$^+$) was resistant to Em (>256 μg ml $^{-1}$), clarithromycin (> 8 μg ml^{-1}), and clindamycin (>4 μg ml^{-1}). *S. thermophilus* BOR-COCZ-T19 (*tet*S/M$^+$) was resistant to Tet (>128 μg ml^{-1}). *Staphylococcus* sp. C202 was resistant to both Em (>256 μg m^{-1}) and Tet (>32 μg ml^{-1}), suggesting the possible possession of both resistance determinants in this isolate. The control strains *L. lactis* ML3, *S. thermophilus* LMD-9, two other commercial *S. thermophilus* starters and *S. aureus* ATCC 29213 were sensitive (< 2 μg ml $^{-1}$) to the above antibiotics.

Lactococcus sp. RMK-T14 (*tet*S/M$^+$) from raw milk was resistant to Tet (>128 μg ml^{-1}), Em (>64 μg ml^{-1}), clarithromycin (>8 μg ml^{-1}), and clindamycin (>4 μg ml^{-1}). Therefore this isolate likely carried multi-drug resistant determinants or multi-drug resistant mechanism(s). The raw milk isolate *Streptococcus uberis* RMK-T22W exhibited resistance to Tet (>128 μg ml^{-1}).

All of the *Pseudomonas tet*A$^+$ isolates recovered from pork and cheese exhibited resistance to Tet (>128 μg m^{-1}) and vancomycin (>32 μg ml^{-1}); The *Pseudomonas* sp. CX-I EM (*erm*C$^+$*tet*S/M$^+$) from packaged sliced chicken lunchmeat was resistant to Tet (>64 μg ml^{-1}) and its tolerance to Em (>256 μg ml^{-1}) was much higher than the control strain *P. aeruginosa* ATCC 27853 (Tet <16 μg ml^{-1}, Em <32 μg ml^{-1}).

Horizontal transfer of the AR gene from food isolates to oral residential bacterium. The *tet*S/M-containing lactococcal isolates CZ-T4 and CZ-T8, recovered from cheese, and RMK-T14, isolated from raw milk, contained a plasmid with an approximate size of 20-25 kb. To assess the potential risk of the foodborne ART bacteria in disseminating AR genes to human microbiota, plasmids isolated from the above strains were used for natural transformation of the oral cariogenic pathogen, *S. mutans* in laboratory media. The *tet*S/M gene was successfully transferred to *S. mutans* UA159 at frequencies ranging from 1.9 $x10^{-7}$ to 2.8 x 10^{-5}, 4.7 $x10^{-7}$ to 2.3 x 10^{-6}, and 3.8 x 10^{-7} to 2.1 x 10^{-6} transformants per recipient cell using CZ-T4, CZ-T8 and RMK-T14 plasmid extracts, respectively. In addition, the multi-drug resistant plasmid pK214 from the cheese isolate *L. lactis* K214 was also successfully transformed into *S. mutans* UA159 at frequencies of 1.1 x 10^{-6} to 1.2 x 10^{-5} transformants per recipient cell. PCR amplification confirmed the presence of the *tet*S/M gene in the streptococcal transformants. MIC test showed that the transformants had significantly increased resistance to Tet (>128 μg ml^{-1}) compared to the parental strain UA159 (<4 μg ml^{-1}). These results illustrated that the *tet*S/M gene from food isolates can lead to resistance in residential host bacteria or pathogens, if acquired by horizontal gene transfer.

A.5 Discussion and Conclusion

Despite the fact that this current study only screened for a limited number of resistance markers, it illustrated the prevalence of ART commensals and AR genes in retail foods. Many ART bacteria-containing ready-to-eat products are consumed without further cooking or processing. Consequently, human are routinely inoculated with ART bacteria

through daily food intake, including opportunistic pathogens and commensals such as *Pseudomonas* sp., *Streptococcus* sp. and *Staphylococcus* sp. The detection of high numbers (i.e., up to 10^8 CFU per serving of food) in several products is alarming, suggesting that food can be a potentially important avenue transmitting ART bacteria. This finding is in agreement with a previous report showing that consuming sterile foods can significantly decrease the presence of ART bacteria in the gastrointestinal system (27). While further research is needed to establish the direct correlation between the ART microbes from foods and the ART population in the host ecosystems, it is evident that a constant supply of ART bacteria, partnered with occasional colonization and horizontal gene transfer, are at least partially responsible for the increased AR profiles seen in human. Such an intrinsic AR gene pool could have significant impact on pathogen resistance in susceptible population, particularly those receiving antibiotic treatment.

Oral cavity could be an important area where many initial interactions between food microbes and human microbiota, including horizontal gene transfer events such as conjugation and transformation, took place during the retention of food residues in the oral cavity. Our data are consistent with results from recent studies showing that the microbiota in children and adults is becoming increasingly resistant to antibiotics, even in the absence of antibiotic treatment (7, 8, 28). In fact, the *tet*S/M and *erm*B genes were found to be abundant in bacteria isolated from foods, which is in agreement with the prevalence of these Tet- and Em-resistance genes in human oral microflora (29). Successful transmission of the resistance genes from the food isolates to the oral residential bacterium *S. mutans*, by natural gene transformation, further confirmed the

functionality of the mobile resistance-encoding elements from food isolates, if acquired by horizontal gene transfer.

Identification of the key pathways in AR gene transfer is critical, but developing a strategy to combat this problem is even more important. The identification of ART bacteria in cheeses often associated with raw milk, such as *Lactococcus* sp., *Streptococcus* sp. and *Staphylococcus* sp., suggests that cheese fermentation is a susceptible process during which ART bacteria could evolve and proliferate. Improving sanitation and milk heat treatment are thereby essential steps in reducing ART bacteria. While it is a major challenge to track the direct and indirect gene transfer events among microbes in complicated ecosystems (1), identifying key AR gene host organisms in foods, and likely in other ecosystems, not only reveals the ultimate consequence of these events in the food chain and the organisms involved in horizontal gene transfer, but opens the door for further characterization of conditions in these ecosystems that might facilitate horizontal gene transfer and features of the organisms that might grant their fitness in such ecological niches (13). Such understanding would be critical for effective counteractive strategies to interfere with the detrimental gene swapping in both natural and host ecosystems. An industrially important lactic acid bacterium, *S. thermophilus*, was found a dominant host for both Tet and Em genes. ART *L. lactis* was also isolated from cheese. Genetic screening and MIC tests of three commercial *S. thermop*hilus starter cultures as well as the control *L. lactis* strain showed that they are free of the above AR genes, suggesting the susceptibility of these starter cultures to horizontal gene transfer during at least certain cheese fermentation processes. The potential health impact of these organisms thus needs to be carefully evaluated. Although it would be a tedious and likely

long-term effort to clean up the AR gene pool in the environment, interrupting the transmission of ART bacteria into human by focusing our efforts on the food chain could be an effective strategy to combat the AR challenge in human.

A.6 Acknowledgements

The authors thank Drs. John Reeve, Steve Schwartz (OSU), Peter Greenberg (UW), Paul Kolenbrander (NIH/NIDCR), Stuart Levy (Tufts) and David White (FDA) for helpful discussions. This project was sponsored by OSU start-up fund for H. H. Wang. M. Lehman is supported by US Air Force Institute of Technology.

The views expressed in this article are those of the author(s) and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the U.S. Government.

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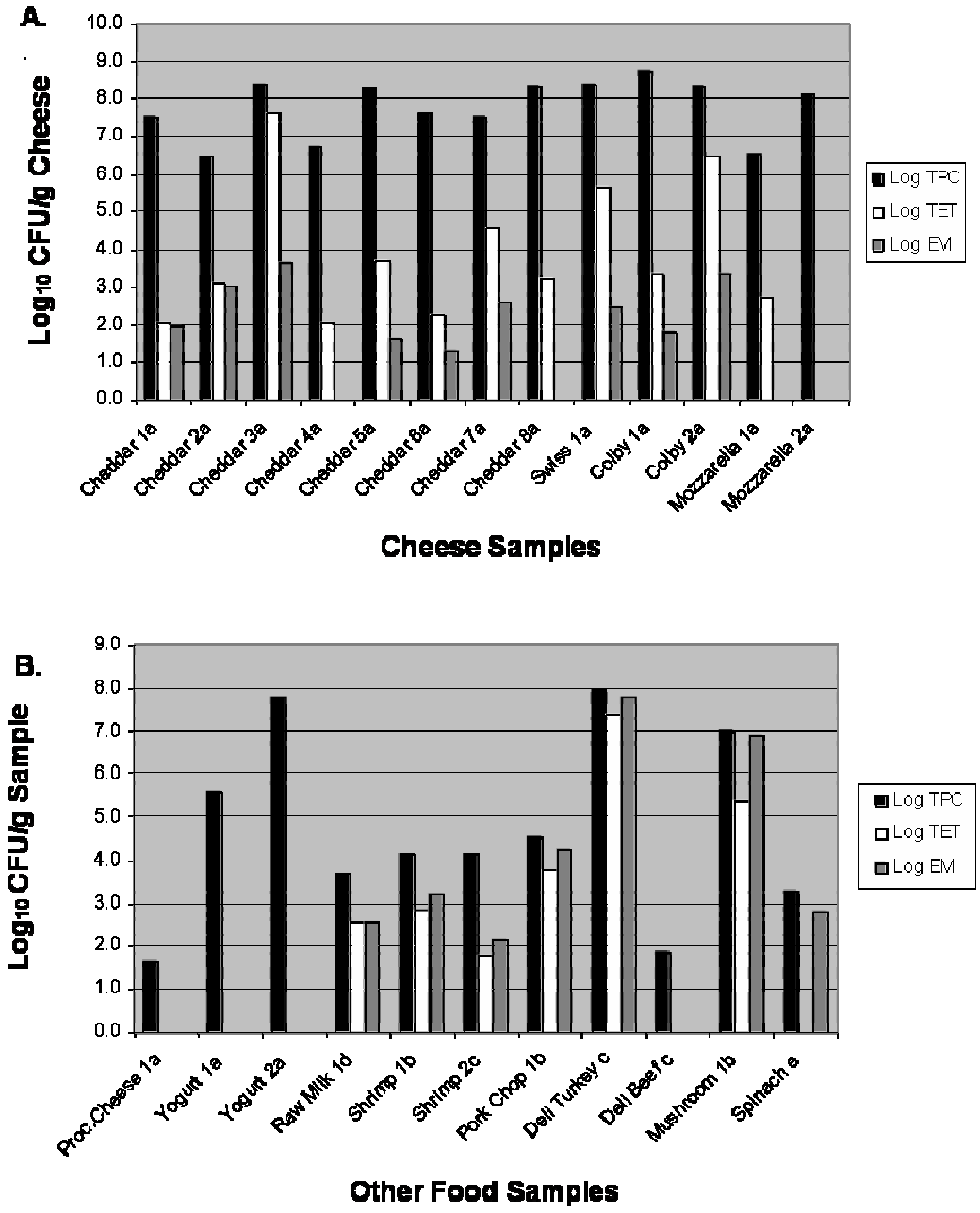


Fig. A.1. Prevalence of ART microbes in retail foods. A) Representative cheese samples. B) [a]Microorganisms were recovered from cheese samples by plating on MRS agar, incubated anaerobically at 30ºC. [b]Microorganisms were recovered by plating on PCA agar, incubated aerobically at 20ºC. [c]Microorganisms were recovered by plating on PCA agar, incubated aerobically at 37ºC. [d]Microorganisms were recovered by plating on MRS agar, incubated anaerobically at 20ºC. [e]Microorganisms were recovered by plating on PCA agar, incubated aerobically at 30ºC.

Food Item	Sample Source	Total Plate Count (CFU/g food)	Tet-resistant Count* (CFU/g food)	Em-resistant Count§ (CFU/g food)
Cheddar Cheese #1[a]	Store I (Brand A)	$3.2x10^{7}\pm1.4x10^{5}$	$1.1x10^{2}\pm1.4x10^{1}$	$9.0x10^{1}\pm1.4x10^{1}$
Cheddar Cheese #2[a]	Store II (Brand B)	$2.9x10^{6}\pm9.1x10^{5}$	$1.2x10^{3}\pm6.7x10^{2}$	$9.5x10^{2}\pm4.2x10^{1}$
Cheddar Cheese #3[a]	Store II (Brand C)	$2.5x10^{8}\pm3.1x10^{7}$	$4.1x10^{7}\pm7.0x10^{5}$	$4.3x10^{3}\pm1.3x10^{3}$
Cheddar Cheese #4[a]	Store II (Brand D)	$5.5x10^{6}\pm3.5x10^{6}$	$1.1x10^{2}\pm1.0x10^{1}$	0
Cheddar Cheese #5[a]	Store I (Brand E)	$1.9x10^{8}\pm4.0x10^{7}$	$8.0x10^{3}\pm3.2x10^{3}$	$4.0x10^{1}\pm4.0x10^{1}$
Cheddar Cheese #6[a]	Store I Brand (B)	$7.0x10^{7}\pm2.8x10^{7}$	$1.8x10^{2}\pm2.0x10^{1}$	$2.0x10^{1}\pm2.0x10^{1}$
Cheddar Cheese #7[a]	Store III Brand (B)	$3.9x10^{7}\pm9.1x10^{6}$	$3.7x10^{4}\pm1.5x10^{4}$	$3.7x10^{2}\pm7.0x10^{1}$
Cheddar Cheese #8[a]	Store I (Brand A)	$2.3x10^{8}\pm3.6x10^{6}$	$1.6x10^{3}\pm2.0x10^{2}$	<1
Cheddar Cheese #9[a]	Store III (Brand C)	$5.2x10^{8}\pm5.2x10^{6}$	<1	$1.0x10^{1}\pm1.0x10^{1}$
Colby Cheese #1[a]	Store I (Brand C)	$5.6x10^{8}\pm4.4x10^{7}$	$2.3x10^{3}\pm6.1x10^{2}$	$6.0x10^{1}\pm0$
Colby Cheese#2[a]	Store II (Brand B)	$2.2x10^{8}\pm8.5x10^{6}$	$2.8x10^{6}\pm8.6x10^{5}$	$2.2x10^{3}\pm1.4x10^{2}$
Swiss Cheese #1[a]	Store I (Brand E)	$2.4x10^{8}\pm1.1x10^{7}$	$4.7x10^{5}\pm4.4x10^{4}$	$2.7x10^{2}\pm1.0x10^{1}$
Mozzarella Cheese #1	Store I (Brand B)	$3.6x10^{6}\pm7.6x10^{5}$	$5.4x10^{2}\pm2.0x10^{1}$	<1
Mozzarella Cheese #2	Store I (Brand A)	$1.3x10^{8}\pm1.7x10^{7}$	<1	<1
Mozzarella Cheese #3	Store I (Brand B)	$4.2x10^{5}\pm4.9x10^{4}$	<1	<1

Continued

Table A.1 Prevalence of AR microbes in selected food samples

Table A.1 Continued

Baby Carrots #1[c]	Store I (Brand F)	$1.2x10^{6}\pm2.5x10^{4}$	-	$6.7x10^{5}\pm1.0x10^{4}$
Baby Carrots #2[c]	Store II (Brand J)	$4.5x10^{7}\pm1.4x10^{6}$	$1.6x10^{4}\pm7.1x10^{2}$	$1.2x10^{7}\pm1.4x10^{5}$
Baby Carrots #3[c]	Store III (Brand K)	$9.0x10^{7}\pm6.6x10^{7}$	$4.1x10^{5}\pm3.5x10^{5}$	$4.0x10^{7}\pm1.9x10^{7}$
Mushroom #1[b]	Store I (Brand G)	$9.3x10^{6}\pm1.4x10^{5}$	$2.4x10^{5}\pm4.2x10^{3}$	$8.1x10^{6}\pm1.4x10^{5}$
Mushroom #1[c]	Store I (Brand G)	$1.5x10^{5}\pm1.1x10^{4}$	$5.0x10^{1}\pm1.4x10^{1}$	$1.0x10^{5}\pm5.7x10^{3}$
Salad #1[b]	Store II (Brand H)	$2.4x10^{6}+8.5x10^{4}$	$1.9x10^{4}+7.1x10^{2}$	$2.2x10^{6}\pm1.4x10^{5}$
Salad #1[c]	Store II (Brand H)	$6.5x10^{5}\pm1.0x10^{5}$	$2.8x10^{3}\pm4.5x10^{2}$	$5.4x10^{5}\pm2.8x10^{4}$
Salad #2[b]	Store I (Brand I)	$3.0x10^{7}\pm2.8x10^{5}$	$4.0x10^{5}\pm1.7x10^{4}$	$1.5x10^{7}\pm5.7x10^{5}$
Salad #2[c]	Store I (Brand I)	$1.7x10^{6}+7.1x10^{4}$	$6.0x10^{2}\pm2.8x10^{1}$	$1.4x10^{6}\pm1.4x10^{4}$
Salad #3[b]	Chain Restaurant A	$4.8x10^{7}\pm5.7x10^{5}$	$5.6x10^{5}+2.8x10^{5}$	$3.7x10^{7}\pm2.3x10^{6}$
Salad #3[c]	Chain Restaurant A	$2.0x10^{7}\pm7.1x10^{5}$	$4.8x10^{3}\pm3.4x10^{2}$	$5.2x10^{6}\pm3.1x10^{6}$
Shrimp #1[b]	Store III (Brand C)	$1.3x10^{4}\pm1.4x10^{2}$	$6.9x10^{2}\pm1.4x10^{1}$	$1.5x10^{3}\pm2.8x10^{2}$
Shrimp #1[c]	Store III (Brand C)	$9.3x10^{2}+9.9x10^{1}$	$3.4x10^{2}+2.8x10^{1}$	$2.1x10^{2}\pm1.4x10^{1}$
Shrimp #2[b]	Store IV	$4.3x10^{3}\pm1.6x10^{2}$	$1.2x10^{2}\pm2.8x10^{1}$	$3.1x10^{2}+4.2x10^{1}$
Shrimp #2[c]	Store IV	$1.3x10^{4}\pm1.8x10^{2}$	$6.0x10^{1}++2.8x10^{1}$	$1.4x10^{2}\pm5.7x10^{1}$
Pork Chop[b]	Store II	$3.2x10^{4}\pm1.6x10^{3}$	$5.7x10^{3}\pm1.4x10^{2}$	$1.8x10^{4}\pm2.8x10^{2}$
Pork Chop[c]	Store II	$4.6x10^{2}\pm2.8x10^{1}$	$1.0x10^{1}\pm1.4x10^{1}$	$6.0x10^{1}\pm2.8x10^{1}$
Raw Milk[d]	Pilot Plant	$4.5x10^{3}\pm2.5x10^{3}$	$3.4x10^{2}\pm3.5x10^{1}$	$3.4x10^{2}+8.0x10^{1}$
Raw Milk[a]	Pilot Plant	$8.5x10^{3}\pm1.5x10^{3}$	$4.8x10^{2}\pm2.0x10^{1}$	$7.6x10^{2}+2.0x10^{2}$
Raw Milk[b]	Pilot Plant	$7.6x10^{2}\pm3.5x10^{1}$	$7.0x10^{4}\pm1.2x10^{4}$	-

[*]Screened on agar plates containing 16µg/ml tetracycline.
[§]Screened on agar plates containing 8µg/ml erythromycin.
[a]Microorganisms were recovered from cheese samples by plating on MRS agar plate and incubated at 30ºC.
[b]Microorganisms were recovered by plating on PCA agar plates and incubated at 20ºC.
[c]Microorganisms were recovered by plating on PCA agar plates and incubated at 37ºC.
[d]Microorganisms were recovered by plating on MRS agar plates and incubated at 20ºC.

Food	*ART trait*	**Resistance gene (# carriers /# isolates screened)**	**16S rRNA gene identity (#organisms/ #identified)**
Cheese	Tet	*Tet*S/M (8/33)	*Lactococcus* sp. (2/8) *Streptococcus thermophilus* (5/8)
		*tet*A (2/33)	*Pseudomonas* sp. (2/2)
	Em	*erm*B (32/56)	*Staphylococcus* sp. (5/28) *Streptococcus thermophilus*. (23/28)
Raw milk	Tet	*tet*S/M (8/108)	*Lactococcus* sp. (1/8) *Streptococcus* sp. (1/8) ++
Salad	Em	*Erm*B (7/20)	*Enterobacter* sp. (3/4) *Pseudomonas* sp. (1/4)+3

Table A.2. Identification of antibiotic-resistant isolates from food based on 16S rRNA gene sequencing.

APPENDIX B

SUPPLEMENTAL RESEARCH

B.1 Introduction.

This appendix presents additional preliminary results obtained during the study. Due to the limitation on time and budget, I am unable to follow up with great details thus the data are not publishable yet. However they do offer further insights as to the breadth of this research as well as the directions where future research will be continued.

B.2 Methods and materials for multiplex PCR using various Em resistant primers.

In order to determine the most efficient technique for screening using PCR, multiplex PCR was compared to conventional PCR techniques using *ere*A, *ere*B, *mph*A, *mef* AE, *erm*B, *erm*C, *erm*F, and *erm*Q primers. Single colonies from pork, cheese, salad, and shrimp Tet and Em plates were streaked for isolation. Bacterial cells from the subsequent isolates were re-suspended in 300 mL sterile dH_2O and placed in 1.5 mL microcentrifuge tubes and placed in boiling water for 10 minutes. Multiplex PCR was performed using 5 µL of the supernatant from each individual sample as the templates in an attempt to screen individual colonies for the presence of the Em resistance-encoding genes. The multiplex primers I used in the study included 8 oligonucleotides to detect 4 sets of Em^r

genes (Sutcliffe et al., 1996): *ere*A-FP 5'-AACACCCTGAA CCCAAGGG AC-3', *ere*A-RP 5'-CTTCACATCCGGATTCGCTCG-3', *ere*B-FP 5'-AGAAATGGAGGTTCATA CTTA-3', *ere*B-RP 5'-CATATAATCATCACCAATGGCA-3', *mph*A-FP 5'-AACTG TACGCACTTGC-3', *mph*A-RP 5'-GGTACTCTTCGTTACC-3', *mef*AE-FP 5'-AGTA TCATTAATCACTAGTGC-3', and *mef*AE-RP 5'-TTCTTCTGGTACTAAAAGTGG-3'. The anticipated PCR products were the 420bp *ere*A, 546bp *ere*B, 837bp *mph*A, and 348 bp *mef*AE fragments. PCR reagents and conditions were followed as outlined by Sutcliffe, et al. (1996).

Results of multiplex PCR.

In these limited experiments, multiple non-specific amplification products were detected by the multiplex PCR. Even when bands appeared at the appropriate size(s), the results were not reproducible when single colonies were screened (Fig. B.3.). Based on these limited experiments, subsequent screening relied on the pooling of DNA from multiple colonies using a single primer pair. Presumptive positives were then individually screened using the same primers, followed by genetic sequencing to validate all positive results. Our results suggest that screening multiple colonies for a single gene was more efficient than multiplexing the resistance gene primers for a single isolate.

Discussion

If multiplexing could be successfully accomplished the time necessary to screen for specific genes could be greatly reduced. The key would be to find the proper combination of primers that are different enough in size and specific enough for the target

genes that they would appear as distinct bands. Combining primers for different mechanisms of antibiotic resistance as well as different antibiotics would be helpful to help determine that prevalence of mechanisms.

B.3 Methods and materials for examination of the efficacy of processing treatments on reducing live ART bacteria in selected produces.

RTE baby carrots and celery sticks were purchased from local retail stores. All samples were tested before the sell-by dates and three samples were examined for each food type. Five treatments were evaluated for their efficacy at different time periods in reducing the number of bacterial colonies on both PCA, and PCA plates with 50 μg-mL^{-1} erythromycin. The five treatments consisted of boiling water, distilled white vinegar (pH2.2), Italian dressing (pH3.0), fresh squeezed lemon juice (pH 2.0), and hydrochloric acid (pH1.5). The pH was measured using a pH meter for all samples except the salad dressing in which case litmus paper was used. Five grams of food sample were aseptically measured and soaked in 10 mL of a treatment solution for designated period of time and then removed from solution and placed in an aseptic bag containing 10 mL of 0.85% NaCl and homogenized for 5 seconds. This obtained saline solution was then serially diluted and plated in duplicates on PCA and PCA-Em plates, incubated for 48 h, aerobically at 30^{0}C. For the boiling water treatment, the food samples were placed in a clean pot of boiling water for 5 sec, 10 sec or 30 sec, respectively. For the rest of the experiments, the food samples were treated for three time intervals, 5 sec, 30 sec, and 60 sec, respectively, using a new food sample each time. The dilutions and plating techniques were the same for all the treatments. The non-treatment base-line counts for

each trial were conducted by soaking the samples in 0.85% sodium chloride in lieu of a treatment. Blank reagent controls without food items were also evaluated by plating on both PCA and PCA-Em plates. The total microbial and ART bacterial counts were converted to CFU/g for each food sample and treatment. Mean CFU/g and standard deviation for each PCA and Em sample were calculated using Excel (Microsoft 2000).

Efficacy of processing treatments on reducing total and ART bacteria in selected foods.

The carrots had less variability in counts than the celery. Overall the acid treatments decreased the number of colonies for both food types (Figs. B.1. and B.2). The decreases in counts were most dramatic in the Em plates. Boiling also reduced the counts but not as much as the acid treatments. There was growth from all treatments when grown in broth indicating that the microorganisms were only damaged by the treatment and not eliminated. Only the dressing and lemon juice had growth on the control plates. In both cases the growth was very low and comprised of eukaryotes as confirmed by microscopic evaluation. Celery had higher eukaryotic colonies when representative colonies were characterized microscopically (approximately 40% vs. <10% for carrots). This could be due to the packaging, since the celery was packed in water and the carrots were not.

Discussion

Treatment with acids reduced the colony counts suggesting that acid treatments could be used as part of a hurdle method of food processing to help reduce the number of antibiotic resistant organisms entering the food chain. Also, because the HCl treatment

reduced the counts as well, it is promising that the stomach acid of healthy individuals will further protect individuals from antibiotic resistant organisms. However that doesn't hold true for acid resistant organisms such as LAB or *Helicobacter*. It should be noted that while the acid treatments would help reduce the number of viable organisms it might not be affective in reducing the amount of DNA available for transformation. This would further warrant the necessity to develop a screening test, such as Real time PCR, which could detect the amount of DNA in a sample rather than merely the DNA from viable cells.

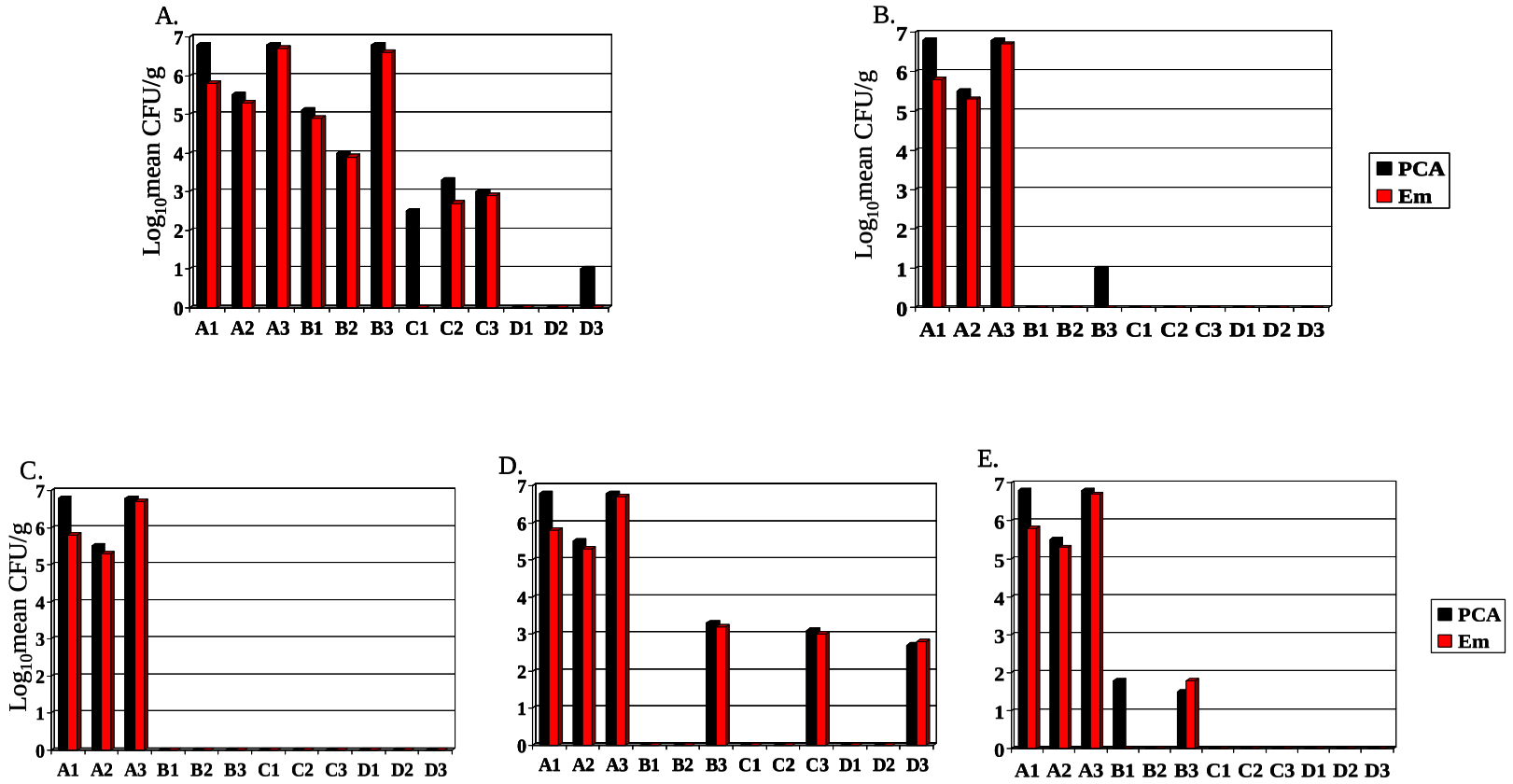


Figure B.1. Prevalence of ART in carrots after various treatments. A) A1-A3, no treatment; B1-B3 boiled 5 sec.; C1-C3 boiled 10 sec.; D1-D3 boiled 30sec. B) A1-A3, no treatment; B1-B3 HCl 5 sec.; C1-C3 HCl 10 sec.; D1-D3 HCl 30sec. C) A1-A3, no treatment; B1-B3 vinegar 5 sec.; C1-C3 vinegar 10 sec.; D1-D3 vinegar 30sec. D) A1-A3, no treatment; B1-B3 dressing 5 sec.; C1-C3 dressing 10 sec.; D1-D3 dressing 30sec. E) A1-A3, no treatment; B1-B3 lemon 5 sec.; C1-C3 lemon 10 sec.; D1-D3 lemon 30sec.

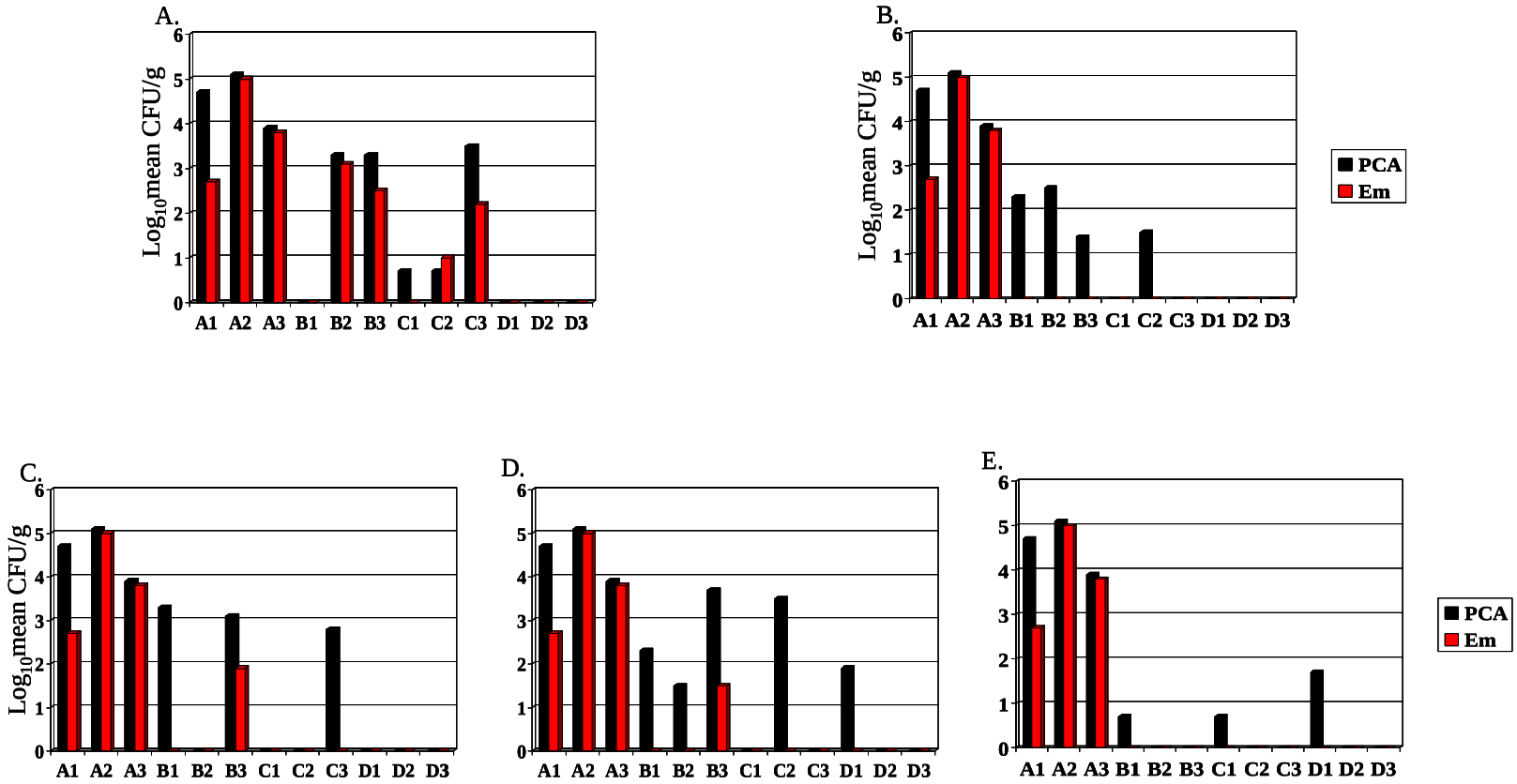
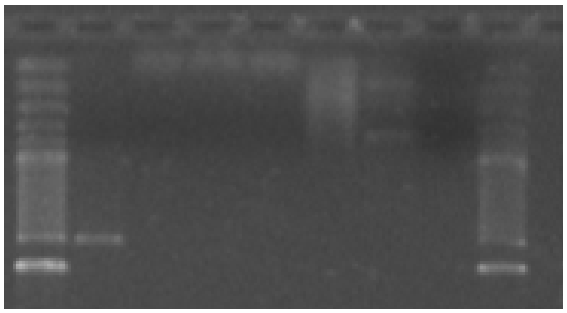
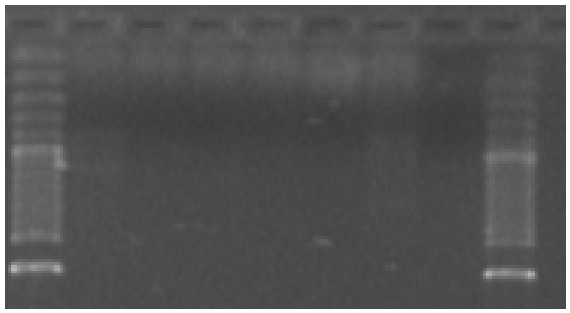


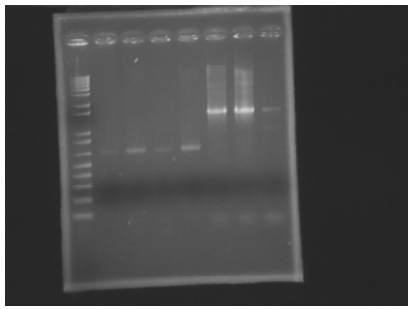
Figure B.2. Prevalence of ART in celery after various treatments. A) A1-A3, no treatment; B1-B3 boiled 5 sec.; C1-C3 boiled 10 sec.; D1-D3 boiled 30sec. B) A1-A3, no treatment; B1-B3 HCl 5 sec.; C1-C3 HCl 10 sec.; D1-D3 HCl 30sec. C) A1-A3, no treatment; B1-B3 vinegar 5 sec.; C1-C3 vinegar 10 sec.; D1-D3 vinegar 30sec. D) A1-A3, no treatment; B1-B3 dressing 5 sec.; C1-C3 dressing 10 sec.; D1-D3 dressing 30sec. E) A1-A3, no treatment; B1-B3 lemon 5 sec.; C1-C3 lemon 10 sec.; D1-D3 lemon 30sec.



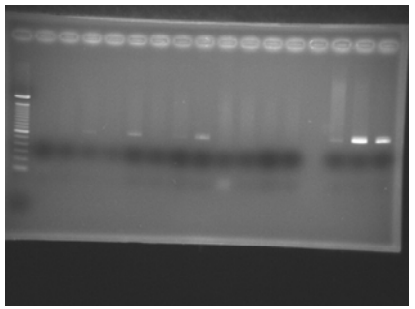
A. Multiplex; using group of colonies.



B. Multiplex using single colonies.



C. Grouping colonies using single *erm*C primer.



D. Single colonies Single primer *erm*C 640bp

Figure B.3. Comparison of multiplexing PCR versus conventional PCR. All samples are from produce.

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