MICROBIAL INDICATORS IN RESTAURANT SALADS: CORRELATION BETWEEN SALAD TYPE, RESTAURANT OWNERSHIP FORMAT, AND CUSTOMER BUSINESS VOLUMES

A Thesis

by

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ABSTRACT

Foodborne illness outbreaks associated with fresh produce have increased over the past decade. Food workers employed at full-service restaurants are found to perform risky food practices more often than food workers employed in other segments of the foodservice industry. The goal of this study was to determine if differences in restaurant ownership format, business volume, and salad type influenced the level of indicator organisms present in restaurant salads. Overall levels of heterotrophic bacteria, total coliforms, *Escherichia coli*, *Enterococcus*, male-specific coliphages, and somatic coliphages were determined by aerobic plate counts (APC), Colilert™, Enterolert™, and U.S. EPA (Method 1601 and 1602), respectively. Molecular methods including automated rep-PCR DiversiLab™ system and reverse transcriptase-PCR were used for the DNA fingerprinting of *E. coli* and the genotyping of male-specific coliphages, respectively.

All of the above mentioned indicator organisms were present in the restaurant salads. Comparisons between restaurant types found that levels of APC, *Enterococcus*, and male-specific coliphages were significantly higher in locally owned restaurants and levels of total coliforms and somatic coliphages were significantly higher in corporate restaurants. The levels for all indicator organisms were significantly higher in specialty salads compared to leafy greens salads. Comparisons between business volumes suggested that indicator organism counts were higher during low customer traffic sampling periods. These results suggested that there were differences in safe food handling practices between locally owned and corporate restaurants. Staffing and labor issues as a result of low customer traffic and the need for additional handling and preparation of specialty salads seemed to increase the risk of cross-contamination issues for fresh produce. DNA fingerprinting for *E. coli* revealed that the same organism was found at multiple restaurants. These results indicate that there was a common source of contamination somewhere between field production and distribution. Genotyping results for male-specific coliphages found that some of the produce had been exposed to human

and animal sources of contamination. Overall, the monitoring for indicator organisms in restaurant salads found that there is still a need for improved education-based programs in the area of safe food handling practice associated with fresh produce for food workers in restaurants.

DEDICATION

I would like to dedicate my thesis to my friends, and family who stood by me and showed their continued love and support.

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NOMENCLATURE

LO	Locally owned
CO	Corporate
LV	Low Volume
HV	High Volume
SS	Specialty Salad
LGS	Leafy Greens Salad

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INTRODUCTION AND LITERATURE REVIEW

I.1 Literature Review

I.1.1 Foodborne Illness Outbreaks in the United States

In the United States, approximately 9.3 million foodborne illness cases, 56,000 hospitalizations, and 1,350 deaths occur annually *(122)*. Unspecified agents cause an additional estimated 38.3 million foodborne illnesses, 72,000 hospitalizations, and 1,700 deaths each year in the U.S *(121)*. The leading causes of bacterial, viral, and parasitic foodborne illnesses are non-typhoidal *Salmonella*, Norovirus, and *Toxoplasma gondii (122)*. Recent estimates of the economic cost of foodborne illness in the United States range from 51.0 billion to 77.7 billion in annual health-related costs *(124)*. These numbers provide economic justification for the importance of programs and other studies whether government, industry, or academia involving the reduction of foodborne illnesses and the associated cost.

I.1.2 Foodborne Illness Outbreaks Associated with Fresh Produce

Fresh produce consumption is on the rise globally due to recently discovered health benefits and increasing availability of fresh produce year round *(78, 100, 109)*. Between 1970 and 2010, in the United States consumption of fresh fruits and vegetables has increased by 27% and 21%, respectively *(78, 151)*. Along with the increase in consumption of fresh produce there has been an increase in the number of foodborne outbreaks and illnesses attributed to fresh produce *(22, 78, 131)*. In the 1970's, fresh produce was reported to be responsible for <1% of the foodborne outbreaks (13 of 1,857) and 1% (708 of 68,712) of foodborne illnesses. Between 1990 and 1997, foodborne outbreaks and illnesses related to fresh produce had risen to 6% (114 of 1,788) and 12% (8,808 of 74,592), respectively *(131)*. Between 1998 and 2007, foodborne outbreaks and illnesses associated with fresh produce had increased again to 15% (684 of 4,638) and 23% (26,735 of 117,136), respectively *(22)*. Scharff *(123)*

estimates foodborne illness associated with fresh produce was responsible for 25% (approximately $39 billion) of the annual health-related costs of foodborne illness. Overall, there is a trend that shows an increase in both produce consumption and produce related foodborne outbreaks.

Since fresh produce consumption and foodborne outbreaks are on the rise it is important to realize why fresh fruits and vegetables are at higher risk for contamination with foodborne pathogens*(100)*. Fresh fruits and vegetables are at higher risk because they are mostly served raw or undergo minimal processing that do not involve a microbicidal kill-step *(74, 100)*. Many pathogens involved in foodborne illness outbreaks associated with fresh produce include Norovirus, *Salmonella spp*., *E. coli O157:H7*, Hepatitis A*, Listeria monocytogenes*, and *Cyclospora cayetanensis (109).* The leading cause for foodborne illnesses associated with fresh produce from 1990 through 2007 were Norovirus*, E. coli O157:H7*, and *Salmonella spp. (22, 50)*. Between 2010 and 2012 Shiga-toxin producing *E. coli* (O157:H7; O145) was identified as the cause of 4 multi-state foodborne outbreaks involving leafy greens, sprouts, and romaine lettuce that resulted in 148 infected individuals and 62 hospitalizations *(23, 26, 32, 33)*. During the same period different serovars of *Salmonella enterica* (Braenderup; Typhimurium; Newport; Enteritidis; Panama; I4,*(122)*,12:i:-) were identified as the cause of 6 multi-state foodborne outbreaks involving mangoes, papayas, cantaloupes, and sprouts that resulted in 723 infected individuals and 150 hospitalizations *(24, 25, 27, 29-31)*. In 2011, a multistate outbreak of *Listeria monocytogenes* in cantaloupe caused 146 cases of listeriosis, and 33 deaths and one miscarriage *(28)*. This outbreak was the largest outbreak to occur of listeriosis involving fatalities in the United States *(93)*.

In 2006, one of the most publicized outbreaks occurred involving *E. coli O157:H7* contaminated spinach that caused 204 cases of foodborne illness across 26 states *(19)*. This outbreaks was responsible for over 100 hospitalizations, 31 people developed hemolytic uremic syndrome (which can result in kidney failure), and 3 deaths *(19)*. Reports of the multistate outbreak brought the problem of fresh produce

contamination to the forefront of consumer awareness much like the Jack-in-the-Box outbreak of *E. coli O157:H7* did for the ground beef industry *(135)*.

I.1.3 Consumer Dining Trends

Today people are dining away from home more frequently and are consuming more ready-to-eat foods such as produce *(63, 73, 78)*. Restaurants and ready-to-eat foods have both been shown to be important locations and sources where foodborne outbreaks occur *(97)*. Modern society continues to foster an "on-the-go lifestyle" in which the American consumer frequently dines away from home. Restaurant sales have grown from $42.8 billion in 1970 to an estimated $660.5 billion in 2013 and contribute 13.1 million jobs to the American economy. Consumers spend approximately 47% of their food dollar dinning out *(107)*. Consumer dining trends of eating away from home and restaurants offering larger portions of food have contributed to less healthy eating habits as well as a rise in obesity rates in the United States *(63, 73)*. In recent years, the health benefits associated with consuming fresh fruits and vegetables daily and governmental health initiatives have led consumers wanting healthier options when dining away from home *(109, 152)*.

Over the past decade, consumers have expressed a growing interest in health and nutrition and want healthier menu choices when dining out *(47, 73)*. Even with expressed interest by consumers looking for healthier menu options, the restaurant industry is faced with the problem of balancing mixed consumer messages based on sales and making profits *(63)*. Consumer demands for listing nutritional facts have increased over the past several years within the quick service fast food industry and have been received positively by consumers *(47, 63)*. In the full service segment of the restaurant industry guiding consumers toward healthier menu items have been met with mixed reviews and in some cases restaurants have seen negative effects *(63)*. Several factors drive menu options in the foodservice industry, and most often, menu options are based mainly on sales and profits *(63)*. Obstacles to healthier menu options include low sales, smaller market appeal, spoilage and short shelf life of fresh fruits and vegetables,

inconsistent supply, and employee training and skill issues *(63)*. Healthier food options in most restaurants involving fresh produce are generally limited to salads and various smaller sized produce side dishes *(63)*.

Although there are many obstacles for restaurants offering healthier menu options, salad sales in full service restaurants have increased 33% from 2000 through 2003 and salad sales between 2001 and 2003 have also increased in quick service restaurants *(47)*. Harneck et al. *(73)* found that salads were the second most ordered items in restaurants. Fresh produce and consumer trends have both shown that consumers are eating more fresh produce and are ordering more healthy food options, such as salads, that are available in restaurants. Restaurants offer a variety of salads for both appetizers and main course entrees *(63)*. Since most salads, leafy greens salads and specialty salads, contain raw fruits and vegetables, they are at higher risk for contamination with foodborne pathogens, especially if safe food handling practices are not followed *(109, 146)*.

I.1.4 Foodborne Illness Outbreaks Associated with Fresh Produce and Restaurants

Since consumers are dining away from home with increasing regularity it is vital to the food industry and food handlers that good food handling practices are followed. Places were food was prepared, such as homes, schools, and workplace cafeterias, it was determined that between 1994 and 2009, restaurants were reported to be the leading location in which people consumed foods that caused foodborne illnesses and outbreaks. Restaurants were involved in 45% (5,721 of 12,567) foodborne outbreaks that happened during this period *(34-39)*. In 2009, a total of 524 foodborne outbreaks were attributed to dining in restaurants, and 83% (435 of 524) of these foodborne outbreaks occurred in full-service restaurants *(39)*. Norovirus is the leading cause of foodborne outbreaks in the United States, and between 2001 and 2007, 46% (2922 of 6355) of all foodborne outbreaks with known etiologies were attributed to Norovirus. It was reported that 83% of these foodborne outbreaks occurred in commercial settings, and 62% of the foodborne outbreaks in commercial settings occurred in restaurants. In 53% of Norovirus

outbreaks reported during this period, food workers were the source of the foodborne outbreaks *(72)*. Norovirus outbreaks were reported to occur commonly in complex foods, such as sandwiches and salads, and simple foods, such as leafy greens vegetables which are foods that usually undergo extensive handling with minimal processing. Boxman et al. *(16)* study obtained environmental swabs from different surfaces in a restaurant involved in a Norovirus outbreak and from an infected employee's hands that was preparing ready-to-eat foods. Norovirus was detected in four of the environmental samples taken. The swabs that were positive were obtained from male and female toilet seats, a handle of a knife used to cut bread, and the hands of the infected food worker. When the Norovirus samples were compared to the fecal sample isolates obtained from the sick patrons, it was found that the genotyped viruses were identical.

I.1.5 Food Workers Role in Produce Contamination

The FDA has reported that food workers are responsible for an estimated 20% of bacteria related foodborne outbreaks *(148)*. Greig et al. *(70)* reviewed a total of 816 foodborne outbreak cases involving food workers between 1927 and 2006. This study found that approximately 40% of the reported cases occurred in restaurants, and that multi-ingredient foods were the most often implicated foods involved in these outbreaks, of which lettuce and leafy green salads were the main food items implicated within this category. Patel et al. *(112)* determined that an outbreak of *Salmonella* Montevideo that occurred in several locations of a restaurant chain in Phoenix, AZ was due to poor hygienic practices and cross-contamination issues when preparing raw chicken that had been contaminated before arrival at the restaurants. Isolates where obtained from uncooked chicken, chopped cilantro, and a cutting board. Food workers that do not follow safe food handling practices increase the risk of contaminating our food and can directly cause foodborne illnesses.

Government, industry, and academia have been involved with ways in which to improve the safety of our food supply. In an effort to improve food safety, the Food and Drug Administration (FDA) has provided the Food Code as a way to address and

minimize the five major risk factors (improper holding temperature, inadequate cooking, contaminated equipment, food from unsafe sources, and poor personal hygiene) responsible for causing foodborne outbreaks within the food service industry *(148)*. State and local authorities have also provided guidelines and regulations to improve food safety such as Texas' version of the Food Code (Texas Food Establishment Rules) *(140)*. Current interventions for risky food handling practices and behaviors within the food service industry are focused around science based educational training, competency based training, and enforcement *(104)*.

In many segments of the food service industry there are many guidelines and risk management programs, such as, Hazard Analysis Critical Control Points (HACCP) program, which if applied to daily operations could mitigate and reduce potential risks involved with food handling practices *(118, 119, 134, 139, 146)*. Academic studies have found that behavioral patterns involving employee's attitudes, beliefs, and behaviors can influence food workers ability to perform good food handling practices *(104)*. Other studies have relied upon microbiological assessments that employ the use of indicator organisms and pathogen testing as a way to assess potential risks and hazards, and can provide information regarding the microbiological quality of a food commodity. Microbiological assessments have been used to show the effectiveness of educational and risk management programs within the food service industry *(6, 7, 16, 40, 44, 51, 52, 86, 87, 99, 105, 133, 134)*. Molecular methods are used extensively by the food industry and governmental health agencies to reduce the risk of foodborne illnesses. Molecular methods have the ability to subtype and compare pathogen strains in food, water, and in clinical cases, and can aid principle investigators with identifying sources of contamination *(13, 53, 58, 60, 108, 137, 156)*. Although there have been several areas of study pertaining to increasing food safety within the food service industry, the FDA has released several reports over the past decade monitoring the five major risk factors involved with risky food handling practices. These reports have found that full service

restaurants had the highest "out of compliance" rating from all of the food service institutions monitored *(147, 149, 150)*.

There are several parameters that can hinder the working relationship within restaurants and the performance of good food handling practices. Full service restaurants have the most complex kitchen working environment. Raw meat products, cooked foods, and raw produce are prepared in close proximity to each other to make a final product which can inherently increase the risk of contamination. Even with more state and local authorities and restaurants requiring employees to receive some food safety training, researchers are still reporting that food handlers are not performing food safety behaviors to the desired levels *(42, 104, 147, 149, 150)*. Commonly cited barriers to safe food handling behaviors in restaurants are time, resources (money and equipment), labor issues (amount of staff), and language barriers *(42, 66, 67, 104)*.

Restaurants depend upon customer sales to be successful. Consumers expect to receive a food product that has been made properly and safely, and they expect it to be served in a timely fashion. Time management plays a huge role in the restaurant industry. During high volumes of business additional resources and labor are needed to ensure that consumer demand is met, and if the working space or appropriate equipment is not available or there is a shortage of labor it can further constrain an already time sensitive situation. Food service workers have reported that time pressure from high volumes of business traffic, staffing issues, and resources affect their abilities to perform proper hygienic practices, avoid cross contamination issues, maintain proper heating and cooling temperatures, and ensuring that foods being served were cooked properly *(42, 66, 67, 104)* . Researchers have found that food workers in restaurants only wash their hands properly between 5% and 25% of the time, and it has been suggested that food workers in restaurants should wash their hands 29 times per hour *(68, 69, 136, 147, 149, 150)*. The minimum amount of time suggested by the FDA outlined in the Food Code for food workers to wash their hands is 20 seconds *(148)*. The demands placed on food

workers to increase food production during a "busy period" will inevitably result in a reduction of food safety behavior to ensure the efficiency of customer service *(104)*.

Hygienic practices such as hand washing and cross-contamination are important causes of contamination in the kitchen *(103)*. Multi-ingredient foods such as specialty salads and leafy greens salads require different levels of preparation. Studies have shown that fresh produce proceeding through the food processing chain can increase the levels of indicator organisms present, and unsanitary food preparation practices of ready-to-eat foods can result in foodborne illnesses and outbreaks *(70, 82, 86, 142)*. There are handling risks that can arise both during high and low volumes of customer traffic, which are dependent upon time and the number of food workers available to prepare food *(42, 66, 67)*. Since specialty salads can contain a greater number of ingredients (leafy greens, cheeses, croutons, tortilla strips, meats etc.) than leafy greens salads, the amount of handling and the number of people handling the salads could play a role in the number of microorganisms present. Management of labor may dictate that a single food worker is present to work several food stations during low customer traffic time periods. Preparing a specialty salad may include working with raw meats and fresh produce to make a single dish. During a "slow period" of business a few orders may need to be prepared with only a single food worker present which could potentially exceed the work pace threshold for this individual. Unhygienic hand washing practices have been responsible for several outbreaks involving food workers *(103)*. Kendall et al. *(89)* found that food workers were more likely to wash their hands prior to engaging in food preparation than between working with raw meats and fresh produce. Pressures from time are common in the food service industry and can influence the ability of a food worker to perform all necessary food safety practices required while trying to meet consumer demand.

Restaurant ownership format can play also play a role in the ability of food workers to properly perform safe food handling practices. Full service restaurants can vary in ownership type which includes independent locally owned restaurants, single

unit franchise restaurants, multiple-unit franchise restaurants, and large chain corporate owned restaurants *(18)*. Differences exist not only in the size and organizational structure of restaurants, but they can also differ in ethnic cuisine, amount of resources available, and operational philosophies. There have been differences reported in the number of critical and non-critical violations received during health inspections, effectiveness of food safety training programs, and the implementation of operational related food safety programs. Several authors have found that large chain corporate restaurants have received fewer health inspection violations than independent and small franchise restaurants *(18, 88, 117-119)*. Roberts et al. *(117)* also found that independent ethnic restaurants had significantly more violations than chain ethnic restaurants and independent and chain non-ethnic restaurants. Kassa et al. *(88)* found that independent to having certified and trained food safety personnel present large chain corporate restaurants had fewer violations than independent and small franchise restaurants, and that independent and small franchise restaurants that had trained and certified food safety personnel received fewer violations than those that did not. Roberts et al. *(118)* found that prerequisite programs and HACCP implementation differed between independent and chain restaurants. This study found that when compared chain restaurants had implemented more food safety practices than independent restaurants. Each of these authors speculated that due to the centralized core structure and resources available, corporate restaurants are able to convey, implement, and monitor the daily operations of each of their individual units more effectively than smaller restaurant operations *(18, 88, 117-119)*.

I.1.6 Indicator Organisms Role in Foods

There are many foodborne pathogens that can contaminate our food, and food can become contaminated anywhere along the "food-to-fork" continuum *(100)*. There are many factors that prohibit the food industry from routine pathogen testing food. The presence of pathogens in food is infrequent, and when present they are not always uniformly distributed within a food matrix. Testing for some pathogens involves

performing complicated assays and there are many different points throughout the food processing chain in which a food product can become contaminated. Testing foods at each point along the food processing chain for pathogens that can occur sporadically would be time consuming, laborious, and costly to analyze *(53)*. Because of the limitations to routine pathogen testing, the food industry uses indicator organisms to monitor foods throughout the food processing chain *(113)*. Indicator organisms are a particular group or species, such as *E. coli*, that are most often fecal in origin, present in higher numbers than pathogens, and are generally regarded as nonpathogenic *(113)*. Testing for indicator organisms should be able to be performed using economical simple laboratory methods that provide results in a relatively short period of time *(113, 120)*. These indicator organisms can infer direct or indirect fecal contamination, the possible presence of enteric pathogens, food quality, and the level of sanitation used in food processing *(113)*. In addition to monitoring indicator organisms in food, the food industry can also monitor the overall bioburden of a food product. Monitoring the bioburden in foods involves assays for assessing the overall populations present such as heterotrophic organisms or monitoring foods for the level of yeasts and molds present. Aerobic plate counts are used to estimate the overall bioburden of a food product by measuring their aerobic and facultative anaerobic mesophilic populations. It is not an indicator of fecal contamination or possible presence of enteric pathogens. This assay is used as an indicator of food quality and to determine the level of sanitation used in food processing *(113, 145)*. It is important to note, that no singular indicator organism can meet all the criteria requirements for an ideal indicator organism and that it suggested that various groups of indicator organisms be used *(113)*.

Total coliforms represents several genera of bacterial genera which include *Escherichia, Enterobacter, Klebsiella, Citrobacter, Aeromonas*, and *Serratia (113, 144, 145)*. Fecal coliforms and *E. coli* are subgroups of total coliforms but can be differentiated from the total coliform group based on either a physiological basis or can be differentiated biochemically. Total coliforms were one of the most commonly used

indicator organisms. In 1914 the U.S. Public Health Services used total coliforms as indicator of fecal contamination in water because this group of bacteria was found to be excreted in the feces of both humans and animals in high numbers, and could be easily isolated and quantified by simple microbiological methods. This group of bacteria share many common characteristics. Coliforms are aerobic and facultative anaerobic, gram negative non-sporeforming rod-shaped bacteria that ferment lactose and produce acid and gas within 24 to 48 hours at 35°C. Total coliforms also produce the enzyme *β*-galactosidase which can be used to differentiate the coliform group from other members of *Enterobacteriaceae (138)*. Fecal coliforms are subgroup of total coliforms and share the same characteristics as total coliforms. Fecal coliforms can be differentiated from the total coliform group due impart to their ability to grow at 44.5±0.2°C within 48 hours *(113, 144, 145)*. Fecal (thermotolerant) coliforms are able to grow at temperatures similar to that found intestinal tracts of both human and animals which provides a higher specificity for this group as a fecal indicator than total coliforms *(113)*. *E. coli* is a subgroup of both fecal coliforms and are present in higher numbers in the feces of humans and animals than other groups of coliforms and fecal coliforms *(138)*. Unlike total and fecal coliforms, *E. coli* conforms to taxonomic and functional identification criteria. *E. coli* can be biochemically differentiated from the other coliform groups. *E. coli* are identified by their IMViC patterns which are + + - - Type I and - + - - Type II. *E. coli* produces indole by its ability to metabolize tryptophan. Fermentation of glucose produces acid and can produce 2,3 butanediol and/or acetoin. *E. coli* has the ability to use citrate as a sole carbon source. *E. coli* also lacks the enzyme urease but produces the enzyme β-glucuronidase. *β*-glucuronidase is used in other assays to differentiate the presence of *E. coli* from other coliforms *(91, 113, 138, 145)*.

Total coliforms, fecal coliforms and *E. coli* are natural residents in the lower intestinal tracts of humans and animals, but each of them has been isolated from naturally occurring environments of non-enteric origins as well *(53, 91, 113, 126, 138)*. Unhygienic conditions in food processing environments can allow coliforms,

Enterococci, *E. coli* to become part of the resident microflora, which can lead to non-fecal contamination of food with this group of indicator organisms *(113, 138)*. Some members of the total and fecal coliform groups have been shown to have the ability to proliferate at refrigeration temperatures *(91)*. Kornacki and Johnson *(91)* have also cited that studies have shown that the value of *E. coli*, coliforms, and fecal coliforms as an index organism does not correlate well with their use as an indicator of the presence of pathogens in foods. Total coliforms are one of the most extensively used indicator organisms *(113)*. The specificity of coliforms as an indicator of fecal contamination is hindered by the fact that coliforms can be found to occur in environments free from enteric contamination, can establish and grow in environments that have become contaminated, and differ in the resistance to stress than many other pathogens. For these reasons total coliforms are mainly used today to assess food quality and determining the sanitary quality of foods that undergo different processing treatments *(91, 113)*.

The ability of fecal coliforms to grow at thermotolerant temperatures similar to that of intestinal tracts of humans and animals adds value to the specificity of this group *(113)*. The fecal coliform group consists mostly of *E. coli* and some non-*E. coli* species such as *Klebsiella*, *Citrobacter* and *Enterobacter*. Fecal coliforms have been shown to be a useful indicator of fecal contamination in shellfish growing waters and shellfish meats. The ubiquitous nature of *Klebsiella* spp. has limited the use of fecal coliforms in certain foods such as produce. In processed foods and some ready-to-eat foods the presence of fecal coliforms at high numbers could be tentatively used as cautious indicator for fecal contamination. This suggests that there is a need to use a more specific indicator such as *E. coli*, when monitoring for fecal contamination in food or water *(113, 138, 144)*.

"Generic" *E. coli* is an indicator organism that is commonly used as an indicator of fecal contamination *(113)*. Most *E. coli* species are natural residents of the gastrointestinal tracts of humans and animals and they are present in high numbers *(138)*. *E. coli* is used for monitor fresh water quality and shellfish waters, and are used

as process verification controls in the food industry *(145)*. Organizations such as the Leafy Greens Marketing Agreement use *E. coli* as an indicator of fecal contamination when present in high numbers to monitor their crops and irrigation waters *(96)*. *E. coli* are more sensitive to some processing stresses than some foodborne pathogens when foods have been dried, frozen, or have a low-pH. *E. coli* is not an indicator that pathogenic organisms are present in foods, but does convey that there might be a higher risk of foods that have been exposed to fecal contaminated *(113)*.

Enterococcus is another indicator organism that can be found in the intestinal tract of both humans and animals. Certain strains of Enterococcus can be species specific such as *E. Faecalis*, *E. Faecium*, in humans and *E. durans*, *E. gallinarum*, *E. avium*, and *E. hirae* in animals *(84)*. Some strains of *Enterococcus* have caused outbreaks of gastroenteritis as an opportunistic pathogen and it also has increased in importance due to its ability to acquire antibiotic resistant *(57)*. *Enterococcus* are more resistant to some environmental stress than coliforms and *E. coli (84, 113)*. *E. faecalis* and *E. faecium* are most often associated with human waste but Ailes et al. *(2)*has found that the most naturally occurring strains on produce belonged to these two species of Enterococci. Ailes et al. *(2)* suggested that testing for total *Enterococci* can be used for both an indicator of produce quality and a potential indicator of fecal contamination. *E. coli* and *Enterococci* have been isolated from several aquatic systems, beach sands, soil sediments, food processing environments, and plant cavities, and they have been reported to have the ability to proliferate and become part of the natural microbiota with in these environments. For these reasons their use as an indicator for fecal contamination has been decreased *(53)*.

Since a single indicator organisms cannot meet all the criteria for an ideal indicator there is a need to use several organisms to ensure that all microbiological parameters are being tested for in a particular environment *(113)*. Pathogenic microorganisms such as bacteria, viruses, and parasites can differ in ecological and survival characteristics, and can react differently when exposed to various environmental

conditions *(53, 120)*. Therefore, monitoring different aquatic and environmental systems and foods there is a need to test for several indicator organisms. Traditional bacterial indicator organisms such as coliforms and *E. coli* have been found not to correlate well with the presences of enteric viruses in water and in shellfish. Alternative indicators of fecal pollution such as male-specific F+RNA and somatic coliphages are viral indicators, and are considered to be reliable alternative fecal indicators for monitor environmental waters, waste waters, sewage, shellfish, and foods *(9, 48, 49, 54, 56, 81, 94, 98, 132, 153)*. Coliphages along with *E. coli* are present in the intestinal tracts of both humans and animals. These viruses use E. coli as a host to replicate, and are similar in size, transport, survival patterns, and are present in higher density to that of enteric viruses *(65, 120)*. Conditions in the environment are more difficult to ascertain for coliphage replication and environmental conditions for replication of the male-specific coliphage are more restrictive *(65, 85)*. Male-specific F+RNA coliphages have been found to share weak correlations with bacterial indicator organisms in water, waste water, and shellfish, and have been found to correlate well with the presence of some enteric viruses in water, shellfish harvest waters, and shellfish meats. Correlations between seasonal patterns and the presence of enteric viruses and coliphages have also been reported whereas bacterial indicators such as *E. coli* have not *(9, 48, 49, 54, 56, 98, 153)*. Male-specific F+RNA coliphages are also of useful for fecal source tracking. Serological typing or genotyping male-specific F+RNA coliphages can differentiate between human and nonhuman fecal sources of contamination adding value to its use as a fecal indicator organism. Male-specific F+RNA coliphages belong to the family *Leviviridae* which contain two genera *Levivirus* (group I and II) and *Allolevivrius* (group III and IV). Genogroups I and IV are generally associated with waste from animals and genogroups II and III are associated with human fecal contamination or human sewage *(65, 126)*. Determining the source of contamination and determining the genetic relatedness of microorganism can be useful for reducing the number of illnesses associated with the fecal contamination in water and food, and can be beneficial for identifying foodborne outbreaks and reducing their durations*(53, 60, 126, 156)*.

I.1.7 Role of Molecular Methods and How They can Aid Traditional Culture Based Methods

Traditional culture based methods rely upon phenotypic traits acquired by microorganisms residing within a particular host or environment which can include a microorganisms ability to metabolize a particular chemical compound or grow on a selective media *(126)*. Identifying foodborne pathogens by culture based methods involve pre-enrichment, selective enrichment, selective plating, biochemical screening, and serological conformation *(101)*. There are a number of phenotypic typing methods such as antibiotic resistance, carbon-source utilization profiling, serotyping, and fatty acid methyl ester profiling that have been used to discriminate among various types of microorganisms *(53, 126)*. Although the use of culture based methods and phenotypic typing methods have been successful for culturing and typing foodborne pathogens there are several drawbacks to their use. Culture based methods are very time and labor intensive requiring several days to obtain results, and there are several viruses that are known to cause foodborne illnesses which cannot be culture *in vitro* or grow poorly under laboratory conditions *(17, 53, 110, 126)*. Bacterial cells that have become stressed can enter a viable but non-culturable state in which some pathogens can still retain their pathogenicity, and if present in low numbers can affect the detection limit sensitivity of culture based assays. This is important with pathogens that have low infective doses. Laboratories performing culture based methods need a wide variety of selective media and reagents and skilled laboratory technicians. Phenotypic typing methods lack the diversity to type a wide range of organisms, suffer from specificity and sensitivity issues, and are less discriminatory than molecular based methods used for genotyping *(17, 53, 62, 126)*.

There are a number of molecular techniques that are used to genotype bacteria, viruses, and parasites. Identification, differentiation, and characterizations of these microorganisms by genotypic typing methods rely upon detecting differences in an organism's DNA or RNA nucleic acid sequences. Molecular methods are rapid and

highly discriminatory, and have the potential to obtain results in less than 24 hours. These methods can be standardized and some are amenable to computer based analysis which aides in interlaboratory reproducibility and comparisons of DNA fingerprints. There are several genotypic typing-based categories which are commonly used for genotyping and DNA fingerprinting foodborne pathogens which include restriction-based methods (plasmid analysis, restriction fragment length polymorphism, PFGE), amplification-based methods (rep-PCR, amplified fragment length polymorphisms, random amplified polymorphic DNA, variable number of tandem repeat, reverse transcriptase-PCR, qPCR), sequencing-based methods (multilocus sequence typing, single nucleotide polymorphism), and microbial characterization techniques (microarray) *(17, 55)*.

I.1.7.1 Molecular Methods uses in Epidemiological Investigations and Microbial Source Tracking

Epidemiological and traceback investigations have successfully used molecular typing techniques to distinguish between non-outbreak and outbreak related strains and have also been able to link foodborne pathogens to a particular environmental source or host using molecular methods. PulseNet and CaliciNet are national molecular subtyping networks for foodborne disease surveillance in the United States *(137, 156)*. These networks use pulsed-field gel electrophoresis (PFGE) and reverse transcriptase polymerase chain reaction (RT-PCR), which are consider the "gold standards", for DNA fingerprinting and viral genotyping foodborne pathogens *(15, 55, 102, 110, 125)*. Microbial source tracking (MST) involves the use phenotypic and genotypic methods to identify and characterize indicator bacteria and enteric pathogens, such as, viruses to identify the host source of origin *(53, 108, 126)*.

Overall MST centers on the general assumption that identifying a specific host marker or a specific characteristic of a microorganism associated with feces can unambiguously identify a specific host source, MST can be divided into culture-base and culture-independent methods of which some methods require a library of data to

compare genotypic and phenotypic traits such as DNA fingerprinting or antibiotic resistance patterns *(53, 126)*. Culture-based, library-dependent methods center around the idea that bacteria have become adapted to a particular host or environmental niche. These hosts or environmental niches can vary in pH, nutrient availability, and receptor specificity and the bacteria and their progeny will inherently share the same phenotypic and genotypic traits. Culture-independent, library independent and culture-based, library-independent methods can also be used as a tool for determining the source of host specific fecal contamination in food or water by using molecular methods to test for the presence of human viruses such as the human adenoviruses and human polyomaviruses or animal viruses such as porcine adenoviruses and bovine polymaviruses. Male-specific F+RNA coliphages are also of useful for fecal source tracking *(14, 53, 126)*. Serological typing or genotyping male-specific F+RNA coliphages can differentiate between human and nonhuman fecal sources of contamination. Male-specific F+RNA coliphages belong to the family *Leviviridae* which contain two genera *Levivirus* (group I and II) and *Allolevivrius* (group III and IV). Genogroups I and IV are generally associated with waste from animals and genogroups II and III are associated with human fecal contamination or human sewage *(65, 126)*.

There is not a single ideal source-tracking method available that can identify fecal pollution from specific sources in food or the environment with complete confidence *(53, 126)*. It is prudent to use different typing methods and target specific areas and microorganisms of concern. There have been several studies combining source-tracking methods that have used combinations of both phenotypic and genotypic methods targeting multiple organisms and host specific markers to determine sources of fecal contamination, but with varied results *(53, 157)*. Rep-PCR and RT-PCR are molecular typing techniques that have been used successful for microbial source tracking. Repetitive extragenic palindromic sequence PCR (rep-PCR) is an amplification-based genotyping method that is used for DNA fingerprinting bacterial isolates. This method has been shown to have comparable but slightly less

discriminatory power than PFGE. The recent standardization and a automated format created by DiversiLab systems (BioMéurex, Durham, NC) has increased interlaboratory reproducibility and results can be obtained in less than 24 hours which can be easily compared with a web-based software. The automated rep-PCR DiversiLab system is also designed for high throughput which allows for several samples to be processed at once using the microfluidic design of their "Lab-on-a-chip" and bioanalyzer to separate amplicons and generate patterns *(41, 46, 75, 128)*. DiversiLab's automated rep-PCR system also has been shown to be capable for building a library of *E. coli* isolates taken from several different environmental and host sources and this analysis tool was used successfully for identifying sources of fecal contamination in water *(157)*.

RT- PCR is a PCR based genotyping method that is used to rapidly detect and genotype enteric RNA viruses such as Norovirus in clinical and environmental matrices *(125)*. Conventional methods such as electron microscopy can be labor intensive to perform and suffers from detection limit issues. Enzyme-linked immunosorbent assays (ELISA) and immunochromatography are rapid tests, but have problems with low sensitivity issues *(102)*. RT-PCR has also been shown to be more reliable than serotyping methods and less labor intensive than amplification-based membrane hybridization techniques for genotyping fecal indicator viruses such as F+RNA coliphages *(58)*.

Increasing trends in fresh produce consumption and foodborne illness outbreaks associated with produce should have lead researchers to find new ways to identify possible routes of contamination and reduce the number of barriers preventing the practice of safe food handling techniques. Ready-to-eat foods such as fresh fruits and vegetables are at greater risk to microbial contamination due to the lack of a microbicidal kill-step before service *(74, 100)*. Restaurants have been found to be places were a majority of foods involved in foodborne outbreaks occur *(34-39)*. Healthy menu option like salads can become easily contaminated by food workers because of the extensive need to handle this food item during preparation *(70, 103, 142)*. Full service

restaurants have been reported as having the highest out of "out-of-compliance" rating involving risky food handling practices throughout all segments of the foodservice industry *(147, 149, 150)*. Restaurant format types, time management, and labor issues have been reported as barriers to safe food handling practices and can affect the level of food safety being practiced in restaurants *(18, 88, 117-119)*. The handling and processing of fresh produce have also been shown to affect the levels of microorganisms present in foods *(70, 82, 86, 142)*. Indicator organisms have been used to evaluate the efficacy of food safety educational programs, evaluate critical control points in HACCP plans, hygienic practices, cross-contamination studies, and produce quality in restaurants *(6, 7, 16, 40, 44, 51, 52, 86, 87, 99, 105, 133, 134)*. Molecular methods have been used to assess host specific sources of fecal contamination in water and in the environment, and have aided epidemiological investigations to identify the sources and organisms involved in foodborne outbreaks *(17, 53, 55, 58, 110, 126, 137, 156)*. Time, labor issues, restaurant ownership formats, and produce handling have been found to have an impact on the ability for food workers to perform safe food handling practices in restaurants.

I.2 Objectives and Hypotheses

I.2.1 Objectives and Hypothesis: Microbiological Survey for Restaurant Salads

The overall objective of this study was to assess the effect of restaurant ownership formats, customer traffic volumes, and salad types on the level of indicator organisms present in fresh produce items obtained from full service restaurants. The primary objective of this study was to examine two different types of salads, leafy greens salads and specialty salads, purchased from two different types of full service restaurants (nationally franchised and locally owned) for the presence of microbial indicators, fecal bacteria indicators, and fecal viral indicators as a function of time pertaining to different volumes of customer traffic. The secondary objective was to assess the genetic relatedness of *E. coli* isolates and to genotype male specific F+RNA coliphages found to be present in these salads. Genotyping male-specific F+RNA coliphages found in the salad samples can provide insight into the source of contamination (human or animal). DNA fingerprinting generic *E. coli* isolates found in salad samples for their genetic relatedness may help to identify possible routes of entry into these restaurants, and assess their potential to persist in foods over long periods of time if proper hygienic and sanitary practices are not followed.

I.2.1.1 Overall Objective and Hypothesis 1

To determine the effect of full service restaurant ownership format, salad type, and business volume have on the presence and levels of total mesophilic populations, total coliforms, *E. coli*, *Enterococcus*, male-specific coliphages, and somatic coliphages in restaurant salads since it has been reported that time, amount of labor, and resources effect the ability of food workers to perform safe food handling practices.

> *Hypothesis 1*: I expected to find that all indicator organisms to be present in restaurant salads and the levels of these indicator organisms to be higher in locally owned restaurants and specialty salads during high volumes of customer traffic.

I.2.1.2 Specific Objectives

1. Determine the presence or absence of indicator organisms in full service restaurant salads
2. Assess the effect of restaurant ownership format on the level of indicator organisms present in full service restaurants
3. Assess the effect of salad type on the level of indicator organism present in full service restaurant salads
4. Assess the effect of customer traffic volumes on the level of indicator organisms present in full service restaurant salads

I.2.2 Objectives and Hypothesis: Microbial Fingerprinting Isolates from Restaurant Salads

I.2.2.1 Overall Objective and Hypothesis 2 and 3

I collected *E. coli* and male-specific coliphages to determine the overall genetic relatedness of *E. coli* isolates and to identify male-specific F+RNA coliphages. *E. coli* isolates and male-specific coliphages were collected from both CO and LO restaurant salads to determine possible routes and sources of fecal contamination present in restaurant salads.

Hypothesis 2: I expected that there will be genetically related *E. coli* present in restaurant salads and that the results will show genetically related *E. coli* will more likely be obtained from a single restaurant than genetically related *E. coli* obtained from several restaurants.

Hypothesis 3: I expected that there were F+RNA coliphages from genogroups I, II, III, or IV present in restaurant salads.

I.2.2.2 Specific Objectives

1. Determine the genetic relatedness of *E. coli* isolates present in full-service restaurant salads by using automated rep-PCR DiversiLab system for DNA fingerprinting
2. Determine the genogroups of male-specific F+RNA coliphages in full-service restaurant salads by using RT-PCR for genotyping

CHAPTER II
MICROBIOLOGICAL SURVEY OF RESTAURANT SALADS FOR INDICATOR ORGANISMS

II.1 Introduction

Most areas of research pertaining to raw produce contamination are based around the prevention of contamination during field production, processing and distribution. Research and management of raw produce safety in these areas are designed to help prevent wide spread outbreaks of foodborne illnesses from occurring. Although raw produce handling and processing in commercial foodservice settings have been shown to be responsible for several foodborne outbreaks there has been little research pertaining the microbiological quality of produce in full-service restaurants and factors that can affect safe food handling practices associated with fresh produce within this setting. The overall objective of this study is to assess the effect of restaurant ownership formats, customer traffic volumes, and salad types on the level of indicator organisms present in fresh produce items obtained from full service restaurants. The primary objective of this study is to examine two different types of salads, leafy greens salads and specialty salads, purchased from two different types of full service restaurants (nationally franchised and locally owned) for the presence of microbial indicators, fecal bacteria indicators, and fecal viral indicators as a function of time pertaining to different volumes of customer traffic.

II.2 Materials and Methods

II.2.1 Microbiological Examination of Salads

II.2.1.1 Sample Collection

The samples to be obtained from restaurants were **specialty salads** and **leafy greens salads**. Specialty salads contain a variation of meats, cheeses, croutons or tortilla strips, nuts, and additional vegetables. In this study green leaf salads were considered to be the stock lettuce leaf mix prepared by a restaurant which may consist of iceberg and/or romaine lettuce alone or have carrots and cabbage added to the stock lettuce leaf mix with no additional ingredients. The salads were obtained from five nationally franchised chain (CO) restaurants and five locally owned (LO) restaurants during periods of high customer traffic and low customer traffic. High customer traffic periods were considered to be lunch and dinner time between the times of 11 AM and 2 PM (lunch) and 5 PM and 9 PM (dinner). Low customer traffic periods were considered to be between 2 PM and 5 PM and 9 PM to close. The restaurants were chosen based on high, moderate, and low health inspection scores obtained from the Brazos Valley Health department. A total of 100 specialty salads and a total of 100 green leaf salads were purchased from both nationally franchised chain full service restaurants and locally owned full service restaurants. Of these, 50 specialty salads and 50 green leaf salads were obtained from high and low customer traffic period. The participation of the restaurants was not known and the salads were purchased on random days throughout this study. The samples were placed in coolers with blue ice and transported immediately to the laboratory for sample processing.

II.2.1.2 Sample Processing

Specialty salads and green leaf salads were processed in a biosafety cabinet (Labconco purifier class II Biosafety Cabinet Delta Series, Kansas City, MO) to prevent laboratory-based contamination of the samples. One hundred grams of each salad sample were aseptically weighed on the analytical balance within the hood and placed

into sterile bags with a membrane filter (VWR, West Chester, PA). Two hundred ml of a 0.1 M sodium phosphate buffer supplemented with 1.0 M NaCl at a pH of 8.0 were added to the stomacher bags. The 0.1 M sodium phosphate buffer supplemented with 1.0 M NaCl was titrated to a pH 8 ± 0.1 using a SevenEasy S20™ pH-meter (Mettler-Toledo, Columbus, OH). The samples were stomached on the (low) setting for 2 minutes. Approximately 200 ml of extract was pipetted from the stomacher bags and placed into 50 ml conical tubes (VWR, West Chester, PA).

II.2.1.3Microbiological Analysis

Microbiological Analysis - The samples were analyzed for different microbiological groupings as described below:

Aerobic Plate Count (APC) - The overall bacterial load of the samples will be assayed by the use of Aerobic Plate Count Agar (Maturin and Peeler., 2001). Serial dilutions were made with 1X PBS and 0.1 ml was plated on plate count agar. The plates were incubated under aerobic conditions for 48 hours at 37°C and the heterotrophic colonies were enumerated.

Total Coliforms and *Escherichia coli* - Total coliforms and *E. coli* were enumerated by Colilert® (IDEXX, Westbrook, ME.) Ten ml, 1 ml, and 10^{-1} dilution of the salad sample extract were placed into sterile centrifuge bottles filled 90 ml, 99 ml, and 99 ml of sterile DI water, respectively. Colilert® snap pack reagents were added to the appropriate labeled bottles and shaken until dissolved. The samples were poured into the appropriately labeled Quanti-Trays® 2000 (IDEXX) and sealed in the Quanti-Tray® sealer (IDEXX). The samples were incubated for 24 hours at 37°C. The results were interpreted by counting the number of wells turning yellow indicating the presence of coliforms and the number of wells fluorescing indicating the presence of *E. coli* and referencing the IDEXX one to 2419/100 mL MPN table provided for enumeration. Positive wells in the Quanti-Tray® 2000 that fluoresced under a long wave (366 nm) UV light were removed by a 1.0 in. 14 gauge needle and a 10 ml syringe (Becton, Dickinson

and Co., Sparks, MC) and enriched overnight in Tryptic Soy Broth in a 37°C water bath under aerobic conditions. Confirmation of *E. coli* positive wells was performed by streaking onto EC-Mug agar. Colonies that fluoresced under a long wave (366 nm) UV light were picked using a sterilized loop and re-streaked onto a Modified mTEC medium (Becton, Dickinson and Co.). Magenta-colored colonies were picked from the modified mTec medium using a flame sterilized loop and placed in Tryptic Soy Broth supplemented with a 20% glycerol and were stored at -80°C.

Enterococcus - *Enterococcus* was enumerated by Enterolert® (IDEXX). Ten ml and 1 ml of the salad sample extract were placed into sterile centrifuge bottles filled 90 ml and 99 ml of sterile DI water, respectively. The Enterolert® snap pack reagents (IDEXX) were added to the appropriate labeled bottles and shaken until dissolved. The samples were poured into the appropriately labeled Quanti-Tray® 2000 pouches and were sealed with the Quanti-Tray® sealer (IDEXX). The samples were incubated for 24 hours at 42°C under aerobic conditions. The results were interpreted by counting the number of positive wells fluorescing for *Enterococci* and referencing the IDEXX one to 2419/100 mL MPN table provided for enumeration. Positive wells in the Quanti-Tray 2000 (IDEXX) that fluoresce under long wave (366 nm) UV light were removed by 1 in. 14 gauge needle and a 10 ml syringe (Becton, Dickinson and Co.) and enriched overnight in Tryptic Soy Broth in a 37°C water bath under aerobic conditions. Confirmation of *Enterococci* positive wells was performed by streaking onto m-*Enterococcus* agar incubated overnight at 37°C under aerobic conditions. Dark red to maroon colonies will be picked using a flame sterilized loop and were placed in Tryptic Soy Broth supplemented with a 20% glycerol and stored at -80°C.

Somatic Coliphages – Somatic coliphages were extracted from the salad sample extracts by centrifugation at 5500 x g for 15 minutes at 4°C, and the supernatant were assayed. Testing for the presence/absence of somatic coliphages were carried out using the Two-step Enrichment and Spot Plate Procedure (Method 1601 EPA, 2001) with the host bacterium *E. coli* strain CN-13. Fifty ml of the sample extract were added to 50 ml

of water and were incubated overnight at 37°C. One ml of the overnight enrichment were taken and centrifuged for 30 seconds at 10,000 rpm. Ten µl of the resulting supernatant were then be spotted onto a Tryptic Soy Agar plate containing 10 ml of a 1% nalidixic acid solution per 1L of TSA. The plates were then be incubated overnight at 37°C under aerobic conditions. Spot plates were analyzed for lysis zone formations indicating the presence or absence of each sample tested for somatic coliphages.

Male-Specific Coliphages – Male-specific coliphages were extracted from the salad sample extracts by centrifugation at 5500 x g for 15 min. 5°C, and the supernatant were assayed. Testing for the presence/absence of male-specific coliphages was carried out using Two-step Enrichment and Spot Plate Procedure (Method 1601 EPA, 2001) with the host bacterium *E. coli* strain F_{amp}^{+}. Fifty ml of the sample extract were added to 50 ml of water and were incubated under aerobic conditions overnight at 37°C. One ml of the overnight enrichment was taken and centrifuged for 30 seconds at 10,000 rpm. Ten µl of the supernatant were be spotted onto a Tryptic Soy Agar plate containing 10 ml of a .15% Ampicillin/Streptomycin per 1L of TSA. The plates were incubated overnight at 37°C under aerobic conditions. Spot plates were analyzed for lysis zone formations indicating the presence of each sample tested for male specific coliphages. Those samples testing positive for male-specific and somatic coliphages were further analyzed to determine the quantitative levels of each.

Enumeration of Somatic Coliphage – Enumeration of somatic coliphages were carried out using the Single Agar Layer Procedure (Method 1602 EPA, 2001) with the host bacterium *E. coli* CN-13. Twenty five ml of the salad sample extract were added to 25 ml of a host solution containing water, log phase *E. coli* strain CN-13, and magnesium chloride. The salad sample extract and host solution were combined for plating with 2X TSA containing 20 ml of 1% nalidixic acid per 1L of 2X TSA. The plates were incubated overnight at 37°C under aerobic conditions. Plates were analyzed for circular zones of clearing and enumerated by counting the number of plaques on each plate.

Enumeration of Male-Specific Coliphage – Enumeration of male-specific coliphages were carried out using the Single Agar Layer Procedure (Method 1602 EPA, 2001) with the host bacterium *E. coli* strain F_{amp}^{+}. Twenty five ml of the salad sample extract were added to 25 ml of a host solution containing water, log phase *E. coli* strain F_{amp}^{+}, and magnesium chloride. The salad sample extract and host solution were combined for plating with 2X TSA containing 20 ml of 0.15% Ampicillin/Streptomycin per 1L of 2X TSA. The plates were incubated overnight at 37°C under aerobic conditions. Plates were analyzed for circular zones of clearing and enumerated by counting the number of plaques on each plate.

Recovery and Storage of Male-Specific Coliphages – In order to genotype male-specific coliphages, the male specific coliphages were recovered from the plaques obtained from the enumeration assay. Flame sterilized scissors were used to remove a portion of a 1 ml pipette tip. The pipette tip was filled with 600 µl of PBS and pressed down over a plaque subsequently removing the plaque from the agar plate. The plaques were vortexed thoroughly and the supernatant was extracted. The supernatant were supplemented with a 20% glycerol and stored at -80°C.

II.2.2 Statistical Analysis

Comparisons for each indicator organism were compared based on the outline listed for the three main categories listed in Table 2-1 by Two-way ANOVA analysis. For categorical groupings with P values >0.050, indicates that there was not a significant relationship observed between the microbial populations. Two-way ANOVA post-hoc analysis was performed using the Holm-Sidak method for all pairwise multiple comparisons and comparisons with P values of >0.05 indicates that there was not a significant relationship observed between the microbial populations. Due to the relatively low numbers of the indicators *E. coli*, *Enterococci*, male-specific coliphage, somatic coliphage present additional statistical analysis were performed by z-test statistical analysis. The z-test did compare the presence/absence of each individual organism to itself within the categorical divisions listed above (π_n – number of positive

samples for a given organism, divided by the total number of samples analyzed). The z-test statistic was calculated for these population proportion comparisons among the same indicator organisms within these different categories (type of restaurant; types of salads; Customer traffic volume). For pairs with P values >0.050, indicates that there was not a significant relationship observed between the microbial populations.

II.3 Results

II.3.1 Microbiological Survey of Restaurant Purchased Salads

A total of 200 salad samples were collected from ten restaurants in Brazos County from September 2009 through May 2010. Among the 200 samples, 100 hundred samples were obtained from 5 nationally franchised corporate restaurants and 100 samples were obtained from 5 locally owned restaurants. Within each restaurant ownership type a total of 50 specialty salads and 50 leafy greens salads were purchased during periods of both high and low customer traffic volumes. Table 2-1 specifies the number of salad samples collected and categorized by restaurant type, salad type, and business volume.

Table 2-1 Number of corporate and locally owned salads categorized by salad type and business volume

Restaurant Type	Salad Type	Business Volume	Sample Number (N)
Corporate			**100**
	Specialty Salad		***50***
		High Volume	25
		Low Volume	25
	Leafy Greens Salad		***50***
		High Volume	25
		Low Volume	25
Locally-Owned			**100**
	Specialty Salad		***50***
		High Volume	25
		Low Volume	25
	Leafy Greens Salad		***50***
		High Volume	25
		Low Volume	25

II.3.2 Overall Quality and Presence of Indicator Organisms in Restaurant Salads

The presence of indicators organisms in restaurant salads in regards to APC and total coliforms is shown in Table 2-2. Both APC and total coliforms were found to be present in all of the salad samples purchased from both locally owned and corporate restaurants. Overall, aerobic plate counts ranged from 4.1 to 9.4 $\log_{10}$ CFU g^{-1} and total coliform counts ranged from 0.3 to 5.7 log10 MPN g^{-1} in the salads purchased from both locally owned and corporate restaurants. The presence of fecal indicator organisms in restaurant salads in regards to *E. coli*, *Enterococcus*, male-specific and somatic coliphages are shown in Table 2-3. This study found that *E. coli*, *Enterococcus*, male-specific and somatic coliphages were present in 19%, 78.5%, 20%, and 22.5% of the restaurant salad samples, respectively. *E. coli* counts ranged from <-0.70 to 2.8 $\log_{10}$ MPN/g. *Enterococcus* counts ranged from <-0.70 to 3.8 log10 MPN/g. Male-specific coliphages counts ranged from <-1.4 to 4.18 $\log_{10}$ PFU/100g. Somatic coliphages counts ranged from <-1.4 to 3.33 $\log_{10}$ PFU/100g (Table 2-2).

Table 2-2 Range of indicator organisms in restaurant salads

	n	APC (log_{10} CFU/g) range	Total Coliforms (log_{10} MPN/g) range	*E. coli* (log_{10} MPN/g) range	*Enterococcus* (log_{10} MPN/g) range	Male-specific Coliphages (log_{10} PFU/100g) range	Somatic Coliphages (log_{10} PFU/100g) range
Total	200	4.1 - 9.4	0.3 - 5.7	<0.7 - 2.8	<0.7 - 3.8	<-1.4 - 4.18	<-1.4 - 3.33
Restaurant Format							
Locally Owned	100	5.1 - 9.4	0.3 - 5.6	0 - 2.12	0 - 3.8	0 - 4.18	0 - 1.38
Corporate	100	4.1 - 9.1	1.8 - 5.7	0 - 2.8	0 - 3.5	0 - 2.20	0 - 3.33
Salad Type							
Specialty Salad	100	5.2 - 9.4	1.3 - 5.6	0 - 2.3	0 - 3.7	0 - 4.18	0 - 3.33
Leafy Greens Salad	100	4.1 - 8.5	0.3 - 5.7	0 - 2.8	0 - 3.8	0 - 2.82	<1
Business Volume							
High Volume	100	4.1 - 9.15	1.6 - 5.7	0 - 2.70	0 - 3.8	0 - 4.07	0 - 2.43
Low Volume	100	4.1 - 9.4	0.3 - 5.6	0 - 2.8	0 - 3.7	0 - 4.18	0 - 3.33

II.3.3 Presence of Indicator Organisms According to Restaurant Type

To determine whether restaurant type affected the concentration and occurrence of indicator organisms in salads, this study compared microbial indicator concentrations between LO and CO restaurant salads. Tables 2-3 and 2-4 show the log mean and the percentage of occurrence of the microbial indicators present in the salads from both restaurant types. The results of the two-way ANOVA analysis showed that salads purchased from LO restaurants had significantly higher ($P = <0.05$) concentrations of APC, *Enterococcus,* and male-specific coliphages than salads purchased from CO restaurants. The presence of total coliforms and somatic coliphages were found to be significantly higher ($P = <0.05$) in CO restaurant salads than salads purchased from LO restaurants. No differences were found for the levels of *E. coli* between the different types of restaurants.

Since some indicator organisms were found to be present less often and in lower numbers a *z* test was performed to determine the significance of the presence/absence of *E. coli*, *Enterococcus*, and male-specific and somatic coliphages. No differences were found for the levels of *E. coli* and male-specific between the different types of restaurants. This analysis did find that *Enterococcus* was present significantly higher ($P = <0.05$) in LO restaurants salads which supports the two-way ANOVA analysis results. Based on presence/absence this test did find that somatic coliphages were present significantly higher ($P = <0.05$) in CO restaurants than LO restaurants.

Table 2-3 APC and total coliform concentrations in restaurant salads

		APC		Total Coliforms	
	n	log CFU/g	Ocr. (%)	log MPN/g	Ocr. (%)
Total	*200*		(100)		(100)
Restaurant Format					
Locally owned	*100*	7.29 ± 0.10[a]	(100)	3.60 ± 0.12	(100)
Corporate	*100*	6.47 ± 0.10	(100)	3.93 ± 0.09[a]	(100)
Salad Type					
Specialty Salad	*100*	7.45 ± 0.09[b]	(100)	4.05 ± 0.09[b]	(100)
Leafy Greens Salad	*100*	6.30 ± 0.09	(100)	3.49 ± 0.11	(100)
Business Volume					
High Volume	*100*	6.85 ± 0.11	(100)	3.87 ± 0.10	(100)
Low Volume	*100*	6.91 ± 0.10	(100)	3.67 ± 0.11	(100)

[a] $P < 0.05$ corporate restaurants compared to locally owned restaurants (2-way ANOVA)

[b] $P < 0.05$ specialty salads compared to leafy greens salads (2-way ANOVA)

Table 2-4 Fecal indicator concentrations in restaurant salads

		E. coli		*Enterococci*		Male-specific coliphages		Somatic coliphages	
	n	log MPN/g	Ocr. (%)	log MPN/g	Ocr. (%)	log PFU/100g	Ocr. (%)	log PFU/100g	Ocr. (%)
Total	*200*		(19)		(78.5)		(20)		(22.5)
Restaurant Format									
Locally owned	*100*	0.07 ± 0.05	(18)	1.53 ± 0.12[a,b]	(92)	0.36 ± 0.10[a]	(23)	0.06 ± 0.03	(14)
Corporate	*100*	0.18 ± 0.06	(20)	0.79 ± 0.10	(65)	0.09 ± 0.04	(17)	0.27 ± 0.07[b]	(31)
Salad Type									
Specialty Salad	*100*	0.2 ± 0.06[c,d]	(29)	1.70 ± 0.11[c,d]	(91)	0.34 ± 0.09[c,d]	(33)	0.33 ± 0.07[c,d]	(40)
Leafy Greens Salad	*100*	0.05 ± 0.04	(9)	0.62 ± 0.10	(66)	0.11 ± 0.05	(7)	0.00 ± 0.00	(5)
Business Volume									
High Volume	*100*	0.06 ± 0.04	(14)	1.09 ± 0.12	(72)	0.21 ± 0.07	(17)	0.17 ± 0.05	(19)
Low Volume	*100*	0.19 ± 0.07	(24)	1.23 ± 0.12	(85)	0.24 ± 0.08	(23)	0.16 ± 0.05	(26)

a P< 0.05 corporate restaurants compared to locally owned restaurants (2-way ANOVA)

b P<0.05 corporate restaurants compared to locally owned restaurants (z test)

c P<0.05 specialty salads compared to leafy greens salads (2-way ANOVA)

d P<0.05 specialty salads compared to leafy greens salads (z test)

To further analyze the trends found, two-way ANOVA post hoc analysis compared several sub-categories within locally owned and corporate owned restaurants as seen in figures 2-1, 2-2, and 2.3. Overall list of sub-categorical comparisons for restaurant type, salad type, and business volume can be seen in Table 2-5. Figure 2.1 shows that levels of APC and *Enterococcus* (P value <0.05) for all comparisons made were significantly higher in LO restaurants. Figure 2.3 shows that specialty salads from LO restaurants had higher levels of male-specific coliphages (P value <0.05) than specialty salads from CO restaurants, and salads from LO restaurants obtained during low customer traffic periods had higher levels of male-specific coliphages (P value <0.05) than salad from CO restaurants obtained during this same customer traffic period. Figure 2-2 and 2-3 shows that the levels of total coliforms and somatic coliphages purchased from CO restaurants during low customer traffic periods were significantly higher (P value <0.05) than salads purchased from LO restaurants during low customer traffic periods, and leafy greens salads purchased from CO restaurants also had significantly higher coliform counts (P value <0.05) than leafy greens salads purchased from LO restaurants. Figure 2-3 shows that specialty salads from CO restaurants had significantly higher levels of somatic coliphages (P value <0.05) than LO restaurants specialty salads. There were no differences observed for the levels of *E. coli* within any of the sub-categories. Further *z* test analysis also found that the number of samples positive for *Enterococcus* were significantly higher (P value <0.05) in salads from LO restaurant than salads from CO restaurants. The number of samples positive for somatic coliphages was found to be significantly higher (P value <0.05) in salads from CO restaurants purchased during low volumes of customer traffic.

Table 2-5 List of sub-categorical comparisons made between salad type, restaurant ownership format, and business volume

Specialty Salad vs Leafy Greens Salads	Corporate Restaurants vs Locally-Owned Restaurants	High Customer Volumes vs Low Customer Volumes
CO-SS vs CO-LGS*	CO-SS vs LO-SS	SS-LV vs SS-HV
LO-SS vs LO-LGS	CO-LGS vs LO-LGS	LGS-LV vs LGS-HV
SS-LV vs LGS-LV	CO-LV vs LO-LV	CO-LV vs CO-HV
SS-HV vs LGS-HV	CO-HV vs LO-LV	LO-LV vs LO-HV

* CO - corporate restaurant; LO - locally owned restaurant; SS - specialty salad; LGS leafy greens salad; HV - high volumes of customer traffic; LV - low volumes of customer traffic

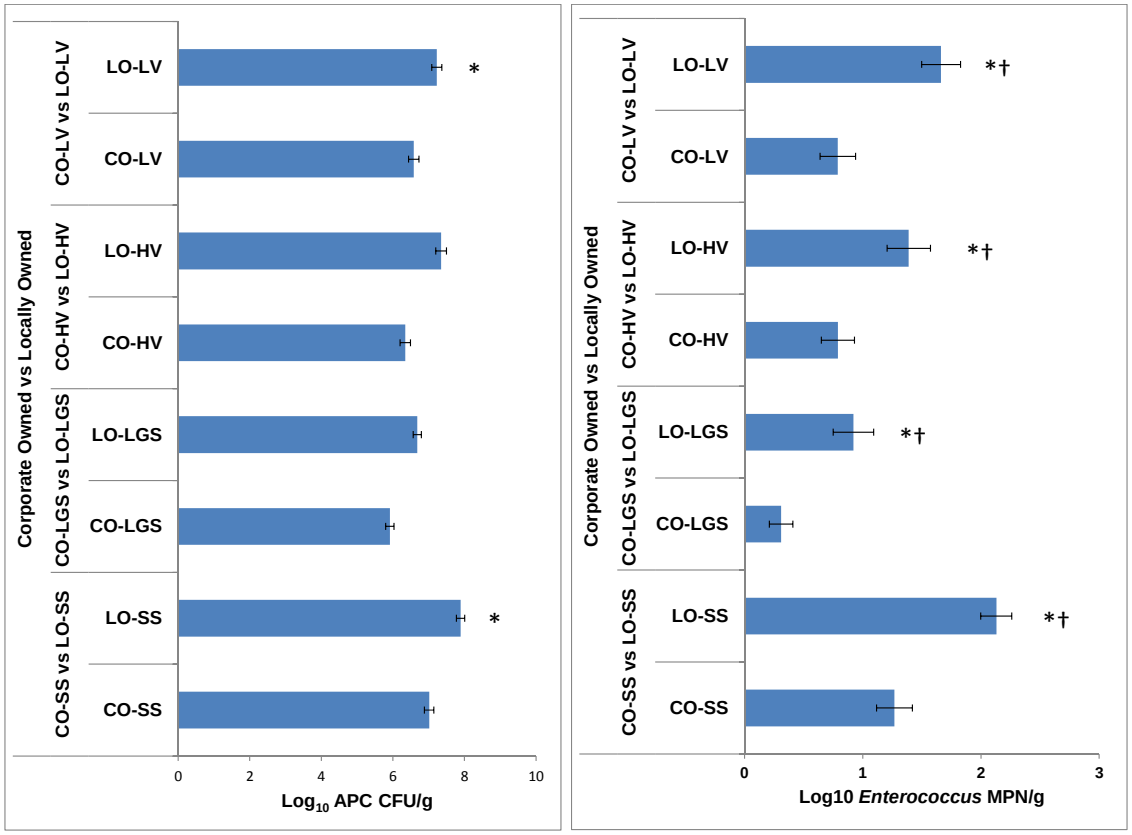

Figure 2-1 Sub-categorical comparisons of APC and *Enterococcus* from corporate and locally owned restaurant salads (CO - corporate restaurant; LO - locally owned restaurant; SS - specialty salad; LGS leafy greens salad; HV - high volumes of customer traffic; LV - low volumes of customer traffic)
* $P < 0.05$ 2-way ANOVA (Holm-Sidak method)
† $P < 0.05$ *z* test

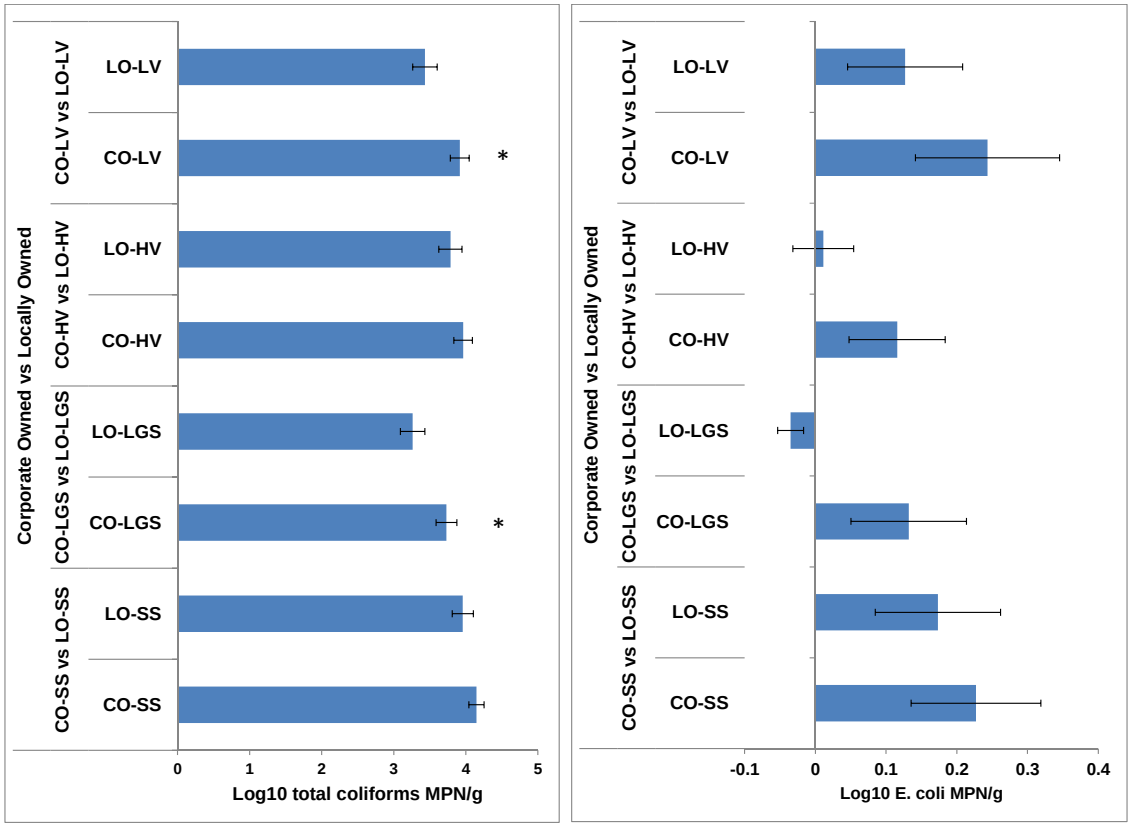

Figure 2-2 Sub-categorical comparisons of total coliforms and *E. coli* from corporate and locally owned restaurant salads (CO - corporate restaurant; LO - locally owned restaurant; SS - specialty salad; LGS leafy greens salad; HV - high volumes of customer traffic; LV - low volumes of customer traffic)
* $P < 0.05$ 2-way ANOVA (Holm-Sidak method)
† $P < 0.05$ *z* test

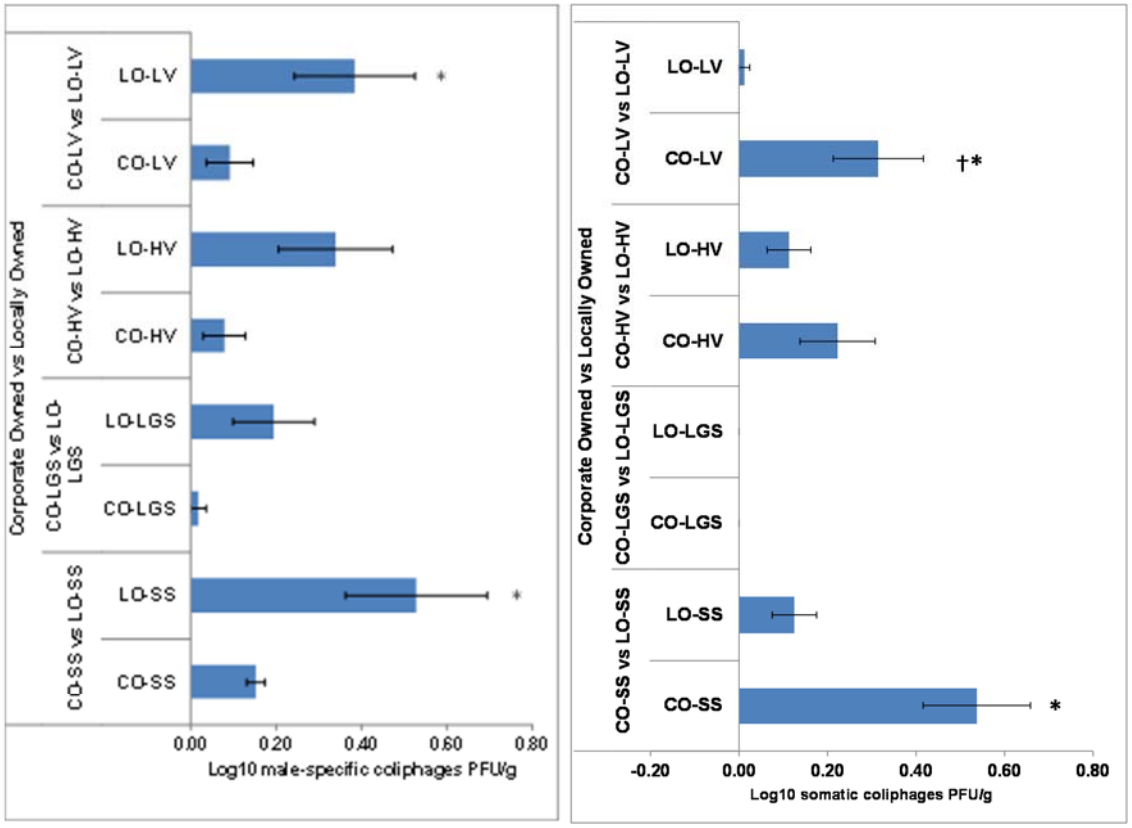

Figure 2-3 Sub-categorical comparisons of coliphages from corporate and locally owned restaurant salads (CO - corporate restaurant; LO - locally owned restaurant; SS - specialty salad; LGS leafy greens salad; HV - high volumes of customer traffic; LV - low volumes of customer traffic)

* $P < 0.05$ 2-way ANOVA (Holm-Sidak method)

† $P < 0.05$ *z* test

II.3.4 Presence of Indicator Organisms According to Salad type

To determine whether salad type affected the concentration and occurrence of indicator organisms in salads, this study compared microbial indicator concentrations between leafy greens salads and specialty salads. Tables 2-3 and 2-4 show the geometric mean and the percentage of occurrence of the microbial indicators present in both specialty and leafy greens salads. The results of the two-way ANOVA analysis showed that specialty salads had significantly higher (P = <0.05) concentrations of APC, total coliforms, *E. coli*, *Enterococcus*, and male-specific and somatic coliphages than leafy greens salads. The *z* test analysis results for *E. coli*, *Enterococcus*, and male-specific and somatic coliphages revealed that the number of samples positive for these indicator organisms were significantly higher (P value <0.05) for specialty salads than leafy greens salads.

To further analyze the trends found, two-way ANOVA post hoc analysis compared several sub-categories within specialty salads and leafy greens salads as seen in figures 2-4, 2-5, and 2-6. APC, total coliforms, *Enterococcus*, and somatic coliphage counts were all found to be significantly higher (P value <0.05) in specialty salads for all comparisons made. Figure 2-5 shows that levels of *E. coli* were found to be significantly higher (P value <0.05) in specialty salads purchased during low volumes of customer traffic than leafy greens salads purchased during the same business volume period. Figure 2-6 shows that male-specific coliphages were found to be significantly higher in specialty salads purchased from corporate restaurants than corporate restaurant leafy greens salads, and LO specialty salads were found to have significantly higher levels (P value <0.05) of male-specific coliphages than LO leafy greens salads. Tables 2-3 and 2-4 also show the results for the z test analysis comparisons of the samples positive for *E. coli*, *Enterococcus*, and male-specific and somatic coliphages. Samples positive for *E. coli*, *Enterococcus*, and male-specific and somatic coliphages were significantly higher (P value <0.05) in specialty salads than leafy greens salads for all comparisons made.

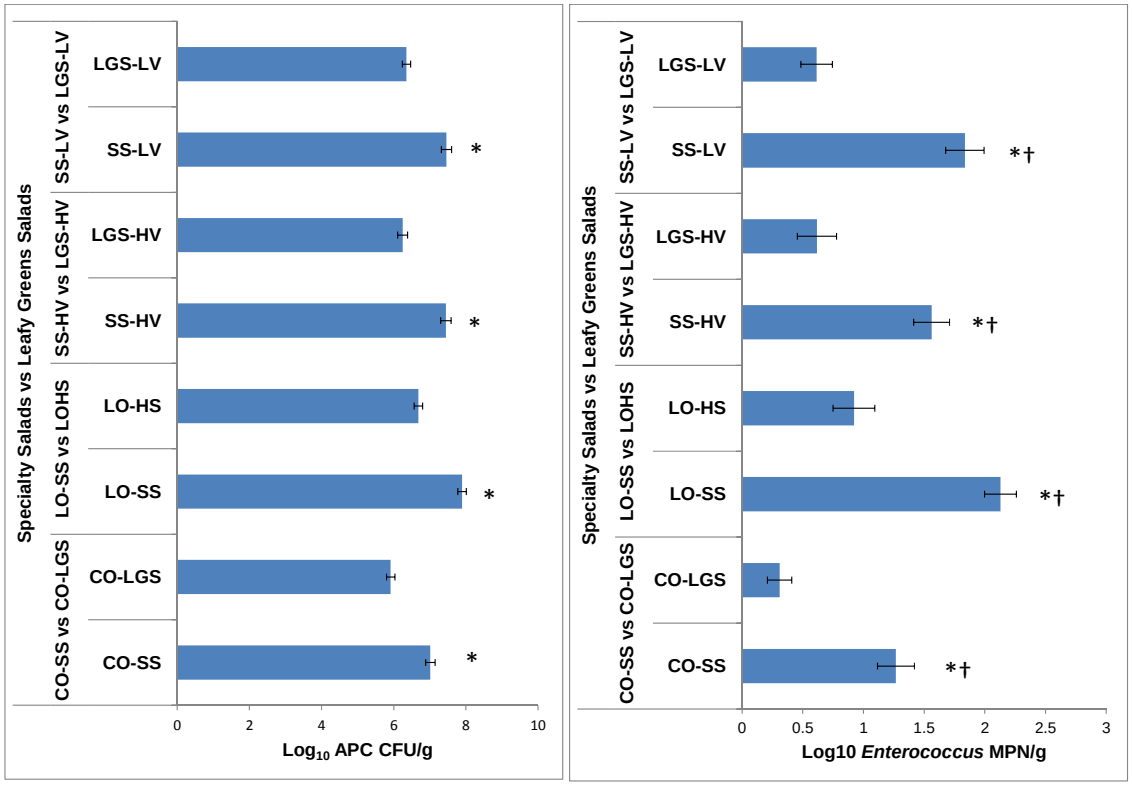

Figure 2-4 Sub-categorical comparisons of APC and *Enterococcus* from specialty salads and leafy greens salads (CO - corporate restaurant; LO - locally owned restaurant; SS - specialty salad; LGS leafy greens salad; HV - high volumes of customer traffic; LV - low volumes of customer traffic)
* $P < 0.05$ 2-way ANOVA (Holm-Sidak method)
† $P < 0.05$ *z* test

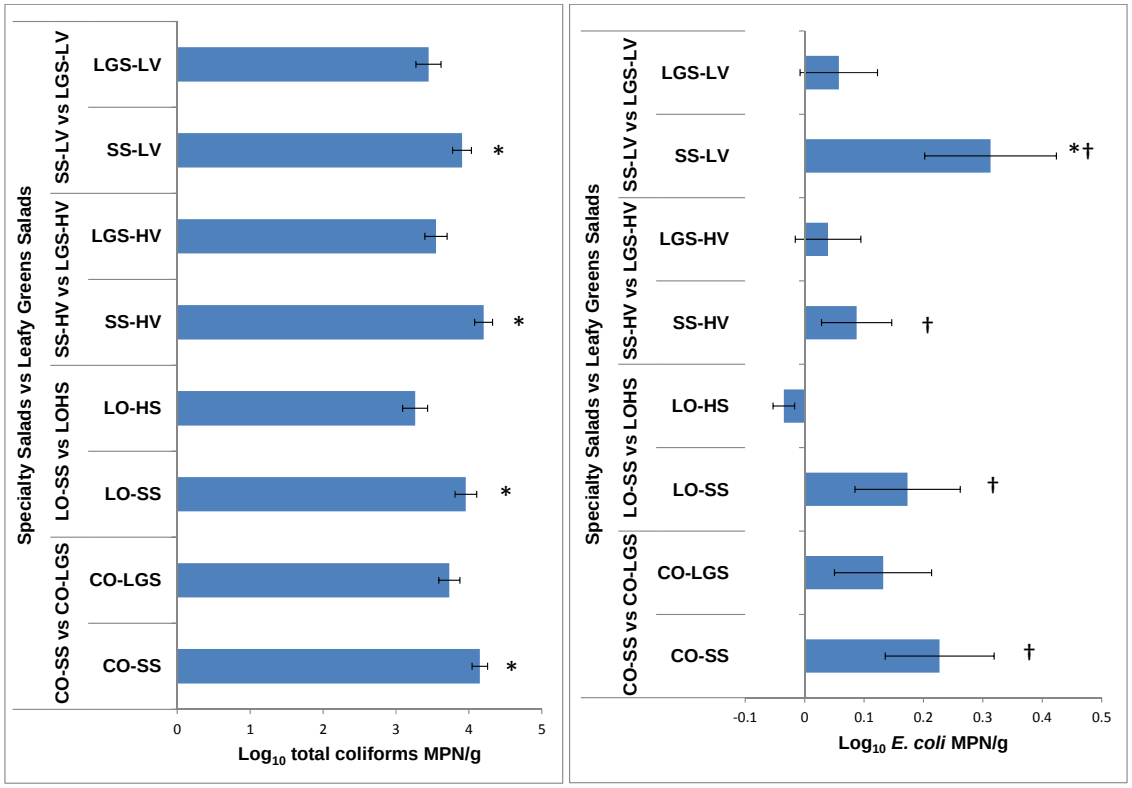

Figure 2-5 Sub-categorical comparisons of total coliforms and *E. coli* from specialty salads and leafy greens salads (CO - corporate restaurant; LO - locally owned restaurant; SS - specialty salad; LGS leafy greens salad; HV - high volumes of customer traffic; LV - low volumes of customer traffic)
* $P < 0.05$ 2-way ANOVA (Holm-Sidak method)
† $P < 0.05$ *z* test

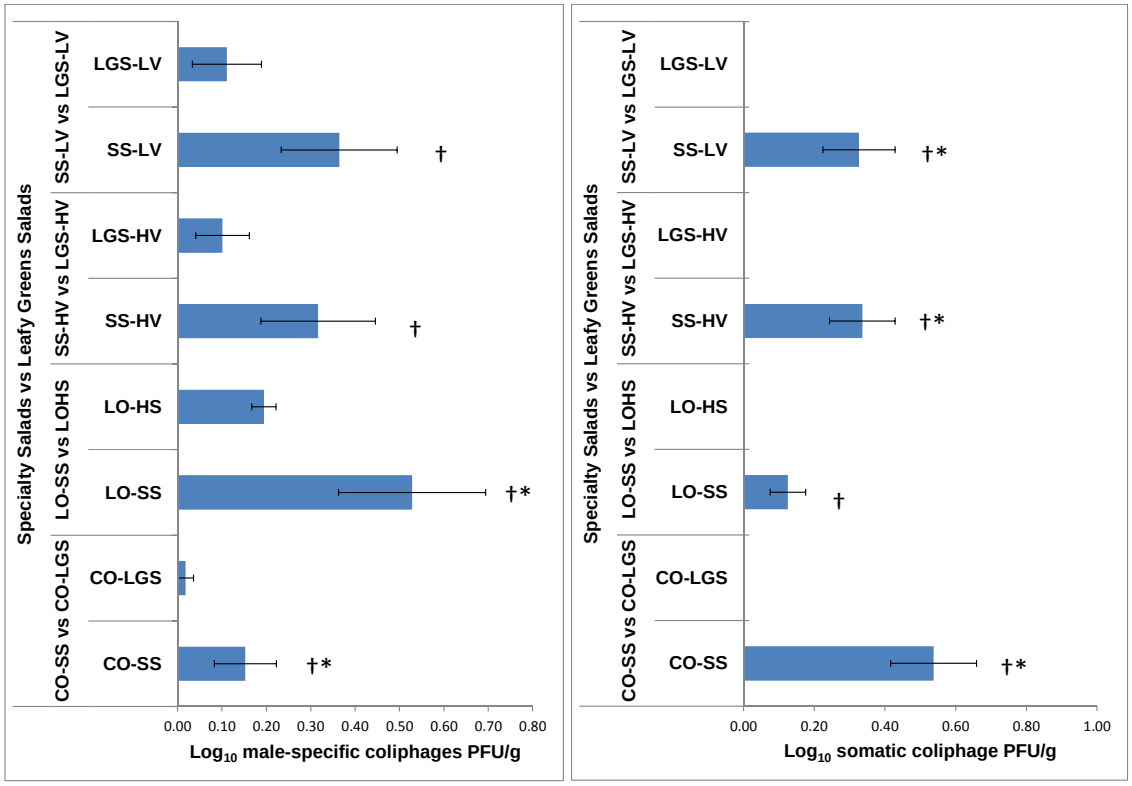

Figure 2-6 Sub-categorical comparisons of coliphages from specialty salads and leafy greens salads (CO - corporate restaurant; LO - locally owned restaurant; SS - specialty salad; LGS leafy greens salad; HV - high volumes of customer traffic; LV - low volumes of customer traffic)
* $P < 0.05$ 2-way ANOVA (Holm-Sidak method)
† $P < 0.05$ *z* test

II.3.5 Presence of Indicator Organisms According to Business Volume

To determine whether business volume affected the concentration and occurrence of indicator organisms in salads, this study compared microbial indicator concentrations between salads purchased during low volumes of customer traffic and high volumes of customer traffic. Tables 2-3 and 2-4 show the geometric mean and the percentage of occurrence of the microbial indicators present in both specialty and leafy greens salads. The results of the two-way ANOVA analysis found that there was no significant differences observed for the concentrations of APC, total coliforms, *E. coli*, *Enterococcus*, and male-specific and somatic coliphages for salads purchased during different volumes of customer traffic. The *z* test analysis results for *E. coli*, *Enterococcus*, and male-specific and somatic coliphages also found that there was no statistical correlation for the number of samples positive for these indicator organisms found in the salads. Furthermore, the two-way ANOVA post hoc test, which can be viewed in figures 2-7, 2-8, and 2-9, revealed only one significant difference. Figure 2-8 shows that the levels of *E. coli* for specialty salads purchased during low volumes of customer traffic periods were found to be significantly higher (P value <0.05) than specialty salads purchased during high volumes of customer traffic periods. *Z* test analysis also did not find any differences in the number of samples positive for *E. coli*, *Enterococcus*, and male-specific and somatic coliphages for any sub-categorical comparisons made for salads purchased during different volumes of customer traffic.

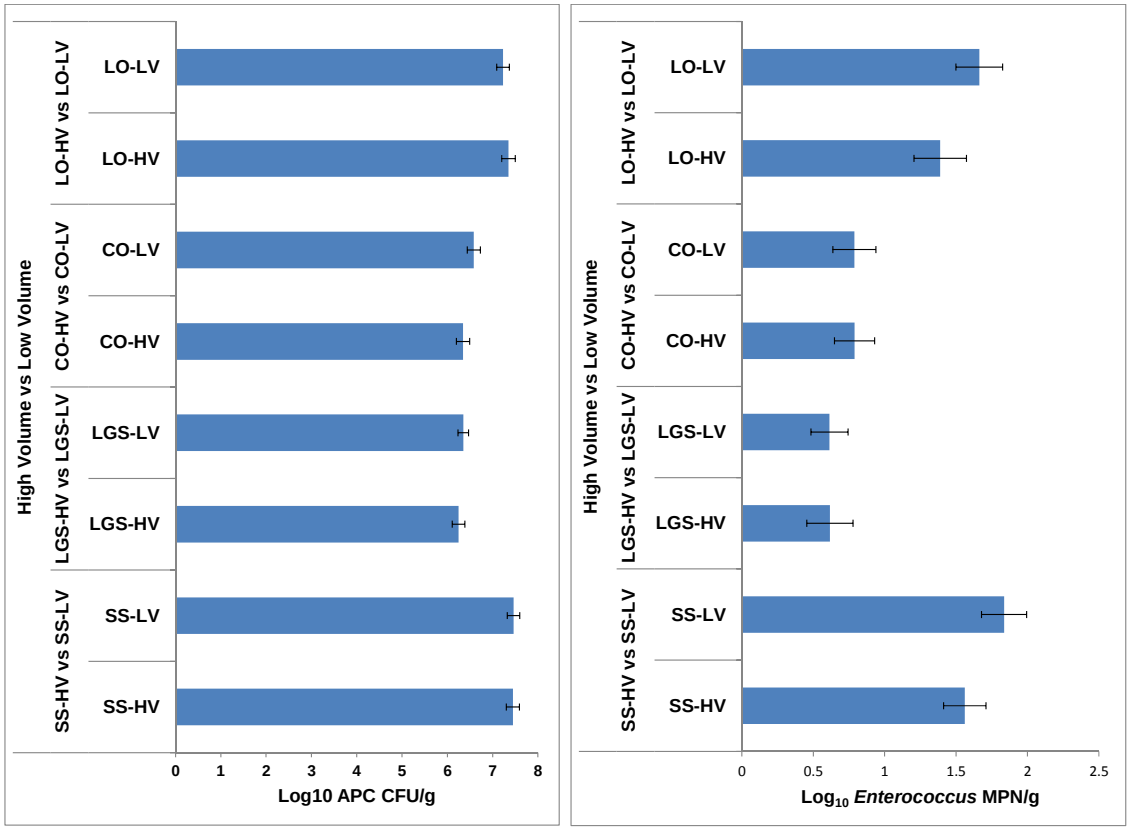

Figure 2-7 Sub-categorical comparisons of APC and *Enterococcus* from salads collected during high and low customer traffic volumes (CO - corporate restaurant; LO - locally owned restaurant; SS - specialty salad; LGS leafy greens salad; HV - high volumes of customer traffic; LV - low volumes of customer traffic)

* $P < 0.05$ 2-way ANOVA (Holm-Sidak method)

† $P < 0.05$ *z* test

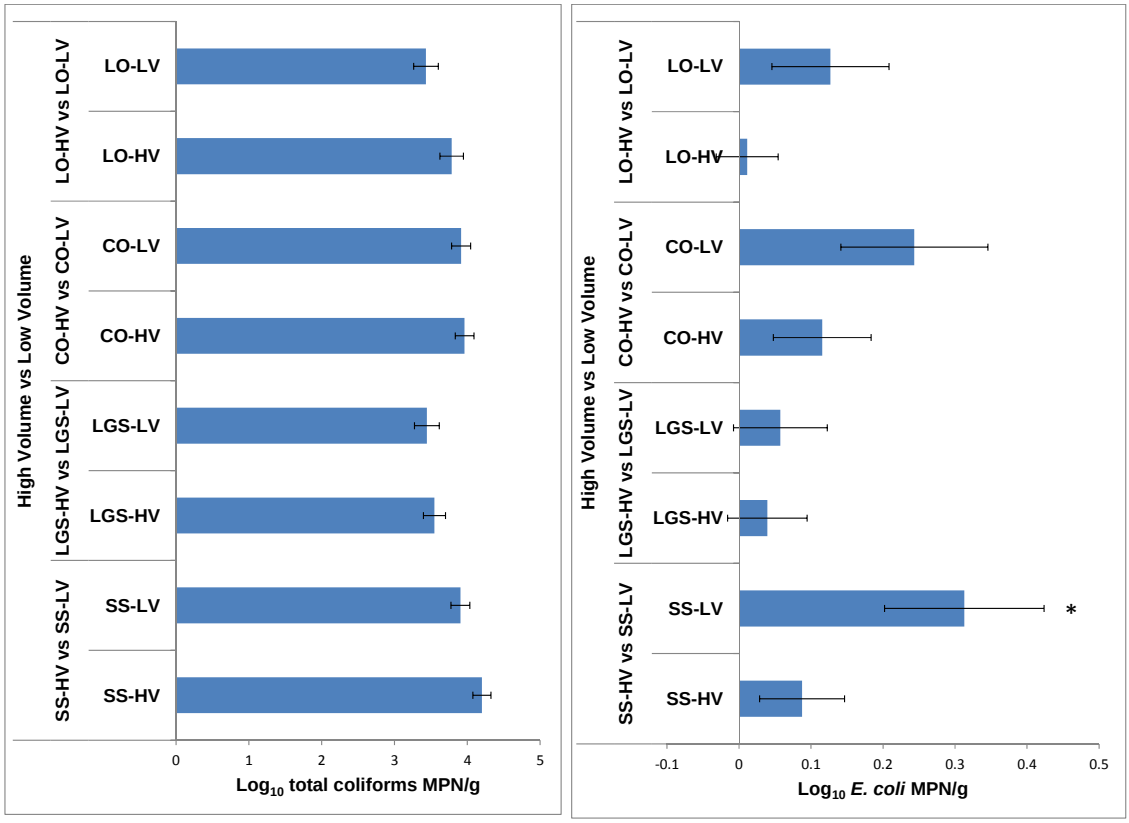

Figure 2-8 Sub-categorical comparisons of total coliforms and *E. coli* from salads collected during high and low customer traffic volumes (CO - corporate restaurant; LO - locally owned restaurant; SS - specialty salad; LGS leafy greens salad; HV - high volumes of customer traffic; LV - low volumes of customer traffic)

* $P < 0.05$ 2-way ANOVA (Holm-Sidak method)

† $P < 0.05$ *z* test

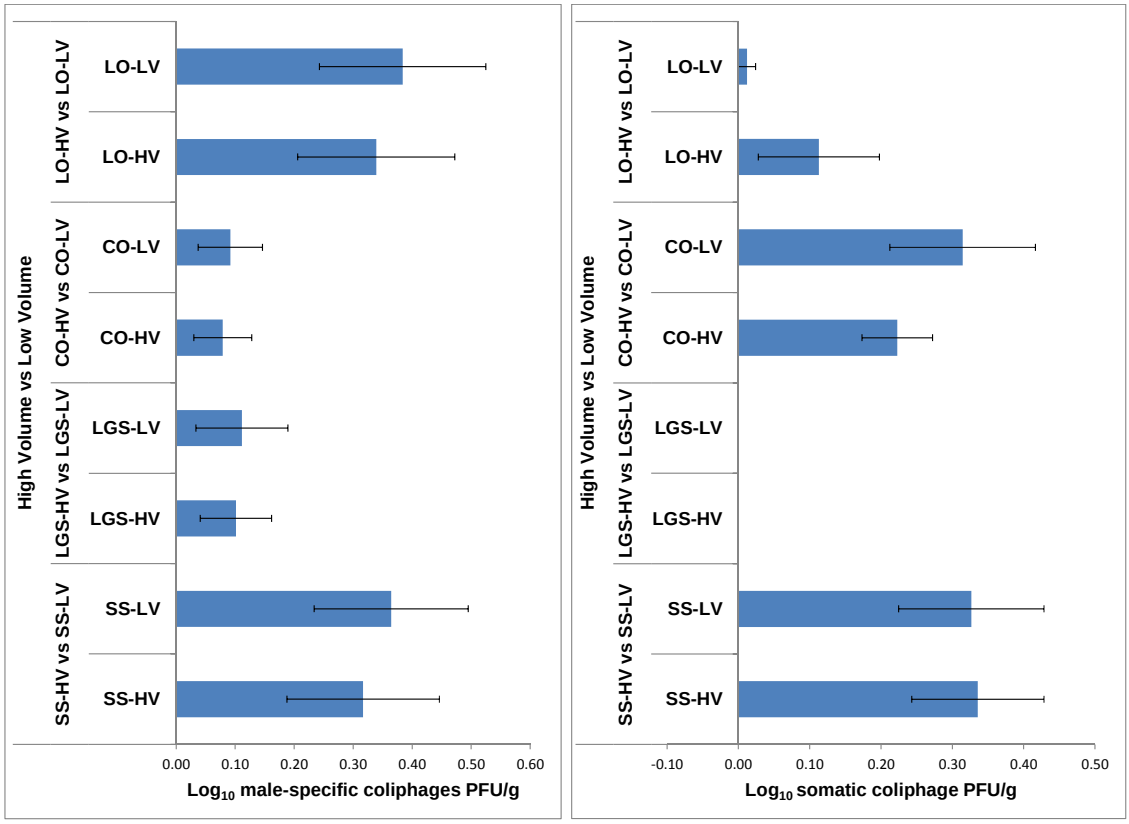

Figure 2-9 Sub-categorical comparisons of coliphages from salads collected during high and low customer traffic volumes (CO - corporate restaurant; LO - locally owned restaurant; SS - specialty salad; LGS leafy greens salad; HV - high volumes of customer traffic; LV - low volumes of customer traffic)
* $P < 0.05$ 2-way ANOVA (Holm-Sidak method)
† $P < 0.05$ *z* test

Overall the results show that salad type does influence the concentration of indicator organisms present in restaurant purchased salads. Restaurant type was shown to influence the concentration of indicator organisms in restaurant salads. Significantly higher levels of APC, *Enterococcus,* and male-specific coliphages were found in salads purchased from LO restaurants. In contrast, the overall levels of total coliforms and somatic coliphages were found to be higher in salads purchased from CO restaurants. Statistical analysis for business volumes and for the effect it had on the concentrations of indicator organisms present in restaurant salads found only one statistically significant difference. The levels of *E. coli* were found to be significantly higher in specialty salads purchased during low customer traffic volumes than specialty salads purchased during high volumes of customer traffic. There were other sub-categorical comparisons observed within salad type and restaurant type that suggests that salads purchased during low volumes of customer traffic had significantly higher levels of indicator organisms. Salad type sub-categorical comparisons found that specialty salads purchased during low volumes of customer traffic had significantly higher levels of *E. coli* than leafy greens salads purchased during low volumes of customer traffic Restaurant type sub-categorical comparisons found that salads purchased from CO restaurants during low volumes of customer traffic had significantly higher levels of total coliforms and somatic coliphages than salads purchased from LO restaurants during low volumes of customer traffic. Salads purchased from LO restaurants during low volumes of customer traffic were found to have significantly higher levels of male-specific coliphages than salads purchased from CO restaurants during low volumes of customer traffic. Sub-categorical comparisons within salad type, restaurant type, and business volume didn't find any statistically significant differences for the levels of E. coli, male-specific coliphages, and somatic coliphages in salads purchased during high volumes of customer traffic.

II.4 Discussion

II.4.1 Overview of the Microbiological Quality of the Restaurant Purchased Salads

There is a growing interest in the microbiological quality and safety of fresh produce throughout the fresh produce processing chain. Monitoring fresh produce for indicator organisms allows researchers and the food industry to identify what steps throughout the fresh produce processing chain impact the level of microorganisms on raw fruits and vegetables, determine the efficacy of disinfectant processes, and can also identify possible routes of pathogen contamination *(1, 2, 11, 12)*. There have been several studies on the microbial quality of fresh produce, but there have been very few studies in regards to the microbial quality of RTE leafy greens and fresh produce entrees purchased from full-service restaurants in the United States. This study found that both leafy greens salads and specialty salads purchased from locally owned and corporate restaurants had detectable levels of APC, total coliforms, *E. coli*, *Enterococcus*, male-specific coliphages, and somatic coliphages. The mean total counts and ranges of the bacterial indicator organisms present in the leafy greens salads purchased from the restaurants in this study were consistent with what other others have reported *(1-4, 8, 77, 86, 114, 141, 154)*. This was expected because restaurants use both prepackaged, prewashed leafy greens and whole unprocessed produce which are washed and minimally processed "in-house".

There are very few studies that have determined the presence of coliphages on fresh produce, and at present there has been very few to report the levels of coliphages found on produce obtained from restaurant establishments in the United States. Hirotani et al. *(77)* reported that vegetables from the United States and Mexico had levels of coliphages that from 0 to approximately 3 logs PFU/g. Allwood et al. *(7)* reported that male-specific coliphages were present in 47% of preprocessed and 19% of unprocessed samples of produce obtained from retail outlets and restaurants. Endley et al. *(51)* reported that 50% of cilantro samples 39% of parsley samples purchased at retail locations were positive for male specific coliphages with a range of 1 to 11 PFU/10g.

Endley et al. *(52)* reported that 25% of carrot samples obtained from the farm, truck, and processing shed were positive for male-specific coliphages. No published studies could be found that reported the presence or level of somatic coliphages on produce. This study found that the enrichment based assay was more sensitive for the detection of coliphages than the quantitative assay. Male-specific coliphages were present in 20% of the salad samples and of those only 50% tested positive by quantitative analysis. Male-specific coliphages found in the salad samples ranged between <1 to 4.18 $\log_{10}$ PFU/100g. Somatic coliphages were present in 22.5% of the salad samples and of those only 23% tested positive by quantitative analysis. The range of somatic coliphages found in the salad samples were <1 to 3.33 $\log_{10}$ PFU/100g.

II.4.2 Microbiological Quality of Salads Determined by Restaurant Ownership Type

The focus of this study was to determine the effect that restaurant ownership format, salad type, and business volume had on the levels of indicator organisms present in restaurant salads. The level of indicator organisms in these salads were found to be influenced by restaurant ownership type although both LO and CO restaurant salads were found to have differing levels of indicator organisms. The results of this study found that LO restaurant salads had significantly higher levels of APC, *Enterococcus,* and male-specific coliphages. Specialty salads from LO restaurants also had significantly higher levels of male-specific coliphages than specialty salads purchased from CO restaurants. Salads from CO restaurants had significantly higher levels of coliforms and somatic coliphages than salads from LO restaurants, and leafy greens salads from CO restaurants were also found to have higher levels of coliforms in salads from LO restaurants. The finding that both restaurants had statistically significant differences in the levels of indicator organisms in their salads suggest that there are difference in produce handling and processing practice between these two full-service restaurant ownership types.

Based on self-reported practices from restaurant managers and visual observations of tomato handling practices Kirkland et al *(90)* found that restaurants

process their produce differently and that food workers in restaurants do not allows follow the recommended safe food handling practices. Most restaurants develop their own food safety programs based upon the FDA Food Code and on state and local laws and regulations. It was not required at the time of this survey that restaurants have written food safety protocols or plans in place for processing fresh produce and mainly relied upon the guidance of the person in charge or corporate mandates to inform them of produce practices for that particular restaurant. Based on personal knowledge and working experience in the restaurant industry as well as personal communications with other employees and managers employed in the restaurant industry here in the Brazos Valley it was found that produce processing is different for each restaurant and that the recommended food safety practices in the Food Code are not always followed. Some examples of what was these communications found where that some restaurants soaked items in ice water baths before processing, and even soak fresh cut produce in ice water baths after processing which is in contrast to what the FDA Food Code recommends. It was also found that more managers of corporate restaurants reported using chemical washes to wash their produce than managers working for locally owned restaurants.

The use or non-use of chemical washes could explain the differences in the levels of indicators organisms found in LO and CO restaurant salads. LO restaurant salads were found to have significantly higher levels of APC and *Enterococcus*. This could suggest that CO restaurants employ produce washing procedures which are more effective at reducing the levels of microorganisms on fresh produce. The current minimum requirement for washing fresh produce in the Brazos valley follows the recommendation of the FDA Food Code that fresh produce should be washed with water to remove soil and contaminants before processing *(148)*. Washing fresh produce with water and sanitizers removes soil and debris, and can reduce and inactivate a number of microorganisms on fresh produce which are responsible for food spoilage and foodborne illness. Washing fresh produce with tap water or with sanitizers have been found to reduce microbial populations by 2 to 3 log units, but microorganisms such as viruses

have been found to be more resistant to disinfection than bacteria *(61)*. Washing with water can remove soil, debris and other contaminates, but is less efficient at inactivating microorganisms present in the wash water or on fresh produce which can allow for the transfer of pathogenic microorganisms from contaminated products to clean products. Washing with sanitizers helps to maintain the quality of the water used to wash fresh produce and based upon the chemical agent and the concentration at which it is applied determines the efficiency of the sanitizing agent's ability to kill or inactivate microorganisms. The use of sanitizing agents are more useful for preventing cross-contamination and when used appropriately can result in a greater microbial reduction than washing with water alone *(61)*.

Although this study found that the levels of total coliforms were significantly higher in CO restaurant salads this could be due to the regrowth of this microorganism after washing. This could be caused by ineffective management of the cold chain in CO restaurants which allowed these microorganisms to proliferate while in storage. Comparisons made between CO leafy greens salads and LO leafy greens salads found that levels of total coliforms were significantly higher in CO leafy greens salads, and no statistically significant differences were observed for the levels of total coliforms between LO and CO specialty salads. Specialty salads usually undergo more processing and handling than leafy greens salads. These results suggest that since there was no statistically significant differences observed for the levels of total coliforms in specialty salads handling and processing may not be a factor for the levels of total coliforms seen in CO salads and in particular CO leafy greens salads. Based on the study by Rediers et al. *(114)* the higher levels of coliforms in these salads could be due to not maintaining the cold-chain in CO restaurants or failing to store foods at the correct temperature.

Rediers et al. *(114)* monitored the cold chain management for fresh-cut endive from the field through delivery and storage in 3 restaurants. This study found that fresh-cut endive stored in restaurants when compared to fresh-cut endive that had been stored under laboratory conditions at 4°C had significantly higher coliform counts. Rediers et

al. *(114)* reported that levels of APC, coliforms, and *Enterobacteriaceae* decreased slightly after delivery to the restaurant and attributed this occurrence to the produce undergoing a produce wash during processing. While APC counts did increase almost 1 log during storage for 7 days at 4°C in these restaurants the differences between the counts observed from the fresh-cut endive stored under laboratory conditions were negligible. Overall the levels observed for both coliforms and *Enterobacteriaceae* counts were higher in fresh-cut endive stored in the restaurants but coliforms counts had increased to 1.1 to 1.6 log CFU/g higher than the fresh-cut endive held under laboratory conditions. It was concluded that the temperature fluctuations impacted the growth of the coliforms on the endive stored in the restaurants because the refrigeration units in which the fresh-cut endive was stored had to be accessed several times throughout the daily operations causing fluctuations in the temperature.

Aerobic plate counts are used as food quality and sanitation indicators, and *Enterococcus* spp. are used as a fecal indicator and more recently has been suggested as a good indicator for the microbiological quality of fresh produce *(2, 84, 86)*. The higher levels of APC and *Enterococcus* observed in salad samples from LO restaurants could also be due to the use of produce that is of poor microbiological quality. Factors which could affect the microbiological quality of fresh produce being served in LO restaurants can include using produce that is past the recommended shelf life, using raw produce and RTE food items that have been exposed to contamination by coming into contact with improperly cleaned and sanitized food contact surfaces, or as a result of unhygienic practices by food workers. In approximately two-thirds of the LO restaurants there was no difference between business volumes with the exception of Friday and Saturday nights. It could go to reason that a decrease in customer traffic flow could lead to the use of less produce in LO restaurants, and based on other studies LO restaurants operate on a smaller budget in which controllable cost play a role

Sensory panels have evaluated bagged, fresh cut leafy greens and determined that bagged, fresh cut leafy greens show little difference in appearance, look visually

acceptable, and can last a week or more after the "sell by" or "use by" date *(154)*. It has been reported that the levels of bacterial indicator organisms such as APC can increase during storage albeit at a reduced growth rate if the cold chain is maintained *(154)*. In order to control cost LO owned restaurants may have used visually acceptable produce which was past the recommended shelf life for the preparation of these salads. This practice potentially could have contributed to the higher levels of APC found in the LO restaurant leafy greens salads.

Other risky food handling practices such as not controlling moisture levels have also been shown to increase the level of APC counts for bagged, fresh cut leafy greens *(154)*. An excess of moisture on produce was observed during a sampling period at one LO restaurant. Iceberg lettuce that had been stored in a storage container had visibly dripping water coming off of it before it was put into a to-go container. Higher levels of APC and *Enterococcus* in LO salads could be a result of failing to reduce the water activity of the produce used in these restaurant salads. Lowering the water activity of food can slow or inhibit bacterial growth rates *(116)*.

Perhaps the most revealing indicator organisms observed in this study were male-specific and somatic coliphages in relation to being able to ascertain certain areas of increased risk in which food handling and processing practices affect the microbiological quality of fresh produce the most. This study found that CO restaurant salads overall, CO restaurant salads purchased during low customer traffic volumes, and CO specialty salads had significantly higher levels of somatic coliphages. LO restaurant salads overall, LO restaurant salads purchased during low customer traffic volumes, and LO restaurant specialty salads had significantly higher levels of male-specific coliphages. Since these trends were observed in salads purchased during low volumes of customer traffic and in specialty salads it suggests that the microbiological quality of these salads were affected by staffing issues during low customer traffic volumes. A lack of time can be a result of not having enough food workers present to handle the work load within a

restaurant which can increase the likelihood of risky food handling practices by food workers during processing and preparation.

It is common practice in full-service restaurants to decrease the amount of staff during low volume customer traffic periods or to reassign food workers responsible for cooking and preparing food for immediate delivery to the customer to help with preparations for the next shift or up-coming events. A decrease in labor or reassignment of labor leaves fewer food workers responsible for the preparation of the in-coming food orders during low volume customer traffic time periods. In order to prevent cross-contamination of food in the kitchen while preparing an order during a lunch or dinner shift food workers are usually designated a particular station in which they are responsible for grilling type operations, salad and sandwich preparation, or desserts. This helps to increase work productivity and decreases the potential for cross-contamination to occur. In between the lunch and dinner shifts only one or two food workers may be present to prepare food orders as they come in. If one person is working the line in the kitchen while preparing orders it will be more difficult to take kitchen utensils to be cleaned, properly clean and sanitize food contact surfaces, properly wash hands, and change gloves. Studies have shown that contaminated food contact surface spiked with enteric viruses such as male-specific coliphages can effectively transfer and contaminate fresh produce *(5)*, and that processed produce have higher levels of coliphage contamination than unprocessed fresh produce *(7, 52)*. Since there are several types of raw produce, RTE food items and meats involved in preparing a restaurant specialty salad these food items can undergo extensive amounts of handling, processing, and come into contact with several food preparation surfaces before consumption. Food contact surfaces can become exposed to coliphages contamination by coming into contact non-potable water, sewage or waste water, raw meats, and other contaminated food items *(81, 100, 109)*.

Studies have found that in some cases regardless of whether or not fresh produce has been washed with tap water or sanitizers the levels of bacteria present on fresh

produce after processing and storage are similar *(61)*. Microorganisms such as viruses have been found to be more resistant to disinfecting wash treatments than bacteria on produce, but unlike bacteria most enteric viruses cannot replicate outside the gastrointestinal tracts of humans and animals. The use of sanitizers and effective washing treatments has been shown to be successful at removing low levels of these organisms from fresh produce. Casteel et al. *(20)* reported that washing fresh produce in tap water achieves a 68% (0.5 log) reduction in the levels of male-specific coliphages washing with water containing 20 to 200 ppm chlorine effectively achieved up to a 96% (1.4 log) reduction. Allwood et al. *(6)* reported that the use of bleach or commercially available sanitizers such as Tsunami10™ could achieve up to a 2.9 log reduction in titers of male-specific coliphages inoculated onto leafy salad vegetables. Legnani et al. *(95)* showed that fresh produce that has been washed, shredded and/or cut, washed with chlorinated water, and rinsed was effective for reducing male-specific coliphages up to 2.48 $\log_{10}$ PFU/10g to undetectable levels. Legnani et al. *(95)* study shows that using a series of chlorinated washes can effectively reduce low levels of coliphage contamination on fresh produce to undetectable levels. Since there were relatively low levels of coliphages reported in this study it could suggest that most of the processing practices in these restaurants were sufficient to remove low levels of coliphage contamination and therefore restaurants with higher levels of contamination is due to risky and unsafe food handling practices.

Tables 2-6 and 2-7 show which restaurants tested positive for male-specific coliphages and which samples had quantifiable counts of male-specific coliphages. In general this study found relatively low counts of male-specific coliphages present in these salads. Out of the 40 salads that tested positive for the presence of male-specific coliphages with the enrichment assay, only 20 tested positive for the presence of male-specific coliphages with the quantitative method. There were only 5 salad samples obtained from CO restaurants that tested positive with the quantitative method, and 14 salad samples that were positive with the quantitative method for locally owned

restaurants. Our results also found that LO restaurants 1 and 5 leafy greens salads and specialty salads had significantly higher levels of male-specific coliphages when compared to all CO restaurant salads and LO restaurants 2, 3, and 4 salads. Locally owned restaurants 1 and 5 were small ethnic Mexican restaurants.

Table 2-6 Corporate restaurant salads positive for male-specific coliphages and *E. coli*

Restaurant	Enrichment F+Specific	Sal F+Specific (PFU/g)	Genogroup (I,II,III,IV)	*E. coli*
Specialty Salads				
Restaurant 1				
HV.C	positive	<1	-	positive
LV.E	positive	1.54E+00	II,III	negative
HV.E	positive	1.38E+00	-	negative
Restaurant 2				
LV.B	positive	<1	-	negative
HV.B	positive	<1	-	negative
LV.C	positive	<1	-	negative
HV.C	positive	<1	-	negative
LV.D	positive	1.93E-01	-	negative
HV.D	positive	<1	-	negative
HV.E	positive	2.32E-01	-	negative
Restaurant 3				
HV.A	positive	<1	-	negative
HV.D	positive	<1	-	negative
Restaurant 4				
LV.B	positive	<1	-	positive
HV.B	positive	<1	-	positive
Restaurant 5				
LV.E	positive	1.20E-01	III	positive
Leafy Greens Salads				
Restaurant 1				
HV.B	positive	<1	-	negative
Restaurant 2				
HV.E	positive	3.48E-02	II	negative
Restaurant 3	negative	-	-	-
Restaurant 4	negative	-	-	-
Restaurant 5	negative	-	-	-

Table 2-7 Locally owned resaurant salads positive for male-specific coliphages and *E. coli*

Restaurant	Enrichment F+Specific	Sal F+Specific (PFU/g)	Genogroup (I,II,III,IV)	*E. coli*
Specialty Salads				
Restaurant 1				
LV.A	positive	<1	-	positive
HV.A	positive	7.78E-02	-	negative
HV.B	positive	<1	-	negative
LV.C	positive	6.05E-01	III	negative
HV.C	positive	2.26E-01	-	negative
LV.D	positive	1.06E+00	III	positive
LV.E	positive	5.09E+00	-	positive
HV.E	positive	5.31E+00	-	negative
Restaurant 2				
HV.C	positive	<1	-	negative
Restaurant 3			-	-
Restaurant 4				
LV.D	positive	<1	-	positive
Restaurant 5				
LV.A	positive	<1	-	negative
HV.A	positive	<1	-	positive
LV.B	positive	<1	-	positive
HV.B	positive	4.83E+01	-	positive
LV.C	positive	1.46E+02	-	negative
HV.C	positive	<1	-	negative
LV.D	positive	8.40E+00	-	negative
HV.D	positive	1.19E+02	I	positive
Leafy greens Salads				
Restaurant 1				
LV.E	positive	5.51E+00	-	negative
HV.A	positive	<1	-	negative
Restaurant 2			-	-
Restaurant 3			-	-
Restaurant 4			-	-
Restaurant 5				
LV.C	positive	6.51E+00	I	negative
HV.A	positive	9.18E-01	-	negative
HV.B	positive	1.49E+00	-	negative

Full-service restaurants have been reported to perform poorly in regards to food safety practices pertaining to the prevention of cross-contamination of food by food workers. In the FDA report on the *Occurrence of Foodborne Illness Risk Factors in Selected Institutional Foodservice, Restaurant, and Retail Food Store Facility Types* (2009) food workers failed to wash their hands appropriately or prevent hand contamination in 75.8% and 46.3% of the observation, respectively. Food contact surfaces and utensils were observed not to be properly cleaned and sanitized in 63.5% of the observations and in 43% of the observation food workers in full-service restaurants failed to separate raw and RTE foods *(147, 149, 150)*. Furthermore, research has found that LO restaurants have performed worse in regards to health department scores and are less likely to have written food safety plans and protocols in place than CO restaurants *(87, 88, 118, 119)*. And as a sub-set of LO restaurants Roberts et al*(117)* found that ethnic LO restaurants performed worse than non-ethnic LO restaurants and ethnic and non-ethnic corporate restaurants on health department scores *(87, 88, 117)*.

The findings that APC, Enterococcus, and male-specific coliphages counts were higher in LO restaurant salads and the higher levels of coliforms and somatic coliphages in CO restaurants salads suggests the produce in these restaurants were a result of unhygienic practices and exposure to improperly cleaned food contact surfaces and utensils which is a result of risky food handling practices by food workers. The significantly higher levels of male-specific and somatic coliphages in LO and CO restaurant specialty salads and salads purchased during low customer traffic volumes highlights the increased risk of produce to cross-contamination issues within a restaurant kitchen during preparation and processing, and that labor issues in the kitchen during low volumes of customer traffic could influence the ability of a food worker to practice safe food handling practices due to time constraints. Even though LO restaurant salads where found to have statistically significant differences for the incidence of male-specific coliphages compared to that of CO restaurant salads this trend cannot be generalized. Upon closer examination of LO restaurant salads from LO restaurants 1

and 5 had consistently higher incidence of male-specific coliphages compared to the rest of the LO restaurant salads, and further analysis of comparing salads from LO restaurants 1 and 5 to that of all CO restaurant salads. Although this data set was performed on a relatively small scale in a localized area results from this study seemingly follow the trends of others which suggest based on comparisons of health department scores that ethnic locally owned restaurants may in fact be at higher risk for unsafe food handling practices designed to prevent foodborne illnesses and suggest that further studies are needed.

II.4.3 Microbiological Quality of Salads Determined by Salad Type

All of the leafy greens salads that were obtained from LO restaurants consisted of either minimally processed iceberg or romaine lettuce. CO restaurants leafy greens salads also consisted of either minimally processed iceberg or romaine lettuce except for 2 of the CO restaurants which contained a mixture of iceberg and romaine lettuce, purple cabbage, and shredded carrots. All CO restaurants sampled offered traditional American cuisine. An overall view of the ingredients that were in the CO restaurant specialty salads were leafy greens (iceberg lettuce, romaine lettuce, cabbage), carrots, tomatoes, shredded cheeses, croutons or tortilla strips, black bean, avocado, corn, cilantro, parsley, onions, pecans, olives, and chicken. Of the 5 LO restaurants sampled 4 were Mexican food restaurants and 1 restaurant served an American based cuisine. Specialty salads purchased from LO restaurants consisted overall of iceberg lettuce, romaine lettuce, cabbage, cilantro, chicken, ground beef, tomatoes, shredded cheese, crumbled feta cheese, black bean, onions, bell peppers, black olives, guacamole, sour cream, carrots, and taco shells, and one salad had a spring mix of leafy greens which included arugula, mesculin, and spinach.

The results for comparison of salad type found that there were significantly higher levels of all indicator organisms present in specialty salads. It was also observed that there were significantly higher levels of APC, total coliforms, *Enterococcus*, and somatic coliphages in specialty salads for every comparison made between specialty

salads and leafy greens salads which can be seen in figure 2-2. For *E. coli* and male-specific coliphages there were only 2 statistically significant differences found between the sub-categorical comparisons made for salad type. Levels of *E. coli* were significantly higher in specialty salads purchased during low customer traffic volumes than leafy greens salads purchased during low customer traffic volumes. Male-specific coliphage counts were significantly higher in CO specialty salads than CO leafy greens salads.

Specialty salads contain several ingredients and this study found that indicator organism counts for specialty salads were higher than what was observed for leafy greens salads. These results were expected based on the wide variety of ingredients, the different microbiological profiles of these ingredients, and the amount of processing and handling that specialty salads undergo before consumption. For instance, dairy products like cheese have naturally high counts of microorganisms, such as lactic acid bacteria and have also been found to sometimes contain *E. coli* and *Enterococcus*. The levels of APC, total coliforms, *E. coli*, and *Enterococcus* have been found to vary between different types of produce. Johnston et al. (06) found that produce obtained throughout the processing chain from the farm to packing had different levels of indicator organisms. Herbs, such as, cilantro and parsley had more than a 1 log CFU/g higher counts of APC, total coliforms, and *Enterococcus* than leafy greens.

Abadias et al *(1)* reported that produce obtained from 4 retail supermarkets had differing levels of APC, lactic acid bacteria, yeast and molds, and *Enterobacteriaceae*, and that the highest counts of these microorganisms were observed in grated carrots, arugula and spinach. The lowest counts were observed with fresh-cut endive and lettuce. Fresh-cut vegetable samples testing positive for *E. coli* were arugula (40%), spinach (20%), lettuce (3.4%), and mixed RTE salads (16.7%). Ailes et al. *(2)* reported on produce obtained throughout the processing chain and found that out of the 14 produce items tested cabbage, cantaloupe, celery, cilantro, mustard greens, and parsley tested positive for *E. coli*. In general these produce items also had significantly higher mean

concentrations of APC, coliforms, and *Enterococcus*. These studies show that produce can differ in the levels of microorganisms. Some leafy greens such as lettuce have lower microbial counts than arugula and herbs such as cilantro and parsley have higher microbial counts than leafy greens.

Processing raw produce can also impact the levels of microorganisms on fresh produce. Johnston et al. *(86)* reported that the levels of indicator organisms for cilantro and parsley where found in higher counts from samples taken from the packing bins after processing than samples taken after harvest. Although it was shown that the levels of indicator organisms did increase for both cilantro and parsley the levels of APC, *Enterococcus*, and coliform counts did not uniformly change between them. The APC counts increased from the field to the packing bin for both cilantro and parsley. *Enterococcus* counts increased for parsley but stayed the same for cilantro. There was a significant increase in coliforms counts for cilantro but for parsley the counts doubled. It was found that indicator organism counts for mustard greens did not change throughout processing and packing. Ailes et al. *(2)* also reported similar findings that APC and coliform counts increased for cilantro and APC, coliform, and *Enterococcus* counts increased for parsley from field to packing. For leafy greens such as arugula and cabbage only *Enterococcus* and APC counts increased, respectively. Ailes et al. *(2)* also found that *E. coli* counts increased significantly for cilantro throughout processing.

Garg et al. *(59)* and Allende et al. *(4)* both reported on the effect that processing had on the level of indicator organisms for fresh-cut produce. They both found that the shredding and cutting step increased the levels of indicator organisms for lettuce and spinach. Allende et al *(4)* reported that psychrotophic bacteria, coliforms, and lactic acid bacteria increased during shredding, significantly decreased during washing, and continued to steadily increase during draining, rinsing, centrifugation, and packaging. The level of indicator organisms at the time of packaging was found to be greater than the levels at the beginning of processing. Produce processors can differ in the way they process raw vegetables. There are different antimicrobial agents that can be used for

washing produce and different concentrations at which they are used *(6, 109)*. Furthermore there can be differences in the number of wash and rinse steps and packaging processes *(4, 95)*. Legnani et al. *(95)* reported on the microbiological quality of minimally processed salads vegetables. This study reported that a series of chlorinated washes were effective in decreasing the levels of indicator organisms throughout processing and the levels of indicator organisms did not significantly decrease increase during storage. This study also found that these chlorinated washes were effective at eliminating low levels of coliphages which have been reported as being more resistant to disinfection treatments than bacteria.

Contamination of fresh produce can occur by coming into contact with utensils and food contact surfaces that have been contaminated with other food products being processed in the kitchen such as raw meats. Kusumaningrum et al. *(92)*found that *Salmonella* spp. and *Campylobacter* spp. *(16)* can be transferred from contaminated food contact surfaces to salad vegetables. Boxmen et al. (16) found that Norovirus can be transferred to the RTE foods and cooking utensils via the hands of a contaminated food worker. Hsu et al. *(81)* found that raw meat can be a source of male-specific coliphage contamination. Allwood et al. *(7)* investigated the occurrence of male-specific coliphages in produce from retail outlets and restaurants and found that fresh produce that had been processed had higher levels of contamination with male-specific coliphages than unprocessed samples. Endley et al. *(52)* all found that more carrots tested positive for the presence of male-specific coliphages after processing than what was found on carrots obtained from the field and transportation truck. These studies highlight the fact that raw produce have different microbiological profiles and microorganisms react differently to washing, processing, and storage, and show that if safe food handling practices are not performed during the handling and processing of fresh produce it can increase the risk of contamination and foodborne illnesses.

The FDA Report on the *Occurrence of Foodborne Illness Risk Factors in Selected Institutional Foodservice, Restaurant, and Retail Food Store Facility Types*

(2009) *(150)* (see Table 2-8), found that food workers in full-service restaurants did not properly maintain correct holding temperatures, wash hands, prevent hand contamination, clean and sanitize food contact surfaces and utensils, and separate raw and RTE foods in 54.7%, 75.8%, 46.3%, 63.5%, and 43% of the observations, respectively. All types of restaurants differ in the size and construction of the kitchens which could limit countertop space, room in refrigeration units, placement of ovens and hand washing sinks, and the number of utility sinks used to thaw meat and wash produce. In a complex working environment of a restaurant kitchen food contact surfaces and sinks are used to process multiple types of food and usually lacks designated areas specifically used for produce processing or have designated food workers that are responsible for processing only produce. All of these can directly affect the safety of fresh produce being served in full-service restaurants. Since specialty salads have the potential to be more prone to cross-contamination issues due to the greater number of ingredients and amount of processing than leafy greens salads it important that safe food handling practices are followed by food workers responsible for the preparation of these salads. Recommended safe food handling practices all take time in an environment that is often short on time, space, and labor, and if the recommended food safety practices are not followed it greatly increases the risk of foodborne illnesses.

Table 2-8 Compilation of FDA reports from 2000-2009: Overview of full-service restaurants "Out of Compliance" percentages for controlling risk factors that increase the risk of foodborne illness outbreaks

Risky Food Handling Practices	2000	2004	2009
Improper Holding Time and Temperature	63.20%	63.80%	54.70%
Poor Personal Hygiene	53.40%	41.70%	40.90%
Proper, Adequate Hand Washing	*81.00%*	*72.70%*	*75.80%*
Prevention of hand contamination	*75%*	*57%*	*46.30%*
Protection from Contamination	43.60%	37.30%	35%
Surfaces, utensils cleaned/sanitized	*33.00%*	*56.60%*	*63.50%*
Raw/RTE foods seperated	*25.00%*	*46.90%*	*43%*
Food from Unsafe Sources	*9%*	*13%*	*12%*
Inadequate Cooking	15.30%	15.80%	15.40%

II.4.4 Microbiological Quality of Salads Determined by Business Volume

Food workers working in the foodservice industry have cited several barriers to safe food handling practices which are time, resources (money and equipment), labor issues (amount of staff), and language barriers *(42, 66, 67, 104)*. Part of this study was to determine the effect that time constraints had on the differences in staffing levels during high and low volumes of customer traffic and the levels of indicator organism in restaurant salads. There was only one statistically significant difference found while comparing the levels of indicator organisms in salads purchased during different volumes of customer traffic. *E. coli* counts in specialty salads purchased during low volumes of customer traffic sampling periods were higher than *E. coli* counts in specialty salads purchased during high volumes of customer traffic sampling periods.

Although only one statistically significant difference was found when comparing levels of indicator organisms in salads purchased during different business volume times comparisons made in full-service restaurant type and salad type found differences in the levels of indicator organisms in salads purchased during low volumes of customer traffic. *E. coli* were found in higher counts in specialty salads than leafy greens salads purchased during low customer traffic sampling periods. Comparisons made between LO and CO salads purchased during low volumes of customer traffic found that CO salads had significantly higher levels of coliforms and somatic coliphages, and LO restaurant salads had significantly higher levels of male-specific coliphages. It is important to note that there were no statistically significant differences found indicating that the levels of indicator organisms were different in salads purchased during high volumes of customer traffic.

High volume customer traffic times were considered to be lunch time, 11 a.m. to 2 p.m., and dinner time, 5 p.m. to closing, Sunday through Saturday. Low volume customer traffic periods were considered to be between 2 p.m. and 5 p.m. Sunday through Saturday. It was observed that busy periods for CO restaurants were consistently busier than LO restaurants. Based on the limited number of sampling times and the short durations of the visits to 3 of the 5 LO restaurants found that there was very little variation in the level of customer traffic between low and high volumes of customer traffic, and it was also observed that there was more business during low volume sampling periods in CO restaurants than for LO restaurants. The only time with similar volumes of high customer traffic for both full-service restaurant ownership types was observed during the sampling periods took place on Friday and Saturday nights. Essentially the comparison of full-service restaurant ownership type and salad type based on business volume was influenced by the lack of business for LO restaurants which allowed us to see this trend.

It is common practice in full-service restaurants to decrease the amount of staff during low volume customer traffic periods or to reassign food workers responsible for

cooking and preparing food to be delivered straight to the customer to help with preparations for the next shift or up-coming events. A decrease in labor or reassignment of labor leaves fewer food workers responsible for the preparation of the in-coming food orders during low volume customer traffic time periods. In order to prevent cross-contamination of food in the kitchen while preparing an order during a lunch or dinner shift food workers are usually designated a particular station in which they are responsible for grilling type operations, salad and sandwich preparation, or desserts. In between the lunch and dinner shifts only one or two food workers may be present to prepare food orders as they come in. This study found that coliforms were present in higher counts in CO restaurant salads purchased during low volumes of customer traffic than LO restaurant salads purchased during low volumes of customer traffic. Somatic coliphages were found to be present more often in CO restaurants salads than LO restaurant salads purchased during low customer traffic volumes.

Since actual business volumes were observed to be busier during low customer traffic sampling times in CO restaurants the likelihood that the higher counts of coliforms and somatic coliphages are a result of produce/RTE foods that have been exposed to a greater variance of temperature fluctuations and risky food handling practices. The higher presence of these indicator organisms could indicate that the food is coming into contact more often with contaminated hands or food contact surfaces and utensils that have not been properly cleaned and sanitized. If one person is working the line in the kitchen while preparing orders it will be more difficult to take kitchen utensils to be cleaned, properly clean and sanitize food contact surfaces, properly wash hands or change gloves. The results also show that risky food handling practices during low volumes of customer traffic were not just related to CO restaurants but for all full-service restaurant types.

When specialty salads purchased during high and low customer traffic volumes were compared specialty salads were found to have higher *E. coli* counts during low customer traffic sampling periods, and *E. coli* were also found in higher counts in

specialty salads than leafy greens salads purchased during low customer traffic sampling periods. *E. coli*, male-specific coliphages, and somatic coliphages were found in significantly higher numbers in salads purchased during low customer traffic time periods from both LO and CO restaurants. Since these are fecal indicators this finding suggests that the food workers responsible for preparing these salads did not follow the recommended hygienic practices and cross contamination occurred as a result of coming into contact with raw meat or unsanitary food contact surfaces and utensils. This strongly suggests that time constraints due to labor issues during low customer traffic volumes can increase the risk of contamination while processing and preparing fresh produce for consumption.

II.4.5 Presence of Male-Specific Coliphages

There have been several studies to suggest that male-specific coliphages can be useful as an additional indicator of fecal contamination and as an indicator of viral contamination. This study found that male-specific coliphages were present in leafy greens and specialty salads from both LO and CO restaurants, and out of the 40 samples testing positive for male-specific coliphages only 30% of these were positive for *E. coli* which can be viewed in Tables 2-6 and 2-7. The absence of *E. coli* in the salad samples that were positive for male-specific coliphages highlights the potential for underestimating the occurrence of fecal contamination, and suggests that male-specific coliphages have value as an additional indicator of fecal contamination and as an indicator of viral contamination.

There are several reviews and epidemiological investigations which show how produce can become contaminated prior to delivery at restaurants *(10, 71, 100, 106, 109, 115)*. In general, raw produce can become contaminated during harvest if exposed to manure, feces from wild animals, fecally contaminated soil, untreated sewage, contaminated irrigation water, or poor hygienic practices by food workers. Postharvest contamination can occur if raw produce comes into contact with contaminated harvesting equipment, poor hygienic practices by food workers, contaminated water

used for washing, chill tanks, sprays, or shipping ice. Contamination that occurs during preparation before consumption can be caused by contact with improperly sanitized food contact surfaces, contact with raw meats, and utensils and poor hygienic practices by food workers *(100, 109)*. It is estimated that only 10% of humans harbor and excrete F+RNA male-specific coliphages *(108)*. F+RNA male-specific coliphages are most often isolated from population based human waste/sewage, feces of animals and animal waste waters *(45)*.

Coliphages and enteric viruses replicate in the gastrointestinal tracts of human and animals. Coliphages are similar in structure, composition, morphology, size, and site of replication. Much like enteric viruses which replicate in mammalian cells coliphages replicate in coliforms which are present in the gastrointestinal tracts of human and animals, and are released into the environment the same way through the feces. Furthermore unlike traditional indicator organisms which can sometimes proliferate and persist in the environment and even grow at refrigeration temperatures coliphages like the F+RNA male-specific coliphage replicate by attachment to bacteria which possess the F-pili. These pili only form on bacteria under certain growth conditions which predominantly occur in the gastrointestinal tracts of warm blooded mammals *(64)*. Coliphages such as the F+RNA male-specific coliphage also share similar characteristics as enteric viruses to water treatment, disinfection processes in water and shellfish depuration, and have been found to share common seasonal trends when present in shellfish growing areas. Coliphages have also been found to better correlate with the presence of enteric viruses in water, waste water, and shellfish than *E. coli*. These characteristics suggest the importance for the need to use additional indicator organisms for screening water and food for fecal contamination and as a suitable indicator for viral contamination. There is additional value for screening for F+RNA coliphages in assessing the microbiological quality of foods. F+RNA coliphages can be genotyped into four genogroups I, II, III, and IV. Genogroups I and IV are indicative of contamination associated with animal waste and genogroups II and

III indicate contamination associated with human sewage or population based human contamination *(9, 48, 49, 54, 56, 81, 94, 98, 132, 153)*.

II.5 Conclusion

Overall the results of this study suggest that there are differences in produce handling and processing practice between locally owned and corporate restaurants. LO restaurants were found to have higher levels of APC, *Enterococcus,* and male-specific coliphages in all of their salads, and higher levels of male-specific coliphage in their specialty salads. CO restaurants were found to have higher levels of coliforms and somatic coliphages in their salads, and CO restaurants were found to have significantly higher levels of somatic coliphages than LO restaurant specialty salads. There were significantly higher levels of *E. coli* in specialty salads purchased during low volumes of customer traffic, significantly higher levels of *E. coli* in specialty salads than leafy greens salads purchased during low volumes of customer traffic, significantly higher levels of coliforms and somatic coliphages in CO restaurant salads than LO restaurant salads purchased during low volumes of customer traffic, and significantly higher levels of male-specific coliphages in LO restaurant specialty salads purchased during low volumes of customer traffic. This suggests that risky food handling practice were more likely to occur when fewer food workers are present to prepare salads during low volumes of business. Specialty salads were found to have significantly higher levels of all indicator organisms. This finding suggest that salads containing more than one fresh produce item requires additional handling and processing and therefore increases the potential risk of cross-contamination.

The differing levels of indicator organisms present in these restaurant salads provide evidence that agree with the 3 reports released by the FDA and the study Kirkland et al. *(90)* which suggests that restaurants process their produce differently and that these restaurants do not allows follow the safe food handling practices recommended. Both full-service restaurant ownership types showed trends which indicated that risky food handling practices could affect the microbiological quality of

their salads. The FDA has reported that full-service restaurants have the highest "out-of-compliance" standing for following the recommended food safety practices that prevent improper holding temperature, inadequate cooking, contaminated equipment, food from unsafe sources, and poor personal hygiene out of 9 institutions responsible for serving food to the public *(102, 147, 149, 150)*.

Roberts et al. *(118)* found that corporate restaurants have more HACCP and prerequisite food safety programs in place than independent-locally owned restaurants. Studies have also found that ethnic locally owned restaurants perform worse on health department scores than non-ethnic independent-locally owned restaurants and ethnic and non-ethnic corporate restaurants *(88, 117)*. The results from LO restaurants 1 and 5 which significantly higher levels of male-specific coliphages supports the trend suggesting that some independent locally owned restaurants are failing to follow the recommended food safety practices which can lead to an increase in risk of foodborne illness outbreaks.

Male-specific coliphages were found in salad samples that did not contain *E. coli* which suggest that there is a need for their use as an additional indicator of fecal contamination. Male-specific coliphages have also been found to be useful as an indicator for viral contamination. Since coliphages are indicators of fecal and viral contamination the finding that they were present in restaurant salads is a strong indication that fecal contamination has occurred and that there is an increased risk for the presence of human enteric pathogens such as Norovirus or Hepatitis A *(7)*.

Several studies have shown that the levels of microorganisms can differ based upon produce type and that processing can also influence the levels of microorganisms dependent upon produce type *(1, 2, 8, 86)*. Fresh produce such as parsley have been found to have higher counts of APC, coliforms, *Enterococcus*, and *E. coli* than leafy greens such as lettuce *(1, 2, 8, 86)*. It could be beneficial for restaurants and other retail food service establishments to have standardized procedures that would suggest an order in which to process produce and at which times it would be the most beneficial to clean

and sanitize utensils and food contact surfaces. This could decrease the risk of cross-contamination between fresh produce items in the kitchen.

One of the shortcomings of this experiment was the times that were chosen to obtain salad samples from both types of restaurants. Since fresh produce is at higher risk for contamination there is a need to design a study that directly looks at the differences between how many people handle a salad under time constrained situations. It would be beneficial for the food industry to implement microbiological testing of produce and produce processing in restaurants from the time of delivery, during storage, after washing, shredding, centrifugation, and storage. These studies need to obtain produce from cold storage in both the walk-in refrigeration units and the table top units before, during and after lunch and dinner shifts.

CHAPTER III
DNA FINGERPRINITING AND GENOTYPING FECAL INDICATOR ORGANISMS IN RESTAURANT SALADS

III.1 Introduction

Today molecular methods are used by the food and water industry as a tool to reliably distinguish or compare foodborne pathogens and fecal indicator organisms from sources such as food, water, environmental matrices, and clinical isolates *(75)*. Microbial source tracking use molecular methods to DNA fingerprint indicator bacteria from unknown sources and compare them against a library data base of fingerprint profiles to determine the source of contamination. PulseNet and CaliciNet are national molecular subtyping networks for foodborne disease surveillance in the United States *(137, 156)*. These networks use pulsed-field gel electrophoresis (PFGE) and reverse transcriptase polymerase chain reaction (RT-PCR), which are consider the "gold standards", for DNA fingerprinting and viral genotyping foodborne pathogens *(15, 55, 102, 110, 125)*. Repetitive extragenic palindromic sequence PCR (rep-PCR) is an amplification-based genotyping method that is used for DNA fingerprinting bacterial isolates. This method has been shown to have comparable but slightly less discriminatory power than PFGE. The recent standardization and a semi-automated format created by DiversiLab systems (BioMéurex, Durham, NC) has increased interlaboratory reproducibility and results can be obtained in less than 24 hours which can be easily compared with a web-based software *(41, 46, 75, 128)*. RT- PCR is a PCR based genotyping method that is used to rapidly detect and genotype enteric RNA viruses such as Norovirus and male-specific coliphages in clinical and environmental matrices *(125)*. This goal of this study was to use rep-PCR and RT-PCR to DNA fingerprint and genotype *E. coli* and male-specific coliphage isolates obtained from leafy greens and specialty salads purchased from 5 locally-owned restaurants and 5 corporate restaurants to identify possible routes and sources of fresh produce contamination.

III.2 Materials and Methods

III.2.1Microbiological Examination of Salads

III.2.1.1 Sample Collection

The samples to be obtained from restaurants were **specialty salads** and **leafy greens salads**. Specialty salads contain a variation of meats, cheeses, croutons or tortilla strips, nuts, and additional vegetables. In this study green leaf salads were considered to be the stock lettuce leaf mix prepared by a restaurant which may consist of iceberg and/or romaine lettuce alone or have carrots and cabbage added to the stock lettuce leaf mix with no additional ingredients. The salads were obtained from five nationally franchised chain restaurants and five locally owned restaurants during periods of high customer traffic and low customer traffic. High customer traffic periods were considered to be lunch and dinner time between the times of 11 AM and 2 PM (lunch) and 5 PM and 9 PM (dinner). Low customer traffic periods were considered to be between 2 PM and 5 PM and 9 PM to close. The restaurants were chosen based on high, moderate, and low health inspection scores obtained from the Brazos Valley Health department. A total of 100 specialty salads and a total of 100 green leaf salads were purchased from both nationally franchised chain full service restaurants and locally owned full service restaurants. Of these, 50 specialty salads and 50 green leaf salads were obtained from high and low customer traffic period. The participation of the restaurants will not be known and the salads were purchased on random days throughout this study. The samples were placed in coolers with blue ice and transported immediately to the laboratory for sample processing.

III.2.1.2 Sample Processing

Specialty salads and green leaf salads were processed in a biosafety cabinet (Labconco purifier class II Biosafety Cabinet Delta Series, Kansas City, MO) to prevent laboratory-based contamination of the samples. One hundred grams of each salad sample were aseptically weighed on the analytical balance within the hood and placed

into sterile bags with a membrane filter (VWR, West Chester, PA). Two hundred ml of a 0.1 M sodium phosphate buffer supplemented with 1.0 M NaCl at a pH of 8.0 was added to the stomacher bags. The 0.1 M sodium phosphate buffer supplemented with 1.0 M NaCl was titrated to a pH 8 ± 0.1 using a SevenEasy S20™ pH-meter (Mettler-Toledo, Columbus, OH). The samples were stomached on the (low) setting for 2 minutes. Approximately 200 ml of extract was pipetted from the stomacher bags and placed into 50 ml conical tubes (VWR, West Chester, PA).

III.2.2 Genotyping of E. coli Isolates

Bacterial culture and DNA extraction - All *E. coli* isolates recovered from the salad samples were confirmed, cultured, and identified on modified mTEC agar (Becton, Dickinson Co.) and incubated overnight at 37°C under aerobic conditions. Colonies presenting the characteristic magenta color will be picked using a flame sterilized loop and streaked onto TSB plates. The TSA plates were incubated overnight at 37°C under aerobic conditions. Colonies from these plates were placed into TSB to incubate aerobically overnight at 37°C.

Total genomic DNA will be extracted using 1.8 ml of broth culture and the UltraClean™ Microbial DNA Isolation Kit (Mo-Bio Laboratories, Solana Beach, CA.) following the manufacturer's protocol. All DNA solutions were standardized to a concentration of approximately 25-50 ng/µl using a NanoDrop (ND-1000) spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

Automated rep-PCR DNA Fingerprinting – Extracted DNA was amplified with the Diversilab® *Escherichia* DNA fingerprinting kit (bioMérieux, Inc., Durham, NC) according to the manufacturer's protocol. Approximately 50 ng/µl of genomic DNA, 2 µl of the kit-supplied proprietary primer mix targeting interspersed repetitive elements within the bacteria's genome, 18.0 µl of the rep-PCR master mix (MM1), 2.5 µl of 10x PCR Buffer (Applied BioSystems, Inc., Carlsbad, CA) and 0.5 µl of the AmpliTaq DNA Polymerase (Applied BioSystems, Inc., Carlsbad, CA) were added for a total of 25 µl

per reaction mixture. The thermal cycling parameters for this assay were as follows: initial denaturation of 94°C for 2 min.; 35 cycles of denaturation at 94°C for 30 s; annealing at 50°C for 30 s; extension at 70°C for 90 s; and a final extension at 70°C for 3 min.

Separation, detection and analysis of the DNA amplicons were performed in a microfluidics DNA LabChip (bioMérieux, Inc., Durham, NC) using the Diversilab system (Bacterial Barcodes, Inc.) and a Bioanalyzer 2100 (Agilent Technologies, Inc., Palo Alto, Ca). Analysis was performed with DiversiLab software v3.41 using the Pearson correlation coefficient to calculate correlations and strengths of correlation and strengths of correlation/similarity among all possible pairs of sample fingerprints. The un-weighted-pair group method with arithmetic mean is used to automatically compare the rep-PCR profiles and create dendrograms for visual comparisons of banding patterns.

III.2.3 Genotyping Male-Specific Coliphages

Coliphage purification - EPA Method 1601 (2001) were used for coliphage enrichment. A log phase culture of *E. coli* F_{amp}^{+} was grown and 1.0 ml were placed into 5 ml of 1X TSB supplemented with a 0.15% Ampicillin/Streptomycin solution and 4 M $MgCl_2$ solution. From this, 1 ml aliquots will be dispensed into microcentrifuge tubes and 200 µl of the male-specific coliphage isolates were added to the appropriate labeled tubes. The isolate were incubated aerobically overnight at 35°C. Isolates were filtered with 0.22 µm filter (Millipore Carrigtwoholl, Co. Cork, Ireland) to obtain a purified phage stock. Ten µl of the phage stock were used for spot plating to ensure the virus enrichment procedure was successful.

Genomic RNA extraction and RT-PCR - The purified phage stock were used to perform the protocol described by Friedman et al (2009) for reverse-transcription-PCR assay to distinguish between the four genogroups of male-specific F^{+} RNA coliphages. Briefly, 1.0 µl of purified phage stock were placed into 49 µl of RNase-free water to make a 1:50 dilution. The tube was placed into the thermocycler and the viral RNA was

heat-released for 5 minutes at 98°C. The heat-released viral RNA were centrifuged at 10,000 x g for 30 seconds at room temperature and chilled on ice for approximately 2 minutes. The heat-released viral RNA was amplified using the Qiagen® One-Step RT-PCR kit (Qiagen, Inc., Valencia, CA). Five µl of the heat released viral RNA, 28.5 µl of sterile water, 10 µl of 5X Qiagen® reaction buffer, 2 µl of 10mM dNTP, 0.5 µl of Rnase SuperAse-In inhibitor (Ambion, Inc Carlsbad, CA), 1 µl of 10 µM FRNA forward primer (group specific), 1 µl of 10 µM FRNA Reverse (group specific) primer (Sigma-Aldrich Co., Woodlands, TX), and 2 µl of the Qiagen® RT-PCR enzyme mix were added together to make a total of 50 µl per reaction mixture.

The primer sequences published by Friedman et al. that were used for the genotyping male-specific coliphages (Appendix).

The RT-PCR reaction were performed using the GeneAmp PCR system 2700 (Applied BioSystems, Foster City, CA). The thermocycler conditions were set to run as follows: 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and to finish with the final extension at 72°C for 10 min. The amplicons were separated by gel electrophoresis in a 1.5% agarose gel, stained with Gel Red Nucleic acid Stain (Phenix Research Products, Chandler, NC) that was diluted 1:10,000. Amplicons were visualized with a MultiDoc-It Digital Imaging System (UVP, LLC Upland, CA). The master mixes were prepared in a PCR hood (PCR Work Station 3560000, Fisher Scientific, Pittsburg, PA) separate from where the template will be added and the gel were run in a separately assigned room to avoid contamination issues.

III.3 Results

III.3.1 Presence of Male-Specific Coliphages and E. coli in Restaurant Salads

A total of 200 salad samples were collected from 10 restaurants located in Brazos County from September 2009 through May 2010. Among the 200 samples, 100 hundred samples were obtained from 5 nationally franchised corporate owned restaurants and 100 samples were obtained from locally owned restaurants. Within each restaurant type a total of 50 specialty salads and 50 leafy greens salads were purchased during periods of both high and low customer traffic volumes. Of the 200 hundred samples collected a total of 40 samples tested positive for male-specific coliphages and a total of 37 samples of tested positive for E. coli.

III.3.2 Male-Specific (F+RNA) Coliphages Genogroups Present in Restaurant Salads

Overall, 40 (20%) of the 200 salad samples collected from locally owned and corporate restaurants were positive for male-specific coliphages according to EPA method 1601 enrichment assay testing for the presence or absence of somatic and male-specific coliphages. Out of 40 samples that tested positive 20 of these samples were able to be enumerated according to EPA Method 1602 enumeration assay for somatic and male-specific coliphages. Of the 20 samples that were enumerated, fourteen samples were genotyped using Friedman et al (45) RT-PCR protocol to identify F+RNA coliphages belonging to genogroups I through IV. There were only 2 locally owned restaurants that had male-specific coliphage samples above the detection limit. A total of 9 coliphage samples from specialty salads and leafy greens salads were genotyped, and of those 4 were positive for F+RNA coliphages belonging to genogroups I and III. Locally owned restaurant 1, identified as 1.LO.SS.LV.C and 1.LO.SS.LV.D, were found to be positive for genogroup III and locally owned restaurant 5, identified as 5.LO.HS.LV.C and 5.LO.SS.HV.D, were found to be positive for genogroup I. Both samples testing positive for genogroup III were from specialty salads purchased from locally owned restaurant 1 during low customer traffic sampling periods. Samples

testing positive for genogroup I was found to be present in both a specialty salad and a leafy greens salad purchased from locally owned restaurant 5 during low customer traffic sampling periods. Samples 1.LO.SS.LV.C and 1.LO.SS.LV.D were purchased exactly one week apart from each other. Locally owned restaurant 1 tested positive for the F+RNA coliphage belonging to genogroup III which is known to be associated with contamination from human waste/sewage. Locally owned restaurant 5 tested positive for the F+RNA coliphage belonging to genogroup I which is known to be associated with contamination from animal waste.

A total of 3 nationally franchised corporate restaurants tested positive for F+RNA male-specific coliphages above the detection limit. There were 4 coliphage samples obtained from specialty and leafy greens salads that were genotyped, and of those, 3 samples were positive for F+RNA male specific coliphages belonging to genogroups II and III. Corporate restaurants 1 and 5, identified as 1.CO.SS.LV.E and 5.CO.SS.LV.E were found to be positive for genogroup III, and corporate owned restaurants 1 and 2, identified as, 1.CO.SS.LV.E and 2.CO.HS.HV.E, were found to be positive for genogroup II. Both samples testing positive for genogroup III were from specialty salads purchased from corporate restaurants during low customer traffic volume periods on the same day. The specialty salad testing positive for genogroup III from the corporate restaurant 1 was also positive for F+RNA male specific coliphage II. A leafy greens salad purchased from corporate restaurant 2 during a high customer traffic period was found to be positive for genogroup II. F+RNA male specific coliphages that were found in these restaurant salads were from genogroups II and III which are associated with contamination from human waste/sewage.

III.3.3 Genetic relatedness of E. coli Isolates from Restaurant Salads

The results of the automated rep-PCR (Diversilab) grouped the *E. coli* isolates into 4 genetically similar clusters (A, B, C, and D). Cluster A contains presents the first observable trend of *E. coli* isolates and contains *E. coli* isolates 41 through 49 which were obtained from 4 CO restaurant salads during one sampling period on the same day.

According to the DiversiLab Analysis Guide the relationship of rep-PCR fingerprints considers bacterial isolates indistinguishable if the samples have a high percentage of similarity of >97% and the individual comparisons of the fingerprinting patterns show no differences. The Pearson's correlation (PC) was chosen to determine the genetic relatedness of these *E. coli* isolates. PC emphasizes peak intensities more than peak presence or absence and this method of statistical comparison is suggested for use when comparing gram-negative organisms. Cluster A, which can be seen in figures 3-2 and 3-3, contains *E. coli* isolates 41 through 49. *E. coli* isolates 41 through 48 were found to have >97% genetic relatedness. *E. coli* isolate 49 when compared to *E. coli* isolates 41 through 48 found that it also had >97% genetic relatedness except for two comparisons in which the percentages were found to be 96.3% and 96.7%. When overlay comparisons were visually observed (data not shown) of the banding patterns it was determined that the differences were negligible and they were considered to be genetically indistinguishable from the other genotyping patterns observed in cluster A.

Clusters B and C showed two trends of produce contamination. Cluster B contained E. coli isolates 28 through 36 which can be viewed in figures 3-1 and 3-2. *E. coli* isolates 28 through 31 were >98.1% genetically related and are considered to be genetically indistinguishable. These *E. coli* isolates were from LO restaurant 5 specialty salads obtained on three separate dates spanning a period of 28 days during the months of April and May. *E. coli* isolate 32 was from LO restaurant 1 specialty salad obtained during a sampling period at the end of April. *E. coli* isolate 32 was found to be genetically similar to *E. coli* isolates 30 and 33 by a similarity percentage of 95.2% and 95.5% respectively. *E. coli* isolates 33 and 34 were also from LO restaurant 2 specialty salad and leafy greens salad and were obtained at the end of March 2010, and are 97.4% genetically related. *E. coli* isolates 35 and 36 were from LO restaurants 2 and 5 specialty salads obtained at the end of March 2010 and at the end of April 2010, respectively. *E. coli* isolates 35 through 36 were >96.1% genetically related

Cluster C contained *E. coli* isolates 15 through 26 which can be viewed in figures 3-1 and 3-3. Within this cluster of genetically related *E. coli* isolates, 19 through 25 were obtained from CO restaurant 5 leafy greens salads and specialty salads with the exception of *E. coli* isolate 20 which was from a salad purchased from CO restaurant 3. The *E. coli* isolates obtained from CO 5 salads were purchased on two consecutive days during mid-May 2010. The *E. coli* isolate from CO restaurant 3 specialty salads was obtained in October of 2009. This cluster of *E. coli* isolates were considered to be indistinguishable with a >99% genetic similarity.

There was a smaller cluster of genetically related *E. coli* isolates in cluster C which were found to be little more genetically diverse. *E. coli* isolates 15 and 16 were 98.5% genetically similar. These *E. coli* isolates were obtained from LO restaurant 5 specialty salads on separate dates occurring 24 days apart during the months of March and April. *E. coli* isolates 17 and 18 had a genetic relatedness of 98.6% and were obtained from a LO restaurant 2 specialty salad and leafy greens salad. These salads were purchased during the same sampling period when *E. coli* isolate 16 was obtained at the end of March. Overall, the genetic relatedness between *E. coli* isolates 15 and 16 and *E. coli* isolates 17 and 18 was >95%. *E. coli* isolates 17 and 18 were also found to be genetically similar (>95%) to several *E. coli* isolates in the cluster containing isolates 19 through 25. *E. coli* isolate 26 was obtained from a LO restaurant 5 specialty salad during a sampling period that took place at the beginning of May 2010. It was found to be genetically related (96.5%) to the *E. coli* isolates in the group containing isolates 19 through 25.

Cluster D contains *E. coli* isolates 1 through 8 which can be viewed in figures 3-1 and 3-3. *E. coli* isolates 1 through 8 were from a CO restaurant specialty salad. The rep-PCR results for this cluster found one *E. coli* isolates that was considered to be genetically indistinguishable and several other *E. coli* isolates that are considered to be genetically similar which indicates that more than one *E. coli* isolate can be present in a single salad sample. Other examples of more than one *E. coli* isolate in a salad sample can be seen in figures 3-1 and 3-2.

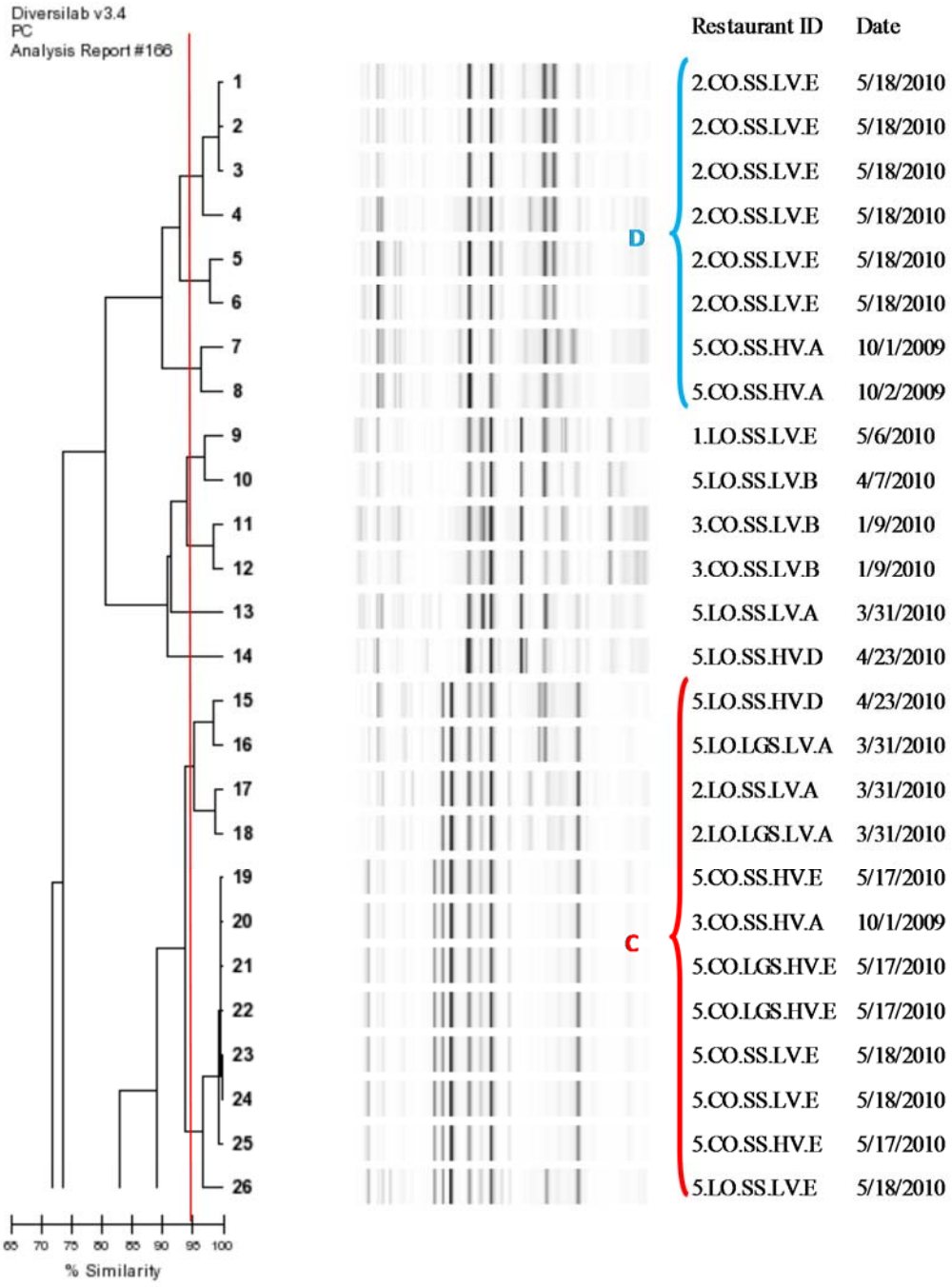

Figure 3-1 Rep-PCR dendrogram of *E. coli* clusters C and D

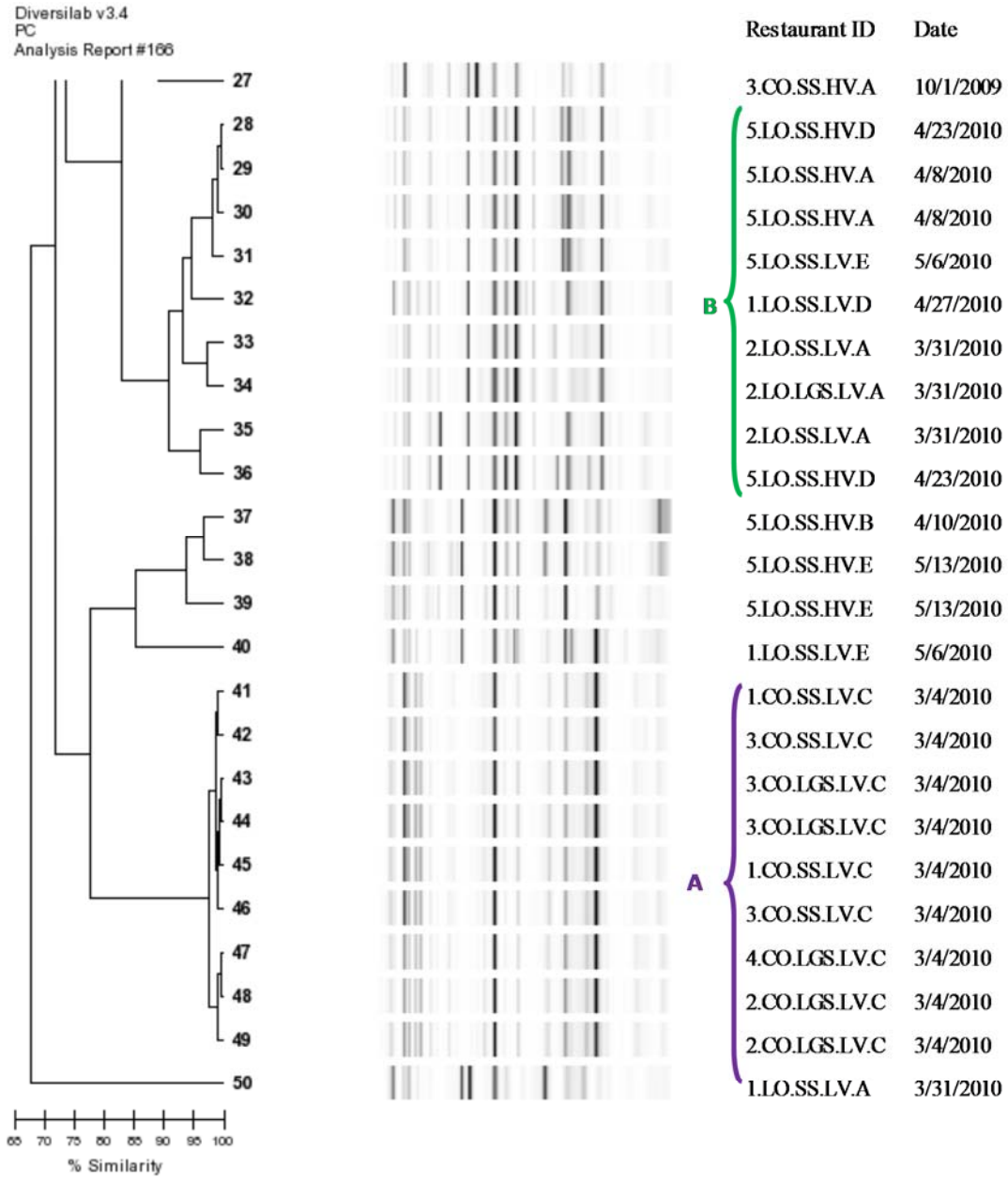

Figure 3-2 Rep-PCR dendrogram of *E. coli* isolate clusters A and B

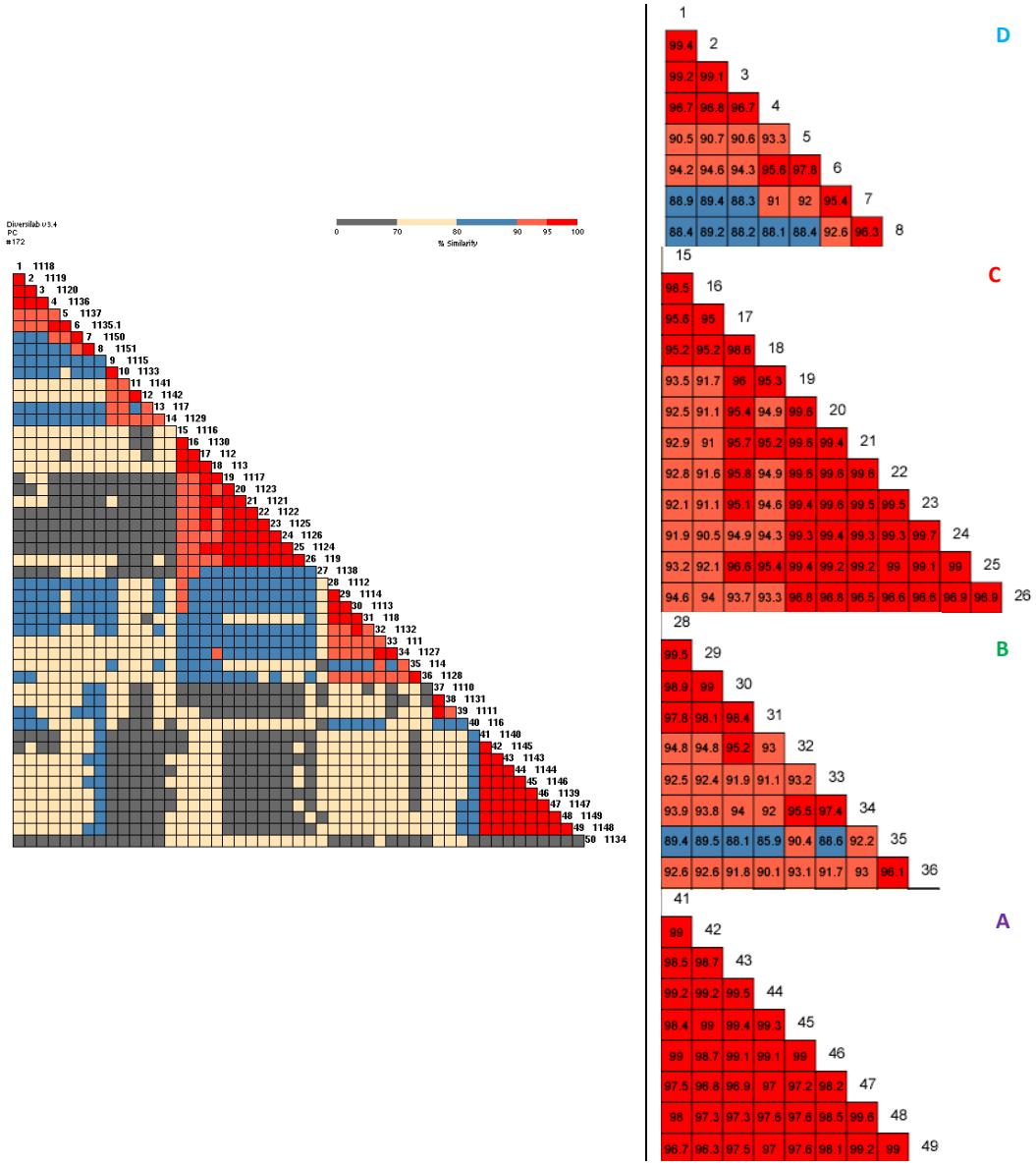

Figure 3-3 Rep-PCR similarity matrix of *E. coli* isolate clusters A, B, C, D

III.4 Discussion

III.4.1 DNA Fingerprinting E. coli

One of the primary goals of this study was to determine whether the *E. coli* found in restaurant salads were genetically related and discern any overall trends which could provide information on routes of contamination or risky food handling practices. Rep-PCR results identified four clusters of genetically related *E. coli* (A, B, C, and, D). *E. coli* isolates in cluster A were from leafy greens and specialty salads collected on the same day from 4 CO restaurants. Although the sources of contamination cannot be determined *E. coli* was found in 3 leafy greens salads from 3 CO restaurants which suggest that the leafy greens used in preparing these salads was contaminated prior to delivery in these restaurants. In general there were very few leafy greens salads that tested positive for *E. coli*. Overall, 19% (19 of 200) of the salad samples in this study were positive for *E. coli* and of those 29% (29 of 100) of the specialty salads and 9% (9 of 100) of the leafy greens salads were positive for *E. coli*. This suggest that the large number of specialty salads contained other ingredients that were contaminated with *E. coli* prior to delivery in the restaurants or the ingredients used to prepare the specialty salads were contaminated during preparation due to risky food handling practices.

Since genetically related *E. coli* in cluster A was found in both leafy greens salads and specialty salads from several restaurants on the same day there is a greater probability that the leafy greens used in these salads were exposed to a common source of contamination prior to delivery in these restaurants. It is unlikely that a single food worker was employed at all four restaurants and was involved with salad preparations on the same day. Raw produce can become contaminated with foodborne pathogens at any point from cultivation through consumption and have been found to be a growing cause of foodborne illness outbreaks. Raw produce can become contaminated during harvest if exposed to manure, feces from wild animals, animal waste waters, fecally contaminated soil, untreated sewage, contaminated irrigation water, or poor hygienic practices by food workers. Postharvest contamination can occur if raw produce comes into contact with

contaminated harvesting equipment, poor hygienic practices by food workers, contaminated water used for washing, chill tanks, sprays, or shipping ice. Contamination that occurs during food preparation before consumption can be caused by contact with improperly sanitized food contact surfaces, contact with raw meats, and dirty utensils as well as poor hygienic practices by food workers. Produce is often consumed raw and lacks a microbicidal step and it is important that growers, processors, distributors, and food preparation practices in the home and in the retail foodservice setting adhere to good agricultural practices, good manufacturing practices, and follow recommended safe food handling practices *(100, 109)*.

Epidemiological studies have shown that produce contaminated prior to delivery at restaurants and retail establishments have been responsible for several foodborne illness outbreaks. These outbreaks can occur in several states or can occur in multiple locations within a single state or metropolitan area *(10, 71, 106, 115)*. Fresh produce outbreaks can occur during field production or during processing. In 2008, an outbreak of *Salmonella* SaintPaul associated with jalapeno peppers caused several cases of foodborne illnesses for patrons dining in one independent Mexican restaurant and 2 chain Mexican restaurants in northern Texas. This investigation revealed that these restaurants had used two different importers to obtain the fresh jalapenos. It was reported that a common practice for these importers was to trade produce amongst each other in order to fill their perspective orders. The investigation eventually revealed that the jalapenos had been imported from a packing facility in Mexico which had obtained the jalapenos from a farm in Mexico. The irrigation water and the peppers from this farm were both positive for this particular strain of *Salmonella* SaintPaul *(10)*.

In 1998 there were two concurrent multistate outbreaks of *Shigella Sonnei* and Enterotoxigenic *E. coli* associated with parsley. The traceback investigation found that a farm in Baja California, Mexico was using a chlorinated wash system in the processing shed which was found not to be functioning properly. The investigators determined that the parsley had been exposed to unsanitary water during washing *(106)*. In 1999, there

was a multistate outbreak of *Salmonella* Newport linked to mangos. These mangos were found to have been imported from Brazil. As a part of a disinfestations treatment these mangos were dipped into unchlorinated hot water before being dipped in a cold chlorinated rinse. This hot water treatment was shown to facilitate the internalization of bacteria from the surface of the fruit which limited the effectiveness of the chlorinated rinse treatment *(130)*.

Contamination of fresh produce can also occur during distribution. In 2001, a distributor was responsible for an outbreak of *Shigella flexneri* Serotype 2a associated with tomatoes. The distributor was found to have hand sorted and packaged over-ripened bruised tomatoes that had not been washed and sold them to five restaurants in the area for a discounted price. Since there was no other outbreaks reported within the area and reports of foodborne illness occurred within a 24 hour period after having eaten at one of these five restaurants it was concluded that the contamination had occurred as a result of the unsafe food handling practices by food workers employed by this distributor to sort the tomatoes *(115)*. These foodborne outbreak investigations have shown that fresh produce can be supplied to different restaurants by different distributors that have purchased produce from the same growers and that produce can become contaminated at any point during the produce processing chain. These studies have implicated numerous causes of produce contamination such as contaminated irrigation water, malfunctioning chlorinated wash systems used in processing, and risky food handling practices, and that genetically related foodborne pathogens can be distributed to several restaurants in a localized area in a city, across a state or across several states. This study found that produce and produce based entrees from several restaurants sampled on the same day were contaminated with indistinguishable genetically related *E. coli*. However, this finding cannot speculate as to how the produce became contaminated, but it does suggest that the fresh produce delivered to these restaurants which were sampled on the same day was exposed to a common source of contamination somewhere along the processing chain.

E. coli isolates in clusters B and C were obtained from CO restaurants 3 and 5 specialty and leafy greens salads and LO restaurants 2 and 5 specialty and leafy greens salads. There are several trends that can be observed from clusters B and C. The *E. coli* isolates grouped together in cluster B suggests that the fresh produce or other RTE food items used for making these restaurant salads had been exposed to a common source of contamination prior to delivery in these restaurants that was recurrent for a long period of time. The six genotyped *E. coli* isolates from CO restaurant 5 leafy greens salads and specialty salads were obtained over a two day sampling period in mid-May 2010. Another one of the genetically related *E. coli* isolates was from a CO restaurant 3 specialty salad which was obtained at the beginning of October 2009. Four *E. coli* isolates were from LO restaurants 2 and 5 specialty and leafy greens salads of which 3 of these *E. coli* isolates were obtained at the end of March 2010 and one of the *E. coli* isolate was obtained at the end of April 2010. Three *E. coli* isolates from LO restaurants 2 and 5 were found in salads approximately five weeks prior to the *E. coli* isolates found in the salads purchased from CO restaurant 5. The most surprising find was the *E. coli* isolate from the CO restaurant 3 specialty salad that was purchase at the beginning of October 2009. This E. coli isolate was genetically related to the E. coli isolates from the contaminated salads obtained from LO restaurants 2 and 5 and CO restaurant 5 which were purchased 6 to 8 months later.

Similar to the finding in cluster A, leafy greens salads from 2 restaurants contained genetically related E. coli in cluster B. Since genetically related *E. coli* was found in very few leafy greens salads compared to that of specialty salads as well as the fact that 6 to 8 months prior there was an *E. coli* isolate from a CO restaurant 3 specialty salad that was genetically related to the *E. coli* isolates found in these leafy greens salads suggests that the leafy greens used in these salads were exposed to a common source of contamination prior to delivery in these restaurants, and that this source of contamination was recurrent over a long period of time. In 2002 and 2005 there were 2 multistate outbreaks of *Salmonella* Newport associated with tomatoes which were traced back to

growers on the eastern shore of Virginia. The epidemiological investigation concluded that a pond that was used for irrigation and application of pesticides was determined to be the source of contamination for these two outbreaks. In this epidemiological investigation irrigation water was a source of contamination which was responsible for causing two outbreaks which spanned a 3 year period *(106)*. The occurrence of genetically related *E. coli* in fresh produce isolated from the salads in this study could be due in part to exposure to a recurrent source of contamination such as irrigation water.

The results for a smaller cluster of genetically related *E. coli* isolates within cluster B and the genetically related *E. coli* isolates in cluster C could suggests that contamination of these salads resulted from risky food handling practices by food workers in the restaurant kitchen or due to a persistent strain of *E. coli* which had been able to effectively occupy a niche within a restaurant kitchen. Two *E. coli* isolates in cluster C and 4 *E. coli* isolates in cluster B were from LO restaurant 5 salads, and were collected over a time span of 5 weeks. With the exception of 1 leafy greens salad obtained at the end of March 2010 the rest of these salads in cluster B and C containing genetically related *E. coli* were found in specialty salads. Since *E. coli* isolate 16 was found in a leafy greens salad at the end of March and the shelf life of leafy green produce such as lettuce is usually 7 *(43)* days finding genetically related *E. coli* in a specialty salad a month later suggests that the produce or other RTE food items used to prepare these salads were either exposed to the same source of contamination prior to delivery at this restaurant or that due to risky food handling practices and cross-contamination issues the produce or other RTE food ingredients used in the preparation of these salads were exposed to common source of contamination in LO restaurant 5's kitchen. Contaminated produce could have been transferred by the hands of a food worker to a RTE food product such as cheese which has a longer shelf life than lettuce, and this cheese could have been used in the subsequent preparation of several salads after the fact. Storage bins that were used to store the contaminated produce may not

have been properly washed and consistently refilled with fresh produce allowing recontamination can occur over a long period of time.

The genetically related *E. coli* isolates obtained over a six week period from LO restaurant 5 salads could have resulted from a common source of contamination during processing before delivery to the restaurant or because of risky food handling practices and cross-contamination issues, but contamination could have also occurred as a result of a persistent strain of *E. coli* that has effectively colonized a niche within their restaurant kitchen. *E. coli* has the ability to acquire phenotypic traits that allows for their survival outside of the commensal tract and colonize niches within food processing environments. In general 1 gram of feces contains greater than 10^6 *E. coli* cells. The secondary habitat for *E. coli* is the environment. *E. coli* is usually excreted through fecal materials which can contaminate soil, water, and food. However the persistence outside of the commensal tract for *E. coli* varies. Environments such as soil, water, sediment, and food vary in range of pH, water and nutrient availability, and temperature fluctuations. *E. coli* is genotypically and phenotypically diverse and has been divided into six different phylogenetic groups. *E. coli* consists of various commensal and pathogenic forms and their genomes can differ up to 20%. These genetic differences can be acquired through gene mutation and acquisition via plasmid or phage mediated gene transfer. These differences can be seen in carbon utilization patterns, antibiotic resistance profiles, flagellar motility, ability to form biofilms, and pathogenicity. Due to phenotypic variations studies have suggested that some *E. coli* strains can become dependent upon the environment in which they have proliferated and lose the ability to adapt to other environments, but other *E. coli* strains can acquire phenotypic and genotypic traits that allow them to persist and survive for longer periods of time within less favorable environments and become a dominant strain *(79, 83, 143, 155)*.

Holah et al. *(80)* wanted to determine if there was a presence of persistent strains of *Listeria monocytogenes* and *E. coli* in chilled food factories located in the UK and if these persistent strains had developed an increased resistance to commonly used

disinfectants employed by the food industry. Approximately 30,000 samples were taken from RTE foods and from the environment in 5 chilled food factories. A total of 181 L. monocytogenes and 176 *E. coli* isolates were obtained from this study and were ribotyped. There were 19 ribogroups for *L. monocytogenes* and 34 ribogroups for *E. coli*. This study found that there were 5 ribogroups of *E. coli* isolated from both the product and environment that persisted for extended periods of time within these facilities. *E. coli* (102-248-S-4) ribogroup was found to be a dominant strain that was isolated on multiple occasions from both the product and the environment. This strain was isolated a number of times over a year's time span, and this particular strain of *E. coli* was found to be present in 3 of the 5 factories tested. Other *E. coli* ribotypes were also found to be persistence in these food processing facilities, but their persistence was found to be only for a few months. These persistent strains of *E. coli* and *L. monocytogenes* were not found to have acquired any increased resistance to commonly used biocides employed by these food processing facilities. These results suggest that these persistent strains can colonize areas within these food processing environments which are not easily accessible to disinfection treatments yet can still contaminate food products.

In a second study by Holah et al. *(79)* a total of 196,000 RTE food and environmental samples were taken over a 3 year period from 4 RTE chilled food factories in the United Kingdom. This study aimed to identify niches in these food factories where *L. monocytogenes* and *E. coli* could persist. This study found that there were 10 *E. coli* ribogroups and 14 *Listeria* spp. ribogroups, and that these ribogroups were isolated from the same site, over a prolonged period of time. It was also found that some of the same *E. coli* ribogroups were isolated from other food factories as well. The ecological niches differed between *E. coli* and *Listeria* spp. Even though *E. coli* and *Listeria* spp. were isolated from both food and environmental samples persistent strains of *E. coli* were most often isolated from food samples as opposed to *Listeria* spp. which were mostly found in the environmental samples.

These facilities are designed to prevent pathogen contamination by low/high risk areas, physical barriers, and management of these facilities implements and adheres to strict food safety programs. Even though these two microorganisms were found to be a persistent source of contamination in both food and the environment *E. coli* and *Listeria* spp. were isolated from <0.5% of the food and environmental samples which suggest that the food safety programs were effective at controlling the presence of these organisms in the 4 RTE chilled food factories. Overall these two studies found that even in the absence of a common source of contamination entering these food processing facilities there were strains of *E. coli* and *Listeria* spp. that had effectively colonized niches in this type of environment and persist for extended periods of time even with proper sanitary practices being followed.

Even though this study was much smaller than both studies undertaken by Holah et al. *(79, 80)* in which *E. coli* was isolated in very few of the samples this study isolated *E. coli* from 19% of the salad samples and results of genotyping these *E. coli* isolates found that genetically related *E. coli* isolates were obtained several times over a six week period in one restaurant. Full service restaurants unlike these food processing facilities have complex working environments, lack physical barriers designed for pathogen prevention, and often lack designated working spaces to separate produce processing from other types of daily activities. Cleaning and sanitation procedures in restaurants may also not be as stringent as what was required by the RTE chilled food factories in Holah et al. *(79, 80)* studies. Full-service restaurants have also been found to engage in more risky food handling practices than other food service institutions *(147, 149, 150)*, and these risky food handling practices such as improperly cleaned and sanitized food contact surfaces and utensils may have allowed *E. coli* to effectively colonize a niche and become a common source of contamination within LO restaurant 5.

From 1998 through 2008, the FDA found that full-service restaurants have the highest “out-of-compliance” observations pertaining to the prevention of risky food handling for 9 distinct foodservice institutions responsible for preparing and serving

food to the public *(147, 149, 150).* Additional studies by other researchers have found that LO restaurants have performed worse on health department scores than CO restaurants, and that ethnic LO restaurants have performed worse on health department scores than non-ethnic LO restaurants, and ethnic and non-ethnic CO restaurants *(18, 88, 117-119).* It has also been found that LO restaurants were less likely to have written food safety plans and protocols in place than CO restaurants *(88, 117).* LO restaurant 5 is a small ethnic restaurant and it was the only restaurant in which genetically *related E. coli* was isolated from several salad samples over an extended period of time. This trend suggest that some ethnic LO restaurants might be more apt for not following food safety practices designed to prevent contamination of foods. Pathogenic strains of *E. coli* such as *E. coli O157:H7* have been shown to have the ability to form biofilms on food contact surfaces and on foods *(129).* If restaurants or any food processing facility do not properly clean and sanitize food contact surfaces and utensils and adhere to good hygiene practices it could provide environments in which pathogenic bacteria can cause prolong outbreaks of foodborne illnesses.

III.4.2 Genotyping Male-Specific F+RNA Coliphages

This study found that male-specific coliphages were present in leafy greens and specialty salads from both locally owned and corporate restaurants, and out the 40 samples testing positive for male-specific coliphages and of these only 30% of these were positive for *E. coli*. The food industry commonly uses *E. coli* as an indicator of fecal contamination, and the absence of *E. coli* in these salad samples highlights the potential for underestimating the occurrence of fecal contamination in the samples that were positive for male-specific coliphages. These findings were similar to Endley et al. *(52)* reported for the presence of *E. coli* and male-specific coliphages on carrots obtained from the field, transportation truck, and processing shed.

Coliphages and enteric viruses replicate in the gastrointestinal tracts of human and animals. Coliphages are similar in structure, composition, morphology, size, and site of replication. Much like enteric viruses which replicate in mammalian cells

coliphages replicate in coliforms which are present in the gastrointestinal tracts of humans and animals, and are released into the environment the same way through feces. Furthermore unlike traditional indicator organisms which can sometimes proliferate and persist in the environment coliphages like the F+RNA male specific coliphage replicate by attachment to bacteria which possess the F-pili. These pili only form on bacteria under certain growth conditions which predominantly occur in the gastrointestinal tracts of warm blooded mammals. Coliphages such as the F+RNA coliphages also share similar characteristics as enteric viruses to water treatments, disinfection processes in water and shellfish depuration, and have been found to share common seasonal trends when present in shellfish growing areas. Coliphages have also been found to be better correlated with the presence of enteric viruses in water, sewage, and shellfish than *E. coli*. F+FNA coliphages have similar survival times in water and on foods to enteric viruses than *E. coli*. These characteristics suggest the importance for the need to use additional indicator organisms for screening water and food for fecal contamination and as a suitable indicator for viral contamination *(9, 48, 49, 54, 56, 81, 94, 98, 132, 153).*

There is additional value for screening for F+RNA coliphages in assessing the microbiological quality of foods. F+RNA coliphages can be genotyped into four genogroups I, II, III, and IV. Genogroups I and IV are indicative of contamination associated with animal waste and genogroups II and III indicate contamination associated with human sewage or population based human contamination. The genotyping results of this study found that 7 of the male-specific coliphages isolates were F+RNA coliphages belonging to groups I, II, and III. These results suggest that raw produce and/or other raw food products used in preparation of these salads were contaminated with human and animal waste.

It is estimated that approximately 10% of the human population harbor and excrete F+RNA coliphages in their feces and F+RNA coliphages have been isolated from the feces of animal feces at variable rates. There are some issues involved with the heterogeneity of these F+RNA genogroups. Exceptions to genogroups and host types

have been seen in the feces from swine and seagulls which contain low concentrations of genogroup II F+RNA coliphages *(45, 53, 64, 65, 108).* Overall the presence and proportion of F+RNA coliphage genogroups I and IV are found in the feces of animals and animal waste waters and F+RNA coliphage genogroups II and III are the predominant groups found in population based human sources such as sewage *(45, 53, 64, 65, 108).*

The genotyping results for this study found that a specialty salad from CO restaurants 1 had male-specific coliphages belonging to II and III, and a specialty salad from CO restaurant 5 had male-specific coliphages belonging to genogroup III. Both of these salads were purchased during the same sampling period in mid-May 2010. A leafy greens salad from CO restaurant 2 was found to have male-specific coliphages belonging to genogroup II and this salad was purchased the day prior to the specialty salads containing genogroups II and III in mid-May. There were 2 specialty salads from LO restaurant 1 that contained F+RNA coliphages belonging to genogroup III and these salads were purchased on two consecutive days at the end of April. The salad samples containing F+RNA coliphages belonging to genogroups II and III indicate that the raw produce in these salads were exposed to human based contamination. A specialty salad and a leafy greens salad from LO restaurant 5 contained F+RNA coliphages belonging to genogroup I. These salad samples were purchased within a one week period of each other at the end of April 2010. F+RNA coliphage genogroups I and IV are found in the feces of animals and animal waste waters. Salad samples containing genogroup I suggest that the produce used in these salad samples were exposed to fecal contamination from animal feces or animal waste waters.

Since isolation of F+RNA coliphages belonging to genogroups II and III directly from human feces is rare, and it is unlikely that the produce positive for genogroup I came into contact with animal feces or animal waste waters in the restaurant this suggests that the produce used in these salads was exposed to fecal contamination in the field or during processing. Although direct exposure in the restaurants can occur as a

result of produce coming into contact with raw meats or improperly cleaned or sanitized food contact surfaces and utensils. Hsu et al. *(81)* isolated F+RNA coliphage belonging to genogroups II, III and IV from raw ground beef and chicken samples obtained from retail locations. Allwood et al. *(7)* and Endley et al. *(52)* found that produce that has undergone processing tested positive more often for male-specific coliphages than unprocessed produce. Allwood et al. *(7)* study is particularly pertinent because this study found that retail samples obtained from retail outlets and restaurants that had been processed were more likely to be contaminated with male specific coliphages than unprocessed samples.

Raw produce can become contaminated with foodborne pathogens at any point from cultivation through consumption and have been found to be a growing cause of foodborne illness outbreaks. Raw produce can become contaminated during harvest if exposed to manure, feces from wild animals, animal waste waters, fecally contaminated soil, untreated sewage, contaminated irrigation water, or poor hygienic practices by food workers. Postharvest contamination can occur if raw produce comes into contact with contaminated harvesting equipment, poor hygienic practices by food workers, contaminated water used for washing, chill tanks, sprays, or shipping ice. Contamination that occurs during food preparation before consumption can be caused by contact with improperly sanitized food contact surfaces, contact with raw meats, and dirty utensils as well as poor hygienic practices by food workers. Produce is often consumed raw and lacks a microbicidal step and it is important that growers, processors, distributors, and food preparation practices in the home and in the retail foodservice setting adhere to good agricultural practices, good manufacturing practices, and follow recommended safe food handling practices *(100, 109)*.

There are several types of produce and other food ingredients involved in preparing a restaurant salad. The produce that restaurants order can be prewashed, precut, and prepackaged or it can be delivered to the restaurants whole and unprocessed. Male-specific coliphages have been found to be more resistant than *E. coli* to chlorinated

washes and commercially available chemical washes. The efficacy of these disinfectants is dependent upon the concentration, the amount of inorganic particulates, and food particles present in the wash waters. Endley et a.l *(52)*reported that more carrots tested positive for male-specific coliphages than *E. coli* after processing, and found that the processing shed used recycled chlorinated wash waters to wash the carrots. If the water used for washing produce is not properly maintained the effectiveness of the wash step can become ineffective at inactivating more resistant microorganisms such as the male-specific coliphage and eventually become a source of contamination.

This study found relatively low counts of male-specific coliphages in these restaurant salads. Out of the 40 salads that tested positive for the presence of male-specific coliphages with the enrichment assay, only 20 tested positive with the quantitative method. Similar results for the presence and quantification of male-specific coliphages were reported by Endley et al. *(51)* for retail samples of cilantro and parsley. Leganani et al. *(95)* reported that 19% of the unprocessed produce samples from a processing facility were positive for coliphages, and quantitative analysis found that male-specific coliphage counts did not exceed 2.48 log PFU/10g. This study reported levels of coliphages as high as log 2.16 PFU/g. Overall salads from CO restaurants did not exceed 0.18 log PFU/g. There were several salad samples from LO restaurants that exceed the level of male-specific coliphages found in CO restaurants salads. Only 7% (7 of 100) of the leafy greens salads were positive for male-specific coliphages, and 33% (33 of 100) of the specialty salads were positive for male-specific coliphages. There were only 5 salad samples obtained from corporate restaurants that tested positive for the quantitative method and 14 salad samples that tested positive by quantitative analysis for LO restaurants.

From 1998 through 2008, the FDA found that full-service restaurants have the highest “out-of-compliance” observations pertaining to the prevention of risky food handling for 9 distinct foodservice institutions responsible for preparing and serving food to the public *(147, 149, 150).* Additional studies by other researchers have found

that LO restaurants have performed worse on health department scores than CO restaurants, and that ethnic LO restaurants have performed worse on health department scores than non-ethnic LO restaurants, and ethnic and non-ethnic CO restaurants *(18, 88)*. It has also been found that LO restaurants were less likely to have written food safety plans and protocols in place than CO restaurants *(117)*. LO restaurant 5 is a small ethnic restaurant and it was the only restaurant in which genetically related *E. coli* was isolated from several salad samples over an extended period of time. A majority of the salad samples testing positive above the detection limit were from LO restaurants 1 and 5, and these samples had the highest male-specific coliphage counts out of all the restaurants tested. There were a total of 5 LO restaurants sampled. There were 60 salads sampled from LO restaurants 2, 3, and 4 and of those salad samples only 2 tested positive for the presence of male-specific coliphages. LO restaurants 1 and 5 were small ethnic restaurants. The high male-specific coliphage counts found in these restaurants suggests several conclusions. The first conclusion is that the raw produce coming into these restaurants was heavily contaminated somewhere along the processing chain prior to delivery, and washing with water or chemical washes was insufficient to remove the high levels of contamination. The second conclusion is that the raw produce processing/disinfection step in these restaurants was inadequate or performed improperly which would normally remove low levels of coliphages from produce. The third conclusion is that the raw produce in these restaurants came into contact with improperly cleaned and sanitized food contact surfaces and utensils that may have been exposed to a another food item which contained male-specific coliphages such as raw meats.

III.5 Conclusion

Testing for indicator organisms with traditional culture based methods helps researchers to determine the overall microbiological quality of our foods, but cannot provide any information about the source of contamination. Molecular methods have been used as a tool to rapidly and effectively differentiate foodborne outbreak and non-outbreak strains and identify sources of food and water contamination. The use of Diversilab™ systems automated rep-PCR and RT-PCR for DNA fingerprinting and genotyping *E. coli* and male-specific coliphage isolates obtained from restaurant salads was able to identify several possible routes of contamination and possible sources of fecal contamination. DNA fingerprinting analysis trends suggest that fresh produce entering these restaurants were exposed to a common source of contamination during field production, processing, or distribution and that the source of contamination could be recurrent for extended periods of time. Another trend suggests that contamination of fresh produce in a single restaurant can occur for an extended period of time as a result of risky food handling practices or the presence of an environmentally persistent strain of bacteria. Genotyping male-specific coliphages revealed that the source of contamination in some of these restaurant salads were from both human and animal sources. This study along with the findings of Endley et al. *(52)* and Allwood et al. *(7)* found that male-specific coliphages were more often present in foods that have been subjected to processing. Additionally, this study found that coliphages could potentially be used as another indicator of sanitary practices in food processing environments.

Having the ability to identify routes of contamination and identify source of contamination can aide researchers and governmental agencies in developing more effective intervention strategies, increase the level of public health, and improve the hygienic quality of our foods. DiversiLab™ systems automated rep-PCR is a relatively simple procedure to perform and allows for several samples to be rapidly typed. Its web-based software allows the user to easily and quickly analyze data and differentiate species, subspecies, and strains of bacteria *(75)*. DiversiLab™ systems automated rep-

PCR tool has been used successfully to build libraries of *E. coli* isolates for microbial source tracking studies *(157).* Future areas of study could include the routine microbiological testing of food and environmental samples obtained during field production, processing, distribution, and preparation in retail and restaurant settings for *E. coli* and use this DNA fingerprinting tool to build libraries for comparison. This would differ from microbial source tracking in that this approach would be trying to effectively identify common areas or common source of contamination that may occur whether it is at particular farm or distributor. Epidemiological and traceback investigations usually occur after the fact this approach could effectively identify problem areas or growers that may be consistently more prone to contamination issues, and based upon these findings targeted interventions could be implemented potentially and preemptively decreasing the risks of foodborne illness outbreaks to occur.

Male-specific coliphages are good alternative indicators that can be used along with *E. coli* to indicate the presence of fecal contamination. Male-specific coliphages have also been found to be a useful as an indicator for viral contamination. F+RNA male-specific coliphages can be genotyped and provide information on whether the source of contamination is human or animal *(45, 64, 65).* Since F+RNA coliphages are indicators of fecal and viral contamination the finding that genogroups II and III were present in restaurant salads is a strong indication that fecal contamination has occurred and that there is an increased risk for the presence of human enteric pathogens such as Norovirus or Hepatitis A *(7).*

Previous results from the microbiological survey of indicator organisms present in restaurant salads suggest that there are differences in produce handling between LO and CO restaurants. LO restaurants 1 and 5 are ethnic LO restaurants which tested positive for male-specific coliphages more often and in higher counts than all the other restaurants sampled. Genotyping results for male-specific coliphages found that the raw produce or other ingredients in these salads were exposed to human and animal source of fecal contamination. This contamination is most likely to occur at some point along the

produce processing chain, but could be a result of cross-contamination issues involving direct contact with raw meats, improperly cleaned food contact surfaces and utensils, or poor hygienic practices by food workers. The DNA fingerprinting results from cluster B suggests that the produce used in the restaurant salads from LO restaurant 5 is being exposed to a common source of contamination within the restaurant and is most likely due to risky food handling practices. These finding suggest that there is a need for better food safety education based programs for ethnic LO restaurants in regards to produce safety, although it could be said that the problem is due to a lack of standardized produce processing practices for the restaurant industry as a whole.

CHAPTER IV
CONCLUSION

IV.1 Conclusion

Overall the results of this study suggest that safe food handling practices are still lacking in both LO and CO restaurants. LO restaurants were found to have higher levels of APC, *Enterococcus,* and male-specific coliphages in all of their salads, and higher levels of male-specific coliphage in their specialty salads. CO restaurants were found to have higher levels of coliforms and somatic coliphages in their salads. There were significantly higher levels of *E. coli* in specialty salads purchased during low volumes of customer traffic than in specialty salads purchased during high volumes of customer traffic. There were significantly higher levels of *E. coli* in specialty salads than leafy greens salads purchased during low volumes of customer traffic. There were significantly higher levels of coliforms and somatic coliphages in CO restaurant salads than LO restaurant salads purchased during low volumes of customer traffic. LO restaurant salads also had significantly higher levels of male specific coliphages in salads purchased during low volumes of customer traffic. This suggests that risky food handling practice were more likely to occur when fewer food workers are present to prepare salads during low volumes of business. Specialty salads were found to have higher levels of all indicator organisms. This finding suggest that salads containing more than one fresh produce item requires additional handling and processing and therefore increases the potential risk of cross-contamination.

Several studies have shown that the levels of microorganisms can differ based upon produce type and that processing can also influence the levels of microorganisms dependent upon produce type *(1, 2, 8, 86).* Fresh produce such as parsley have been found to have higher counts of APC, coliforms, *Enterococcus,* and *E. coli* than leafy greens such as lettuce *(1, 2, 8, 86).* It could be beneficial for restaurants and other retail food service establishments to have standardized procedures that would suggest an order in which to process produce and at which times it would be the most beneficial to clean

and sanitize utensils and food contact surfaces. This could decrease the risk of cross-contamination between fresh produce items in the kitchen. Time constraints due to increased business volumes during high customer traffic volumes and a lack in labor during low customer traffic volumes can decrease the amount of time and hinder the abilities of food workers to perform safe food handling practices. Since fresh produce is at higher risk for contamination there is a need to design a study that directly looks at the differences between how many people handle a salad under time constrained situations. It would be beneficial for the food industry to implement microbiological testing of produce and produce processing in restaurants from the time of delivery, during storage, after washing, shredding, centrifugation, and storage. These studies need to obtain produce from cold storage in both the walk-in refrigeration units and the table top units before, during and after lunch and dinner shifts.

The use of DiversiLab systems automated rep-PCR and RT-PCR for DNA fingerprinting and genotyping E. coli and male-specific coliphage isolates obtained from restaurant salads was able to identify several possible routes of contamination and possible sources of fecal contamination. DNA fingerprinting analysis trends suggest that fresh produce entering these restaurants were exposed to a common source of contamination during field production, processing, or distribution and that the source of contamination could be recurrent for extended periods of time. Another trend suggests that contamination of fresh produce in a single restaurant can occur for an extended period of time as a result of risky food handling practices or the presence of an environmentally persistent strain of bacteria. Genotyping male-specific coliphages revealed that the source of contamination in some of these restaurant salads were from both human and animal sources. This study along with the findings of Endley et al. *(52)* and Allwood et al. *(7)* found that male-specific coliphages were more often present in foods that have been subjected to processing. Additionally, this study found that coliphages could potentially be used as another indicator of sanitary practices in food processing environments.

Having the ability to identify routes of contamination and identify source of contamination can aide researchers and governmental agencies in developing more effective intervention strategies, increase the level of public health, and improve the hygienic quality of our foods. DiversiLab systems automated rep-PCR is a relatively simple procedure to perform and allows for several samples to be rapidly typed. Its web-based software allows the user to easily and quickly analyze data and differentiate species, subspecies, and strains of bacteria *(75)*. DiversiLab systems automated rep-PCR tool has been used successfully to build libraries of *E. coli* isolates for microbial source tracking studies *(157)*. Future areas of study could include the routine microbiological testing of food and environmental samples obtained during field production, processing, distribution, and preparation in retail and restaurant settings for *E. coli* and use this DNA fingerprinting tool to build libraries for comparison. This would differ from microbial source tracking in that this approach would be trying to effectively identify common areas or common source of contamination that may occur whether it is at particular farm or distributor. Epidemiological and traceback investigations usually occur after the fact. The approach of identify problem areas or growers that are consistently prone to contamination issues using molecular methods to identify genetically related indicator organism could effectively help with targeted interventions. The implementation of these targeted interventions could preemptively decrease the risk of foodborne illness outbreaks to occur.

Male-specific coliphages were found in salad samples that did not contain *E. coli* which suggest that there is a need for their use as an additional indicator of fecal contamination. Male-specific coliphages have also been found to be useful as an indicator for viral contamination. Since coliphages are indicators of fecal and viral contamination the finding that they were present in restaurant salads is a strong indication that fecal contamination has occurred and that there is an increased risk for the presence of human enteric pathogens such as Norovirus or Hepatitis A *(7)*.

Previous results from the microbiological survey of indicator organisms present in restaurant salads suggest that there are differences in produce handling between LO and CO restaurants. LO restaurants 1 and 5 are ethnic LO restaurants were found to have significantly higher levels of male-specific coliphages than all the other restaurants sampled. Genotyping results for male-specific coliphages found that the raw produce or other ingredients in these salads were exposed to human and animal source of fecal contamination. This contamination is most likely to occur at some point along the produce processing chain, but could be a result of cross-contamination issues involving direct contact with raw meats, improperly cleaned food contact surfaces and utensils, or poor hygienic practices by food workers. The DNA fingerprinting results from cluster B suggests that the produce used in the restaurant salads from LO restaurant 5 is being exposed to a common source of contamination within the restaurant and is most likely due to risky food handling practices. This suggests that LO restaurants may need a better understanding of the microbiological risk factors involved with fresh produce processing.

Overall this study highlights that there are continued problems with risky food handling practices by food workers in the full-service restaurant industry. There is a need for better food safety education based programs pertaining to safe produce processing in the restaurant industry and that there is a need for food safety education based programs better suited for ethnic LO restaurants in regards to produce safety. The fresh produce industry could also benefit greatly from routine monitoring and apply molecular based techniques for microbial source tracking to quickly and easily identify problem growers, distributors, and processors who might not be practicing GAPs, GMP, and safe food handling practices.

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APPENDIX

Locally Owned Specialty Salads Low Volume Customer Traffic

Sample ID	APC (CFU/g)	Coliforms (MPN/g)	*E. Coli* (MPN/g)	*Enterococcus* (MPN/g)	Male-Specific Coliphage (PFU/g)	Somatic Coliphage (PFU/g)
Restaurant 1A	6.88E+08	>2419.6	2.97E+01	2.96E+03	BD*	0
Restaurant 1B	3.25E+07	8.50E+02	0	2.76E+02	0	0
Restaurant 1C	1.75E+08	2.85E+04	0	9.55E+01	6.05E-01	0
Restaurant 1D	1.62E+07	>2419.6	1.32E+02	1.41E+03	1.06E+00	0
Restaurant 1E	5.09E+08	5.11E+04	2.76E+01	6.73E+02	5.09E+00	0
Restaurant 2A	1.35E+08	5.88E+03	1.49E+00	3.08E+03	0	0
Restaurant 2B	8.31E+06	1.36E+03	0	5.04E+02	0	BD
Restaurant 2C	2.59E+07	1.20E+04	0	1.73E+03	0	BD
Restaurant 2D	1.06E+08	2.01E+05	0	4.82E+02	0	3.84E-02
Restaurant 2E	3.86E+08	5.03E+03	0	9.00E+01	0	0
Restaurant 3A	4.02E+08	>2419.6	0	2.56E+02	0	0
Restaurant 3B	9.06E+06	1.18E+04	0	2.10E+00	0	0
Restaurant 3C	2.13E+08	1.96E+02	2.97E+01	4.93E+00	0	0
Restaurant 3D	1.12E+08	>2419.6	0	1.92E+01	0	0
Restaurant 3E	2.79E+09	>2419.6	0	1.76E+02	0	BD
Restaurant 4A	3.54E+07	3.87E+05	0	1.04E+02	0.00E+00	0
Restaurant 4B	2.96E+06	1.97E+01	0	4.90E+02	0.00E+00	0
Restaurant 4C	4.41E+08	6.28E+02	0	5.28E+02	0.00E+00	0
Restaurant 4D	2.88E+07	>2419.6	4.00E-01	4.84E+03	BD*	0
Restaurant 4E	1.65E+07	9.81E+04	0	6.49E+01	0.00E+00	0
Restaurant 5A	1.60E+08	8.77E+04	0	2.24E+02	BD	0
Restaurant 5B	9.79E+05	2.02E+02	1.00E-01	1.42E+01	BD	0
Restaurant 5C	4.29E+07	1.62E+05	0	4.86E+02	1.46E+02	0
Restaurant 5D	3.01E+06	2.58E+05	0	7.98E+01	8.40E+00	0
Restaurant 5E	1.47E+08	>2419.6	1.35E+02	3.14E+02	0	0

* Below Detection Limit

Locally Owned Specialty Salads High Volume Customer Traffic

Sample ID	APC (CFU/g)	Coliforms (MPN/g)	*E. Coli* (MPN/g)	*Enterococcus* (MPN/g)	Male-Specific Coliphage (PFU/g)	Somatic Coliphage (PFU/g)
Restaurant 1A	7.78E+07	3.60E+04	0	3.87E+00	7.78E-02	BD
Restaurant 1B	4.88E+08	2.40E+04	0	1.05E+02	BD*	0
Restaurant 1C	3.58E+07	2.61E+04	0	8.91E+00	2.26E-01	2.26E-01
Restaurant 1D	3.60E+07	6.00E+01	0	8.16E+00	0	BD*
Restaurant 1E	9.78E+07	>2419.6	0	3.96E+03	5.31E+00	0
Restaurant 2A	2.40E+08	4.80E+03	3.96E-01	1.70E+00	0	0
Restaurant 2B	5.70E+08	5.11E+03	0	5.82E+02	0	8.00E-02
Restaurant 2C	3.32E+07	4.05E+03	0	1.22E+01	BD*	0
Restaurant 2D	2.71E+08	1.22E+04	0	1.86E+03	0	0
Restaurant 2E	4.88E+08	4.04E+04	0	9.91E+02	0	0
Restaurant 3A	8.82E+08	3.46E+05	0	1.76E+02	0	0
Restaurant 3B	1.28E+09	>2419.6	0	5.33E+00	0	0
Restaurant 3C	6.45E+08	3.92E+05	0	5.11E+01	0	0
Restaurant 3D	2.54E+08	1.97E+04	0	4.50E+00	0	0
Restaurant 3E	1.42E+09	2.72E+05	0	1.94E+03	0	0
Restaurant 4A	2.31E+07	1.16E+03	0	3.10E+03	0	0
Restaurant 4B	1.06E+07	3.43E+04	1.98E-01	1.62E+01	0	0
Restaurant 4C	5.64E+06	8.50E+02	0	7.45E+01	0	0
Restaurant 4D	5.52E+06	2.96E+02	0	3.11E+01	0	BD
Restaurant 4E	7.46E+07	1.60E+05	0	1.79E+02	0	1.18E-01
Restaurant 5A	9.60E+07	1.24E+05	7.37E+00	1.62E+02	BD	0
Restaurant 5B	4.61E+08	5.64E+04	2.25E+00	4.66E+02	4.83E+01	2.25E-01
Restaurant 5C	2.05E+06	1.59E+05	0	3.03E+01	BD	0
Restaurant 5D	8.49E+06	1.23E+04	4.03E-01	1.21E+02	1.19E+02	8.06E-02
Restaurant 5E	2.44E+08	>2419.6	3.44E+01	1.89E+02	0	0

* Below Detection Limit

Locally Owned Leafy Greens Salads Low Volume Customer Traffic

Sample ID	APC (CFU/g)	Coliforms (MPN/g)	*E. Coli* (MPN/g)	*Enterococcus* (MPN/g)	Male-Specific Coliphage (PFU/g)	Somatic Coliphage (PFU/g)
Restaurant 1A	3.01E+07	4.00E+04	0	1.41E+01	0	0
Restaurant 2A	5.32E+06	2.02E+00	0	2.02E+00	0	0
Restaurant 3A	2.76E+07	2.20E+04	0	8.06E-01	0	0
Restaurant 4A	2.16E+07	>2419.6	0	4.22E+01	0	0
Restaurant 5A	3.49E+07	2.34E+02	0	1.02E+00	5.51E+00	0
Restaurant 2A	3.02E+06	1.47E+02	3.96E-01	1.43E+01	0	0
Restaurant 2B	2.07E+05	3.85E+01	0	4.59E+02	0	0
Restaurant 2C	2.37E+07	1.90E+01	3.79E-01	9.80E+02	0	0
Restaurant 2D	4.46E+06	5.52E+04	0	6.16E+02	0	BD*
Restaurant 2E	1.36E+07	5.71E+02	0	2.12E+01	0	0
Restaurant 3A	6.49E+06	3.97E+02	0	3.15E+00	0	0
Restaurant 3B	6.29E+05	>2419.6	0	1.94E+00	0	0
Restaurant 3C	7.25E+07	3.54E+04	0	5.88E+02	0	0
Restaurant 3D	4.55E+06	5.81E+03	0	2.42E+00	0	0
Restaurant 3E	4.87E+06	9.22E+03	0	2.50E-01	0	0
Restaurant 4A	8.25E+05	4.18E+02	0	7.11E+00	0	0
Restaurant 4B	4.55E+05	7.54E+00	0	7.56E+01	0	0
Restaurant 4C	1.10E+06	7.60E+02	0	1.46E+01	0	0
Restaurant 4D	3.74E+06	2.33E+04	0	3.21E+01	0	0
Restaurant 4E	1.67E+06	4.68E+03	0	3.33E+01	0	0
Restaurant 5A	3.37E+07	>2419.6	5.81E-01	6.67E+00	0	0
Restaurant 5B	1.60E+05	6.21E+02	0	1.29E+00	0	0
Restaurant 5C	7.14E+07	7.26E+03	0	0	6.51E+00	0
Restaurant 5D	5.78E+05	3.05E+04	0	0	0	0
Restaurant 5E	4.11E+06	1.80E+02	0	3.93E-01	0	0

* Below Detection Limit

Locally Owned Leafy Greens Salads High Volume Customer Traffic

Sample ID	APC (CFU/g)	Coliforms (MPN/g)	*E. Coli* (MPN/g)	*Enterococcus* (MPN/g)	Male-Specific Coliphage (PFU/g)	Somatic Coliphage (PFU/g)
Restaurant 1A	4.99E+07	3.04E+04	0	2.00E-01	BD*	0
Restaurant 2A	3.67E+08	4.57E+03	0	0	0	0
Restaurant 3A	2.15E+07	3.90E+01	0	0	0	0
Restaurant 4A	1.09E+08	2.15E+02	0	1.96E-01	0	0
Restaurant 5A	2.54E+07	3.34E+05	0	6.06E+00	0	0
Restaurant 2A	2.01E+07	3.66E+02	0	5.35E+00	0	0
Restaurant 2B	3.47E+06	7.95E+03	0	8.52E+02	0	0
Restaurant 2C	3.43E+06	4.58E+01	0	4.08E+00	0	0
Restaurant 2D	6.27E+06	6.87E+02	0	>2419.6	0	0
Restaurant 2E	2.70E+06	3.04E+04	0	8.31E+02	0	0
Restaurant 3A	1.54E+07	2.39E+04	0	0	0	0
Restaurant 3B	1.08E+06	2.98E+03	0	2.27E-01	0	0
Restaurant 3C	1.39E+05	1.02E+02	0	0	0	0
Restaurant 3D	2.84E+05	1.22E+03	0	0	0	0
Restaurant 3E	4.79E+07	2.31E+05	0	5.71E+03	0	0
Restaurant 4A	1.75E+06	9.99E+01	0	3.82E+03	0	0
Restaurant 4B	9.53E+05	1.14E+03	2.05E-01	3.02E+00	0	0
Restaurant 4C	7.48E+05	6.89E+02	0	3.26E+01	0	0
Restaurant 4D	2.07E+05	1.15E+02	0	6.54E+00	0	0
Restaurant 4E	2.80E+06	1.39E+04	0	6.20E+00	0	1.52E-01
Restaurant 5A	8.03E+06	7.57E+03	0	3.04E+00	9.18E-01	0
Restaurant 5B	2.25E+07	1.51E+05	0	1.02E+00	1.49E+00	0
Restaurant 5C	1.15E+07	3.24E+03	0	3.85E-01	0	0
Restaurant 5D	4.33E+05	7.75E+02	0	1.12E+01	0	0
Restaurant 5E	4.22E+06	1.72E+05	0	0	0	0

* Below Detection Limit

Corporate Owned Specialty Salads Low Customer Traffic Volume

Sample ID	APC (CFU/g)	Coliforms (MPN/g)	*E. Coli* (MPN/g)	*Enterococcus* (MPN/g)	Male-Specific Coliphage (PFU/g)	Somatic Coliphage (PFU/g)
Restaurant 1A	5.98E+06	1.42E+03	0	1.90E+02	0	0
Restaurant 2A	1.47E+07	7.49E+02	0	3.49E+01	0	0
Restaurant 3A	5.39E+05	1.01E+04	2.11E+02	8.72E+01	0	0
Restaurant 4A	2.14E+05	1.53E+03	0	1.48E+01	0	0
Restaurant 5A	1.50E+08	4.99E+03	0	2.11E+01	1.54E+00	0
Restaurant 2A	6.17E+06	6.93E+04	0	1.20E+02	0	0
Restaurant 2B	1.52E+07	3.20E+04	0	1.69E+01	BD*	0
Restaurant 2C	6.74E+07	7.71E+03	0	>2419.6	BD	1.57E-01
Restaurant 2D	2.79E+07	1.89E+05	0	3.88E+00	1.93E-01	1.29E-01
Restaurant 2E	1.32E+09	4.74E+04	1.76E+01	6.08E+00	0	2.05E+01
Restaurant 3A	1.99E+07	8.79E+03	1.77E+00	1.54E+02	0	BD
Restaurant 3B	9.27E+06	4.93E+03	1.99E+00	1.25E+01	0	2.39E-01
Restaurant 3C	3.21E+07	1.16E+05	2.09E+02	0	0	0
Restaurant 3D	1.76E+07	1.53E+05	0.00E+00	1.18E+00	0	BD
Restaurant 3E	4.69E+07	3.44E+04	0	2.75E+01	0	7.35E-02
Restaurant 4A	7.90E+05	2.95E+03	1.82E+00	1.56E+01	0	BD
Restaurant 4B	3.84E+07	2.26E+03	2.14E+02	4.72E+01	BD	2.47E+00
Restaurant 4C	3.76E+08	>2419.6	4.02E-01	>2419.6	0	BD
Restaurant 4D	1.81E+07	>2419.6	0	>2419.6	0	4.03E-02
Restaurant 4E	1.32E+08	2.77E+04	0	2.77E+01	0	2.29E-01
Restaurant 5A	2.47E+06	2.14E+03	0	2.04E+02	0	0
Restaurant 5B	3.46E+06	6.96E+04	0	0	0	2.84E-01
Restaurant 5C	8.33E+05	3.21E+04	0	1.92E-01	0	0
Restaurant 5D	3.47E+05	1.38E+04	0	1.89E-01	0	8.29E-01
Restaurant 5E	1.58E+08	1.53E+03	4.01E-01	2.21E+00	1.20E-01	0

* Below Detection Limit

Corporate Owned Specialty Salads High Customer Traffic Volume

Sample ID	APC (CFU/g)	Coliforms (MPN/g)	*E. Coli* (MPN/g)	*Enterococcus* (MPN/g)	Male-Specific Coliphage (PFU/g)	Somatic Coliphage (PFU/g)
Restaurant 1A	2.80E+06	1.36E+03	0	1.23E+01	0	0
Restaurant 2A	2.13E+05	6.79E+03	0	2.53E+01	0	BD
Restaurant 3A	1.11E+06	1.54E+04	3.41E+01	8.88E+00	BD*	BD
Restaurant 4A	1.84E+07	5.81E+04	0	0	0	0
Restaurant 5A	2.72E+08	1.40E+04	0	2.41E+01	1.38E+00	0
Restaurant 2A	6.08E+06	2.47E+03	0	1.04E+01	0	BD
Restaurant 2B	2.25E+07	6.90E+04	0	0	BD*	0
Restaurant 2C	6.99E+06	1.83E+04	0	0	BD	BD
Restaurant 2D	1.66E+06	8.27E+04	0	0	BD	BD
Restaurant 2E	3.85E+08	2.32E+05	0	2.26E+01	2.32E-01	2.32E-01
Restaurant 3A	1.62E+07	1.65E+04	0	3.31E+03	BD	0
Restaurant 3B	5.02E+06	3.13E+04	0	1.09E+01	0	2.39E-01
Restaurant 3C	9.88E+06	9.02E+04	0	0	0	0
Restaurant 3D	1.11E+06	6.42E+02	0	1.67E+02	BD	BD
Restaurant 3E	1.67E+08	1.26E+05	1.80E+00	8.21E+01	0	2.46E+00
Restaurant 4A	1.57E+05	1.03E+04	0	4.42E+02	0	0
Restaurant 4B	2.10E+07	7.00E+03	3.33E+00	7.60E+01	BD	1.27E+00
Restaurant 4C	2.77E+07	>2419.6	0	2.98E+02	0	1.48E-01
Restaurant 4D	2.20E+07	9.67E+04	0	2.43E+01	0	0
Restaurant 4E	2.62E+08	>2419.6	2.00E-01	2.19E+02	0	4.01E-02
Restaurant 5A	1.50E+06	1.80E+03	1.70E+00	1.70E+01	0	BD
Restaurant 5B	2.85E+06	1.08E+04	0	1.78E+00	0	1.07E+00
Restaurant 5C	3.00E+06	1.64E+05	0	0	0	0
Restaurant 5D	4.80E+06	6.68E+04	0	0	0	0
Restaurant 5E	2.99E+06	3.34E+05	1.91E+01	7.68E+01	0	0

* Below Detection Limit

Corporate Owned Leafy Greens Salads Low Customer Traffic Volume

Sample ID	APC (CFU/g)	Coliforms (MPN/g)	*E. Coli* (MPN/g)	*Enterococcus* (MPN/g)	Male-Specific Coliphage (PFU/g)	Somatic Coliphage (PFU/g)
Restaurant 1A	5.94E+06	1.92E+02	0	1.92E+00	0	0
Restaurant 2A	1.37E+04	1.43E+03	0	0	0	0
Restaurant 3A	8.69E+05	2.33E+04	0	2.11E+00	0	0
Restaurant 4A	2.61E+05	1.53E+02	0	1.43E+00	0	0
Restaurant 5A	3.56E+06	3.66E+02	0	2.09E+00	0	0
Restaurant 2A	3.84E+05	1.30E+03	0	0	0	0
Restaurant 2B	9.94E+04	1.08E+05	0	0	0	0
Restaurant 2C	1.01E+06	7.07E+04	5.80E+02	0	0	0
Restaurant 2D	1.46E+05	4.67E+04	0	3.91E-01	0	0
Restaurant 2E	6.03E+06	3.53E+04	0	0	0	0
Restaurant 3A	5.62E+05	4.90E+03	0	0	0	0
Restaurant 3B	7.21E+05	1.01E+04	0	0	0	0
Restaurant 3C	2.27E+06	2.03E+05	1.94E+01	0	0	0
Restaurant 3D	1.64E+06	5.44E+04	0	2.89E+00	0	0
Restaurant 3E	7.74E+06	4.92E+02	0	0	0	0
Restaurant 4A	1.31E+05	1.20E+02	0	1.53E+01	0	0
Restaurant 4B	3.41E+05	6.33E+03	0	0	0	0
Restaurant 4C	4.71E+06	2.24E+05	4.01E+00	1.48E+02	0	0
Restaurant 4D	8.94E+05	7.31E+04	0	1.85E+01	0	0
Restaurant 4E	3.48E+06	2.04E+03	0	1.95E-01	0	0
Restaurant 5A	1.02E+06	9.40E+01	0	1.88E+00	0	0
Restaurant 5B	2.32E+06	8.68E+02	0	1.92E+00	0	0
Restaurant 5C	8.92E+05	2.89E+04	0	0	0	0
Restaurant 5D	5.66E+06	4.24E+04	0	3.80E-01	0	0
Restaurant 5E	4.63E+07	4.40E+04	1.93E-01	6.35E+00	0	0

* Below Detection Limit

Corporate Owned Leafy Greens Salads High Customer Traffic Volume

Sample ID	APC (CFU/g)	Coliforms (MPN/g)	*E. Coli* (MPN/g)	*Enterococcus* (MPN/g)	Male-Specific Coliphage (PFU/g)	Somatic Coliphage (PFU/g)
Restaurant 1A	3.76E+06	6.91E+02	0	0	0	BD
Restaurant 2A	1.19E+04	2.51E+03	0	0	BD*	0
Restaurant 3A	2.39E+05	1.29E+03	0	0	0	0
Restaurant 4A	3.04E+04	1.48E+03	0	0	0	0
Restaurant 5A	7.27E+07	4.65E+05	0	4.03E+01	0	0
Restaurant 2A	8.94E+05	6.29E+01	0	2.34E+01	0	0
Restaurant 2B	2.78E+06	>2419.6	0	0	0	0
Restaurant 2C	1.31E+06	1.43E+04	0	0	0	0
Restaurant 2D	3.63E+06	8.55E+03	0	0	0	BD
Restaurant 2E	6.91E+06	1.32E+05	0	1.92E-01	3.84E-02	0
Restaurant 3A	1.41E+06	7.69E+02	0	0	0	0
Restaurant 3B	1.17E+05	2.54E+04	0	0	0	0
Restaurant 3C	5.36E+05	3.78E+04	0	0	0	0
Restaurant 3D	3.05E+05	3.14E+03	0	0	0	0
Restaurant 3E	2.78E+06	1.90E+04	0	0	0	0
Restaurant 4A	5.14E+04	2.19E+02	0	2.47E+02	0	0
Restaurant 4B	2.13E+04	8.10E+02	0	4.42E+01	0	0
Restaurant 4C	2.55E+05	9.47E+03	0	5.96E+00	0	0
Restaurant 4D	3.69E+05	3.93E+04	0	1.92E+00	0	0
Restaurant 4E	2.96E+06	7.16E+03	0	3.92E-01	0	0
Restaurant 5A	1.74E+06	6.63E+03	0	0	0	BD
Restaurant 5B	1.31E+05	1.99E+02	0	1.96E+00	0	0
Restaurant 5C	9.56E+05	6.60E+04	0	0	0	0
Restaurant 5D	1.81E+05	2.51E+02	0	0	0	0
Restaurant 5E	4.15E+06	1.07E+04	4.57E+02	1.71E+02	0	0

* Below Detection Limit

Locally Owned Restaurants vs Corporate Owned Restaurants

Sample ID	*n*	*E. Coli* Positive	*E. Coli* %	*Enterococcus* Positive	*Enterococcus* %	Male-Specific Coliphage Positive	Male-Specific Coliphage %	Somatic Coliphage Positive	Somatic Coliphage %
Locally Owned	*100*	18	18%	92	92%	23	23%	14	14%
Corporate Owned	*100*	20	20%	65	65%	17	17%	31	30%
Locally Owned House Salad	*50*	4	8%	42	84%	5	10%	2	4%
Corporate Owned House Salad	*50*	5	10%	24	48%	2	4%	3	6%
Locally Owned Specialty Salad	*50*	14	28%	50	100%	18	36%	12	24%
Corporate Owned Specialty Salad	*50*	15	30%	41	82%	15	30%	28	56%
Locally Owned Low Customer Traffic Volume	*50*	11	22%	48	96%	11	22%	5	10%
Corporate Owned Low Customer Traffic Volume	*50*	13	26%	37	74%	6	12%	14	28%
Locally Owned High Customer Traffic Volume	*50*	7	14%	44	88%	12	24%	9	18%
Corporate Owned High Customer Traffic Volume	*50*	7	14.00%	28	56%	11	22%	17	34%
Locally Owned House Salad High Volume Customer Traffic	*25*	1	4%	19	76%	3	12%	1	4%
Corporate Owned House Salad High Customer Traffic Volume	*25*	1	4%	10	40%	2	8%	3	12%
Locally Owned House Salad Low Customer Traffic Volume	*25*	3	12%	23	92%	2	8%	1	4%
Corporate Owned House Salad Low Customer Traffic Volume	*25*	4	16%	14	56%	0	0%	0	0%
Locally Owned Specialty Salad High Customer Traffic Volume	*25*	6	24%	25	100%	9	36%	8	32%
Corporate Owned Specialty Salad High Customer Traffic Volume	*25*	6	24%	18	72%	9	36%	14	56%

Locally Owned Specialty Salad Low Customer Traffic Volume	*25*	8	32%	25	100%	9	36%	4	16%
Corporate Owned Specialty Salad Low Customer Traffic Volume	*25*	9	36%	23	92%	6	24%	14	56%

Leafy Greens Salad vs Specialty Salad

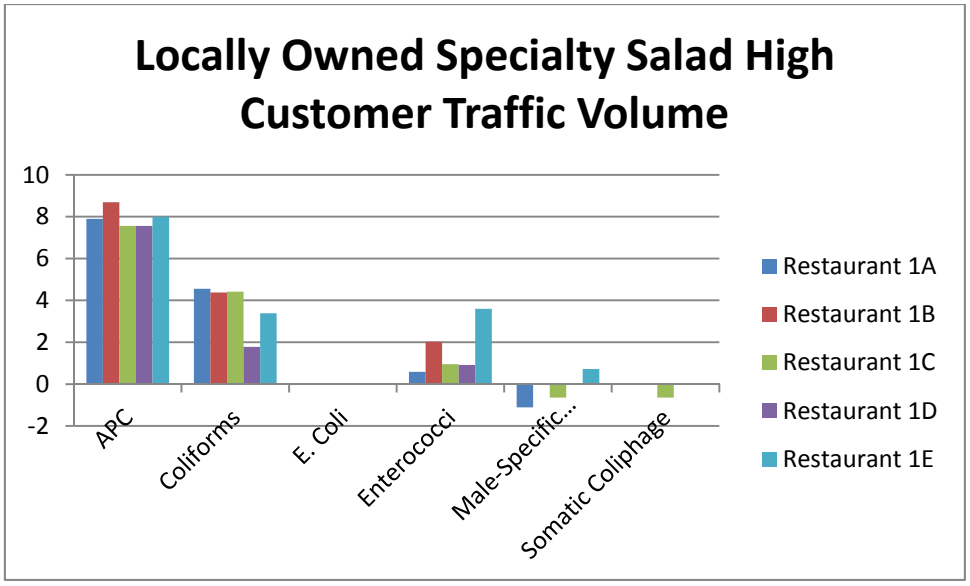
		E. Coli		*Enterococcus*		Male-Specific Coliphage		Somatic Coliphage	
Sample ID	*n*	Positive	%	Positive	%	Positive	%	Positive	%
Specialty Salad	*100*	29	29%	91	91%	33	33%	40	40%
Leaf Greens Salad	*100*	9	9%	66	66%	7	7%	5	5%
Locally Owned Specialty Salad	*50*	14	28%	50	100%	18	36%	12	24%
Locally Owned Leafy Greens Salad	*50*	4	8%	42	84%	5	10%	2	4%
Corporate Owned Specialty Salad	*50*	15	30%	41	82%	15	30%	28	56%
Corporate Owned House Salad	*50*	5	10%	24	48%	2	4%	3	6%
Specialty Salad Low Customer Traffic Volumes	*50*	17	34%	48	96%	15	30%	18	36%
Leafy Greens Salad Low Customer Traffic Volumes	*50*	7	14%	37	74%	2	4%	1	2%
Specialty Salad High Customer Traffic Volumes	*50*	12	24%	43	86%	18	36%	22	44%
Leafy Greens Salad High Customer Traffic Volumes	*50*	2	4%	29	58%	5	10%	4	8%
Locally Owned Specialty Salad Low Customer Traffic Volume	*25*	8	32%	25	100%	9	36%	4	16%
Locally Owned Leafy Greens Salad Low Customer Traffic Volume	*25*	3	12%	23	92%	2	8%	1	4%
Locally Owned Specialty Salad High Customer Traffic Volume	*25*	6	24%	25	100%	9	36%	8	32.00 %
Locally Owned Leafy Greens High Customer Traffic Volume	*25*	1	4%	19	76%	3	12%	1	4%
Corporate Owned Specialty Salad Low Customer Traffic Volume	*25*	9	36%	23	92%	6	24%	14	56%
Corporate Owned Leafy Greens Salad Low Customer Traffic	*25*	4	16%	14	56%	0	0%	0	0%

Volume									
Corporate Owned Specialty Salad High Customer Traffic Volume	*25*	6	24%	18	72%	9	36%	14	56%
Corporate Owned Leafy Greens High Customer Traffic Volume	*25*	1	4%	10	40%	2	8%	3	12%

Low Customer Traffic Volume vs High Customer Traffic Volume

Sample ID	*n*	*E. Coli* Positive	*E. Coli* %	*Enterococcus* Positive	*Enterococcus* %	Male-Specific Coliphage Positive	Male-Specific Coliphage %	Somatic Coliphage Positive	Somatic Coliphage %
Low Customer Traffic Volume	*100*	24	24%	85	85%	17	17%	19	19%
High Volume Customer Traffic	*100*	14	14%	72	72%	23	23%	26	26%
Locally Owned Low Customer Traffic Volume	*50*	11	22%	48	96%	11	22%	5	10%
Locally Owned High Customer Traffic Volume	*50*	7	14%	44	88%	12	24%	9	18%
Corporate Owned Low Volume Customer Traffic	*50*	13	26%	37	74%	6	12%	14	28%
Corporate Owned High Volume Customer Traffic	*50*	7	14%	28	56%	11	22%	17	34%
Leafy Greens Salad Low Volume Customer Traffic	*50*	7	14%	37	74%	2	4%	1	2%
Leafy Greens Salad High Volume Customer Traffic	*50*	2	4%	29	58%	5	10%	4	8%
Specialty Salad Low Customer Traffic Volume	*50*	18	36%	48	96%	15	30%	18	36%
Specialty Salad High Customer Traffic Volume	*50*	11	22%	43	86%	18	36%	22	44%
Locally Owned Leafy Greens Salad Low Customer Traffic Volume	*25*	3	12%	23	92%	2	8%	1	4%
Locally Owned Leafy Greens High Customer Traffic Volume	*25*	1	4%	19	76%	3	12%	1	4%
Locally Owned Specialty Salad Low Customer Traffic Volume	*25*	8	32%	25	100%	9	36%	4	16%
Locally Owned Specialty Salad High Customer Traffic Volume	*25*	6	24%	25	100%	9	36%	8	32.00%
Corporate Owned Leafy Greens Salad Low Customer Traffic	*25*	4	16%	14	56%	0	0%	0	0%

Volume									
Corporate Owned Leafy Greens High Customer Traffic Volume	*25*	1	4%	10	40%	2	8%	3	12%
Corporate Owned Specialty Salad Low Customer Traffic Volume	*25*	9	36%	23	92%	6	24%	14	56%
Corporate Owned Specialty Salad High Customer Traffic Volume	*25*	6	24%	18	72%	9	36%	14	56%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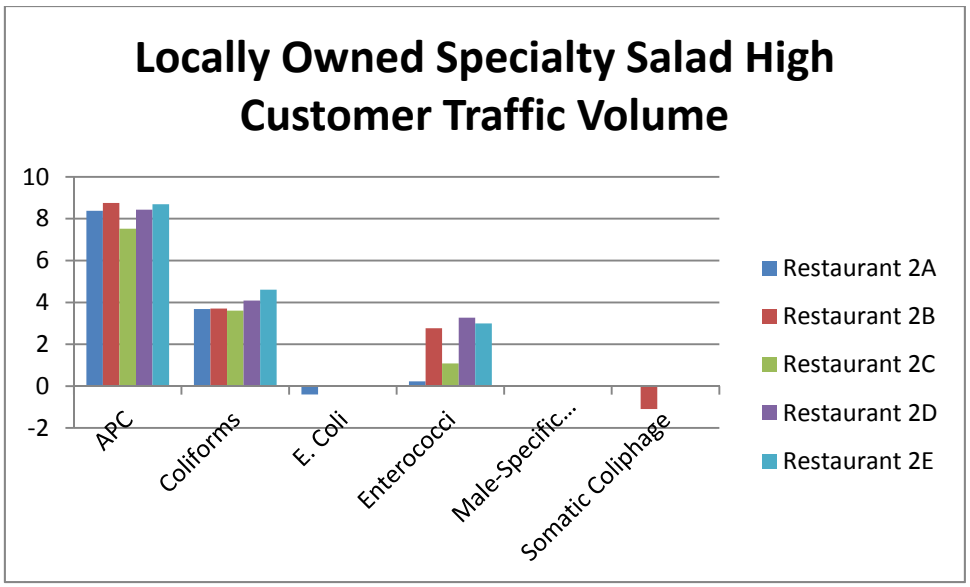
Restaurant 1B
Restaurant 1C
Restaurant 1D
Restaurant 1E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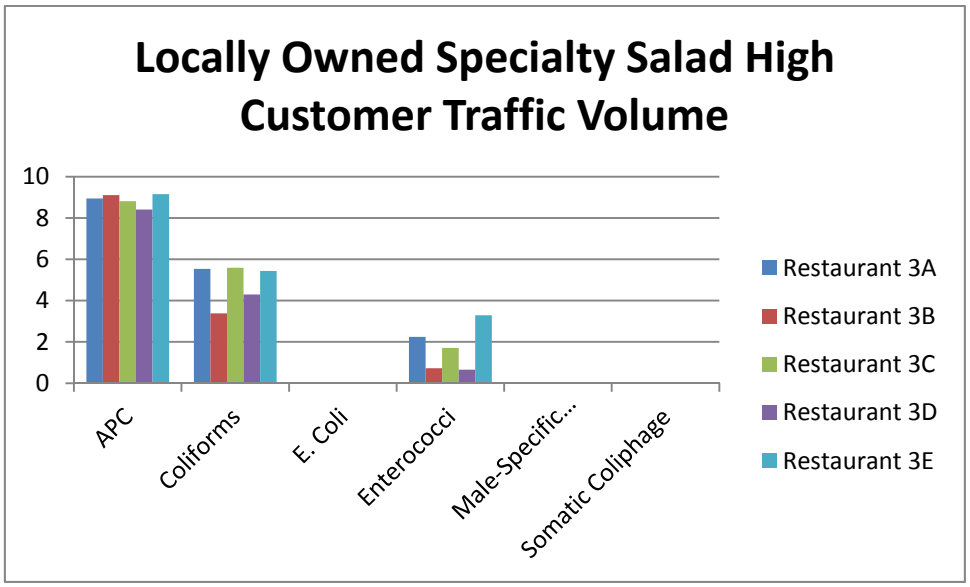
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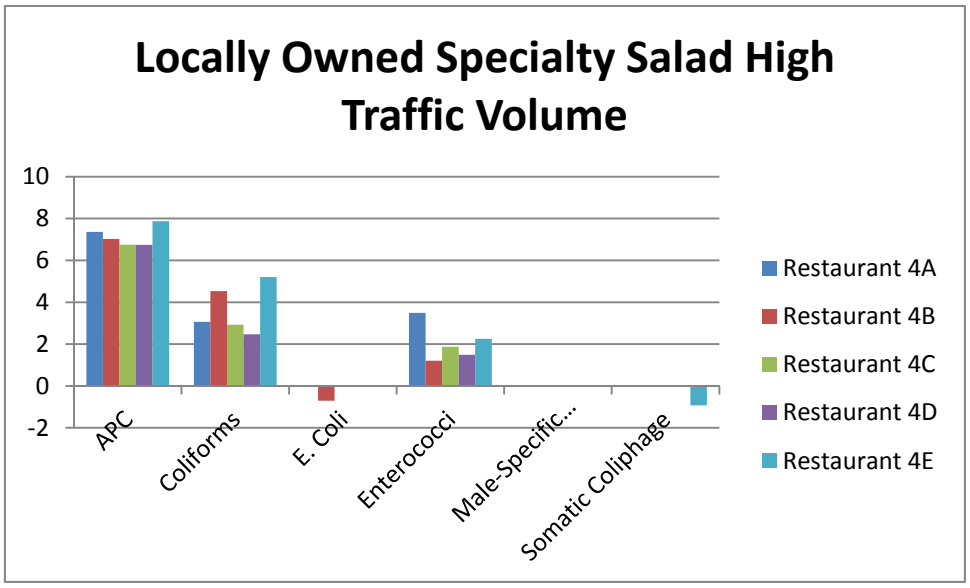
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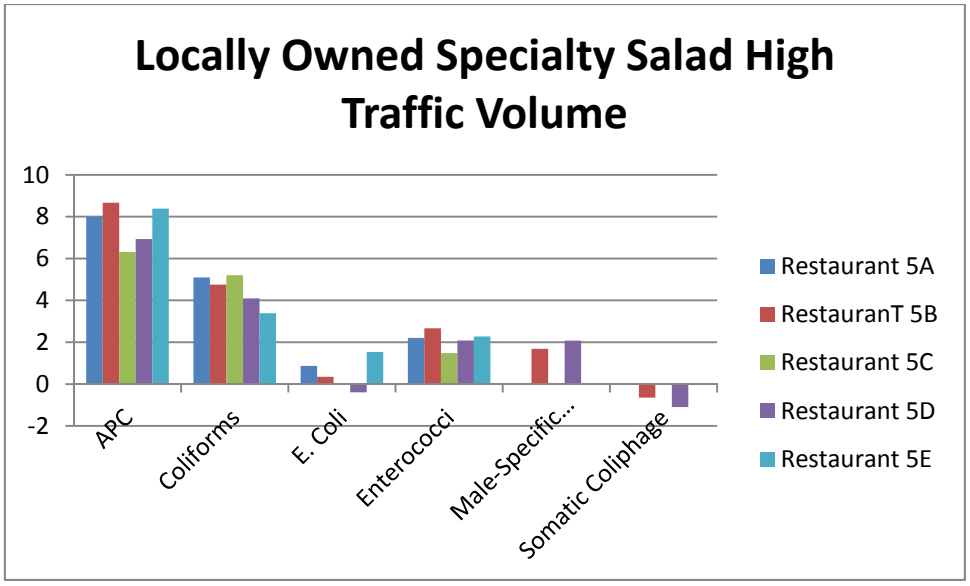
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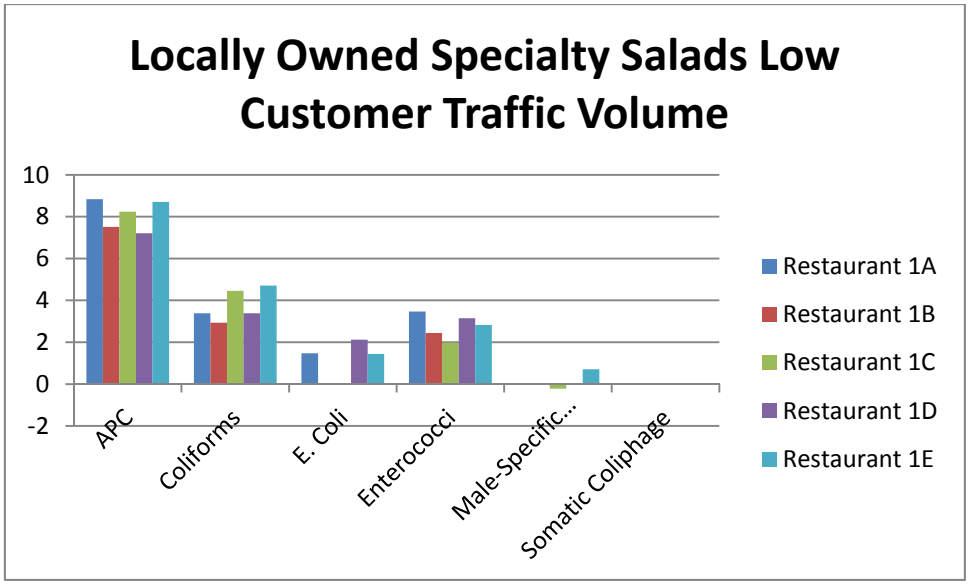
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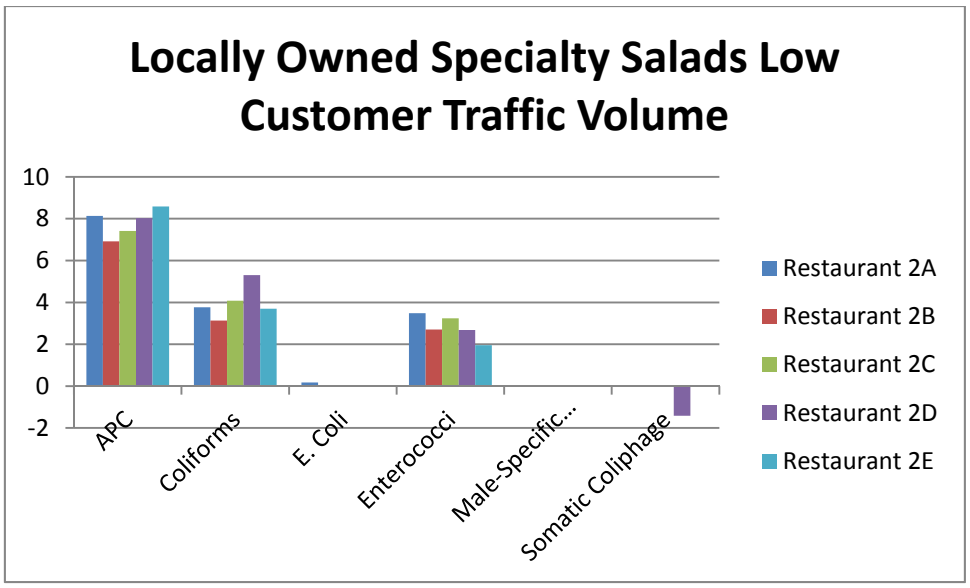
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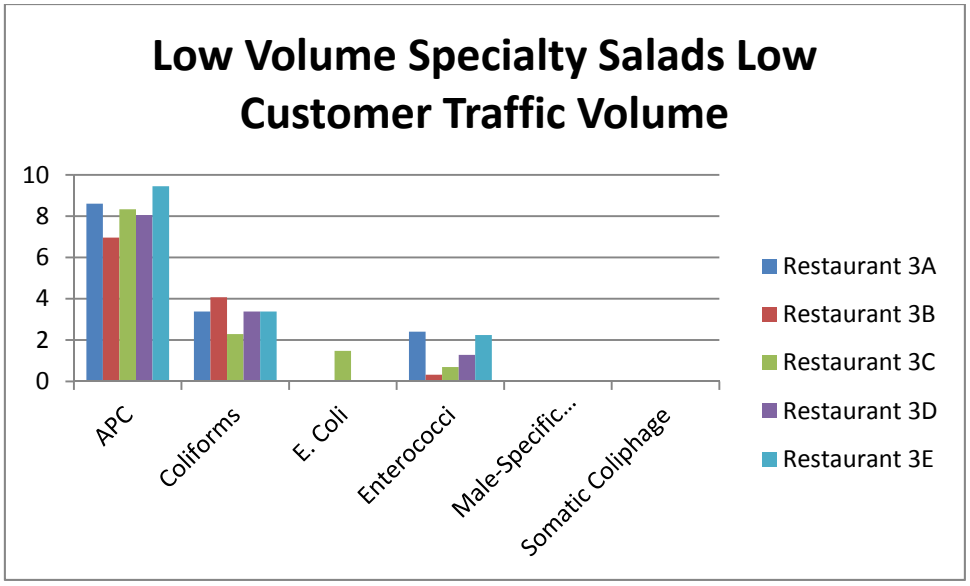
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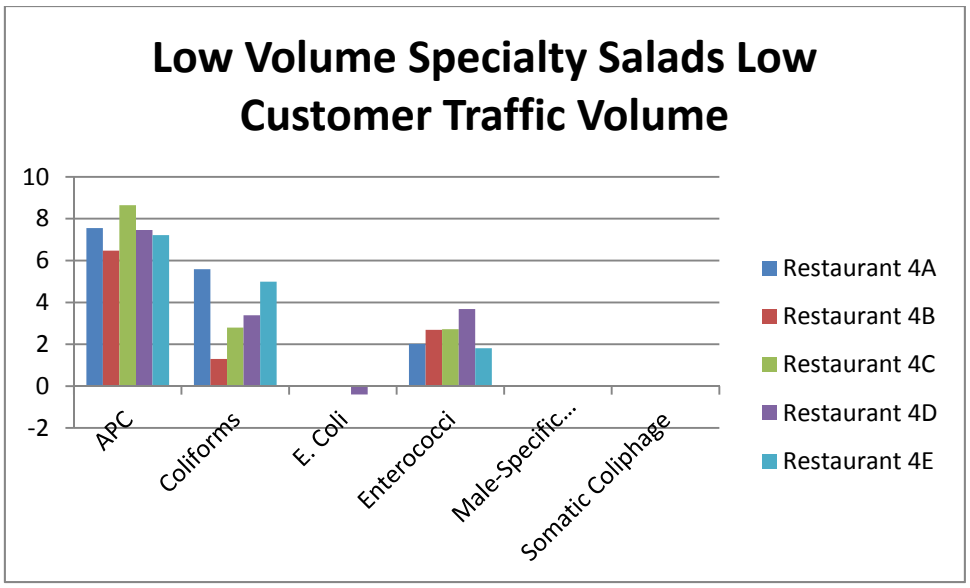
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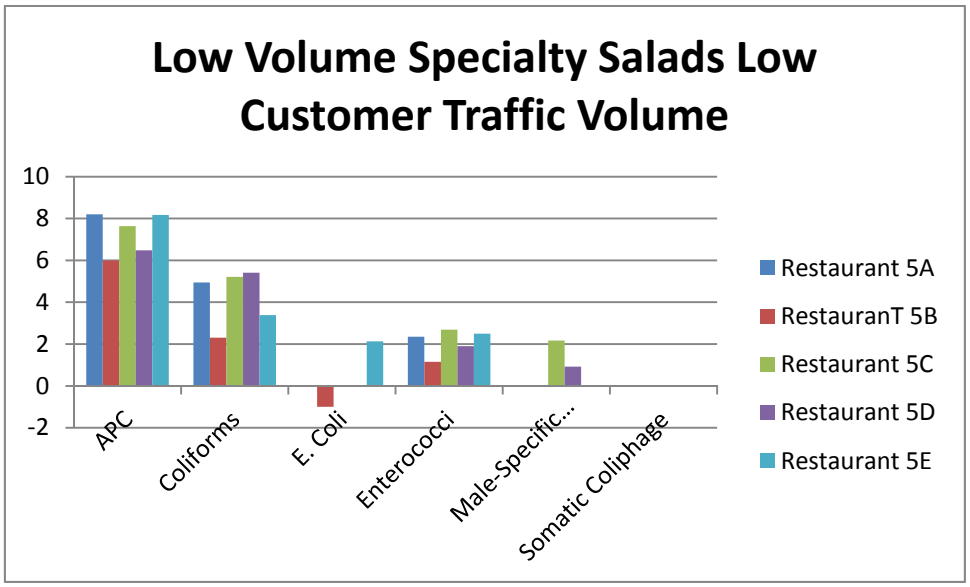
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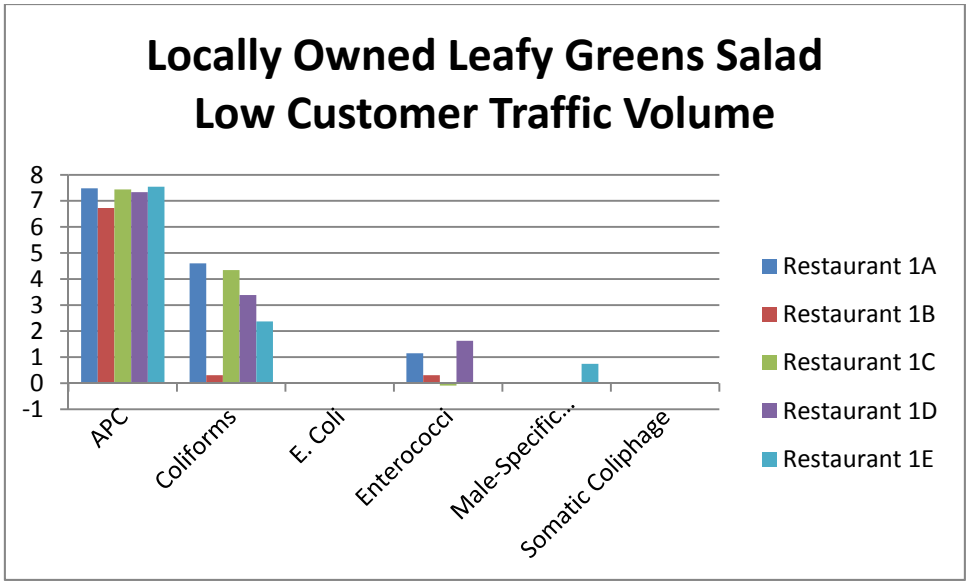
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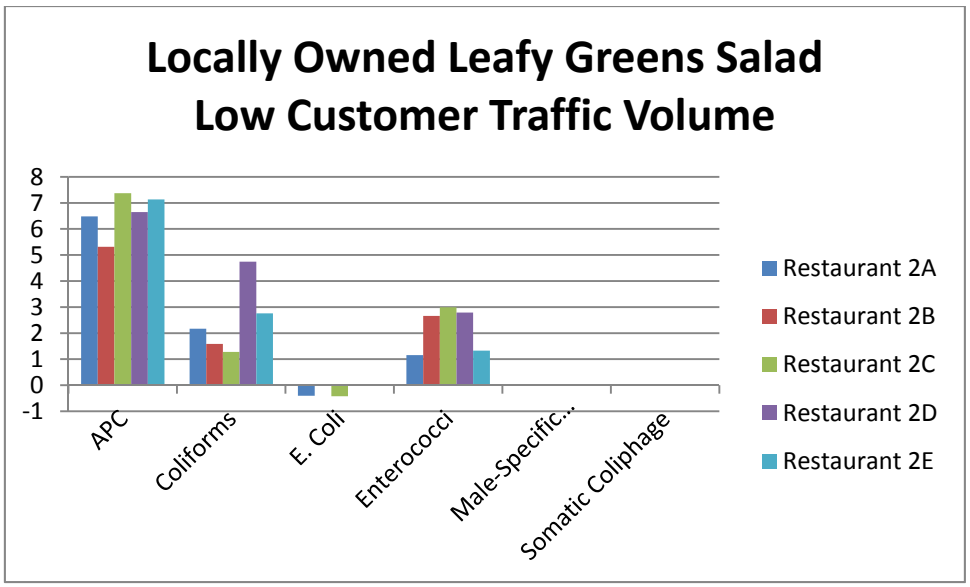
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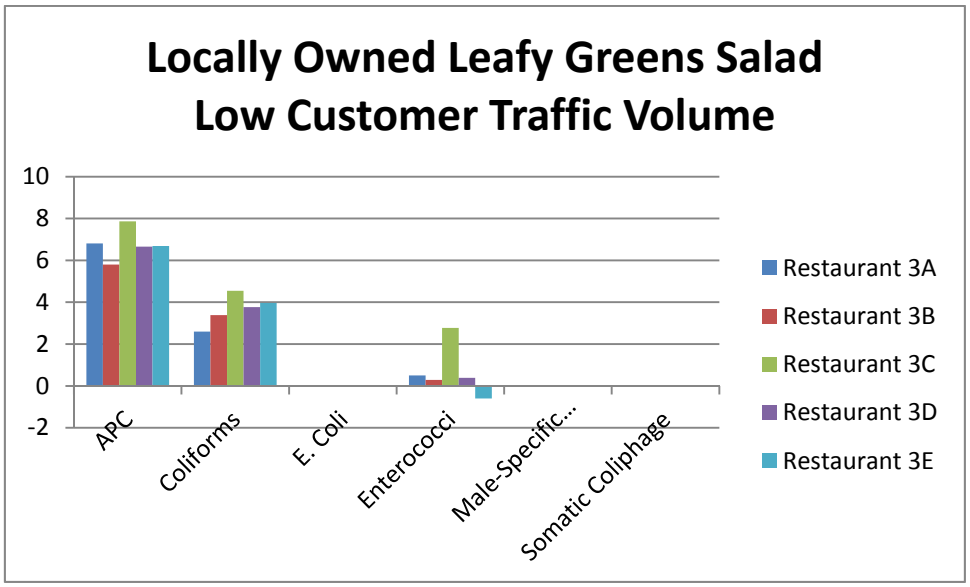
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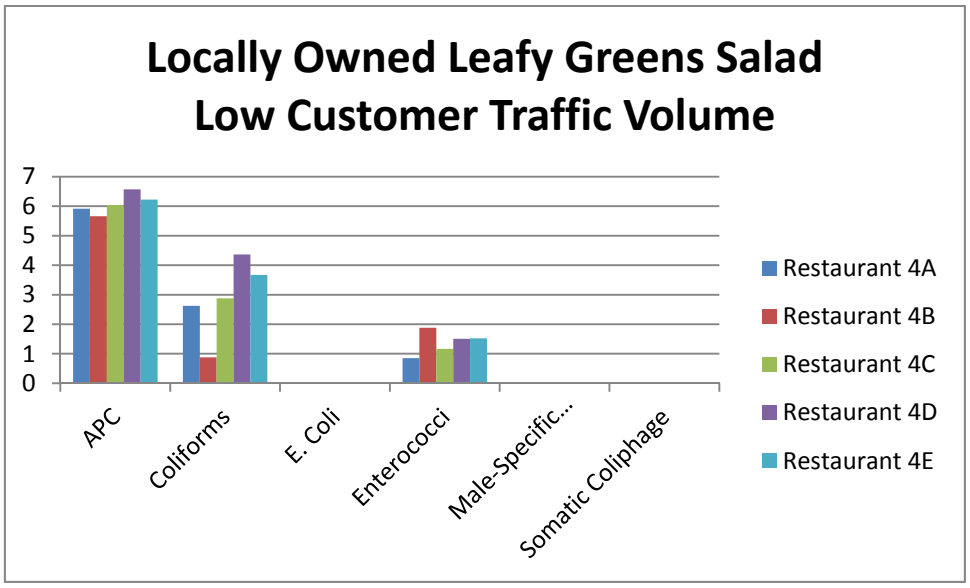
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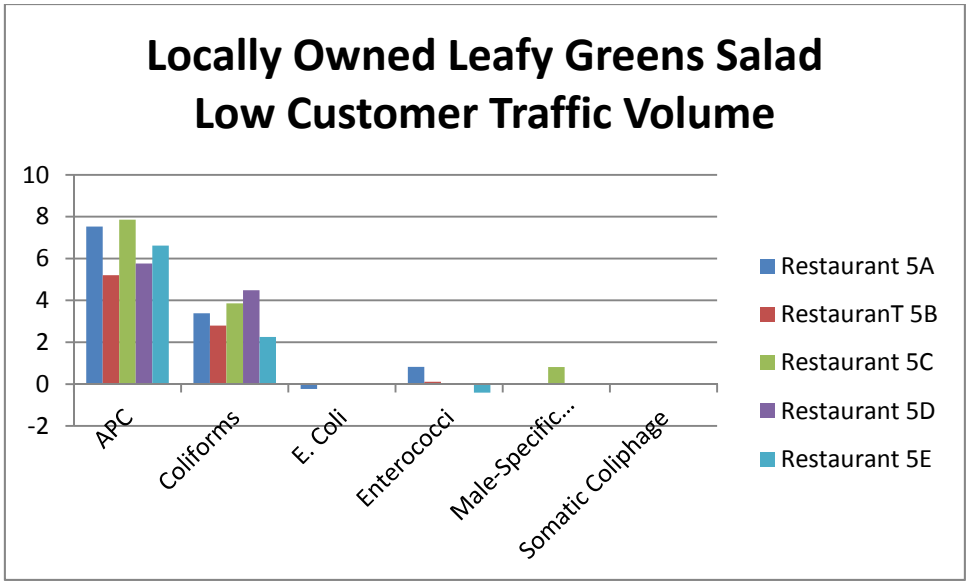
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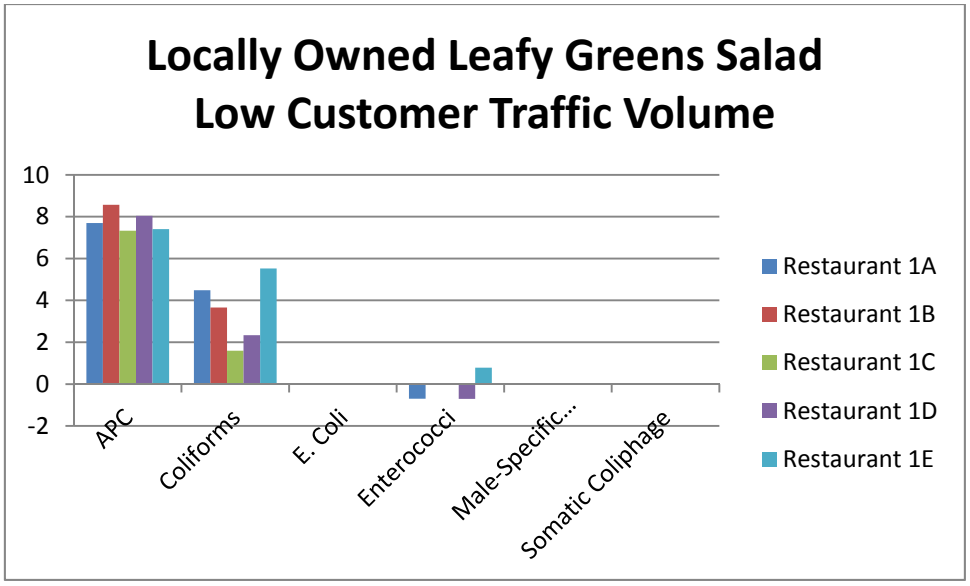
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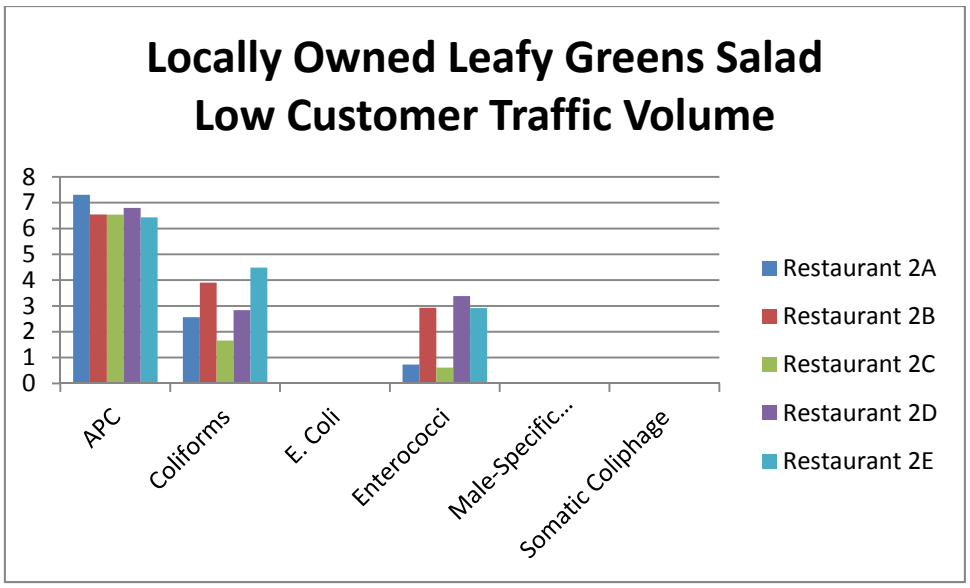
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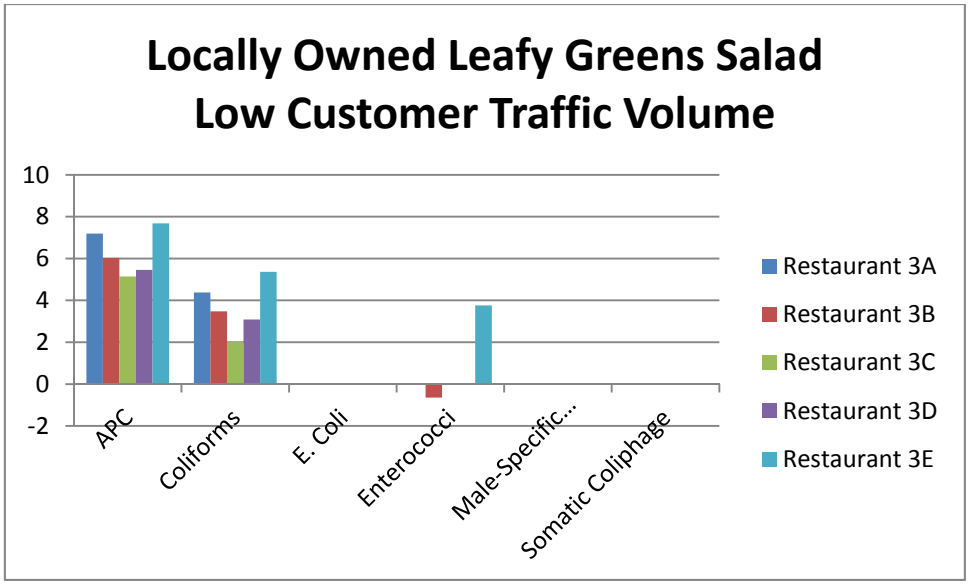
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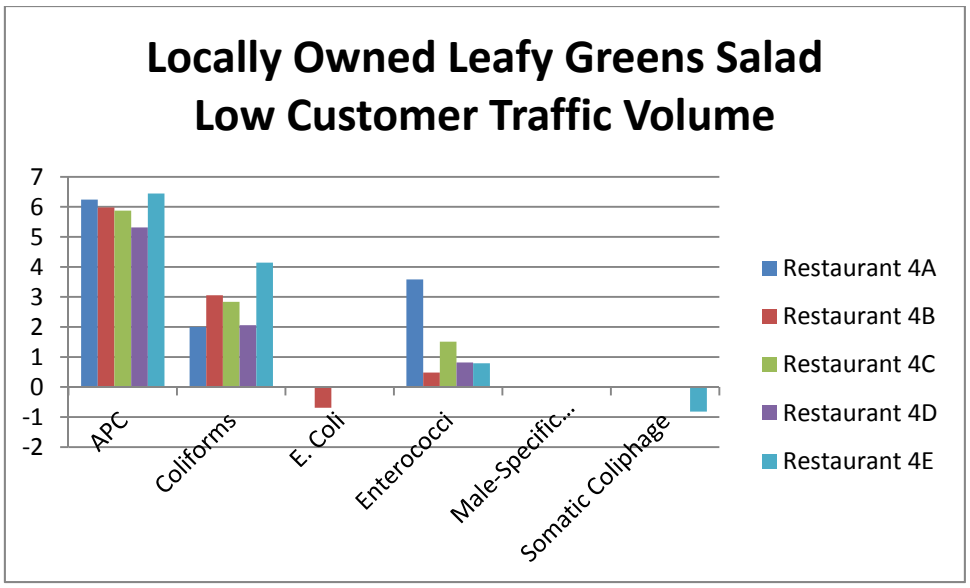
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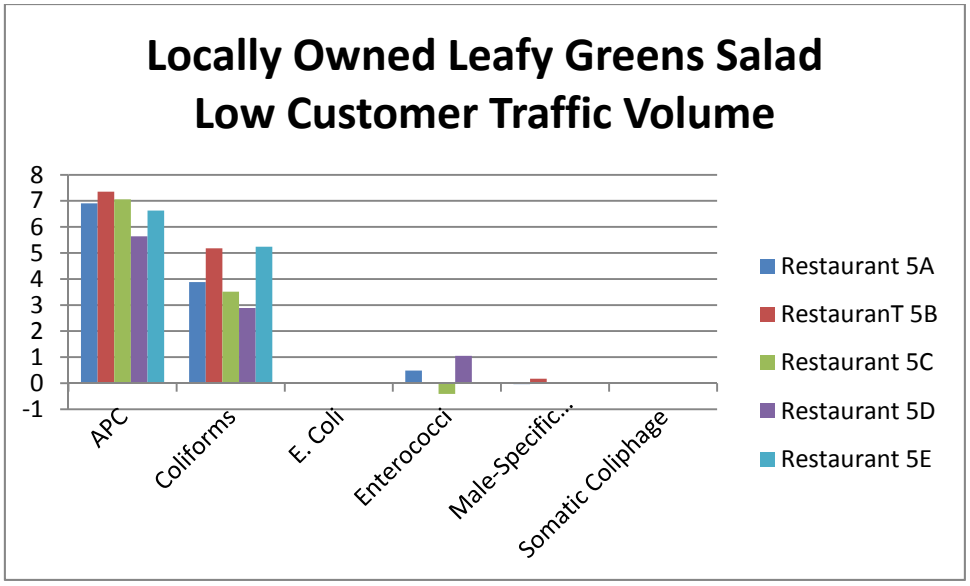
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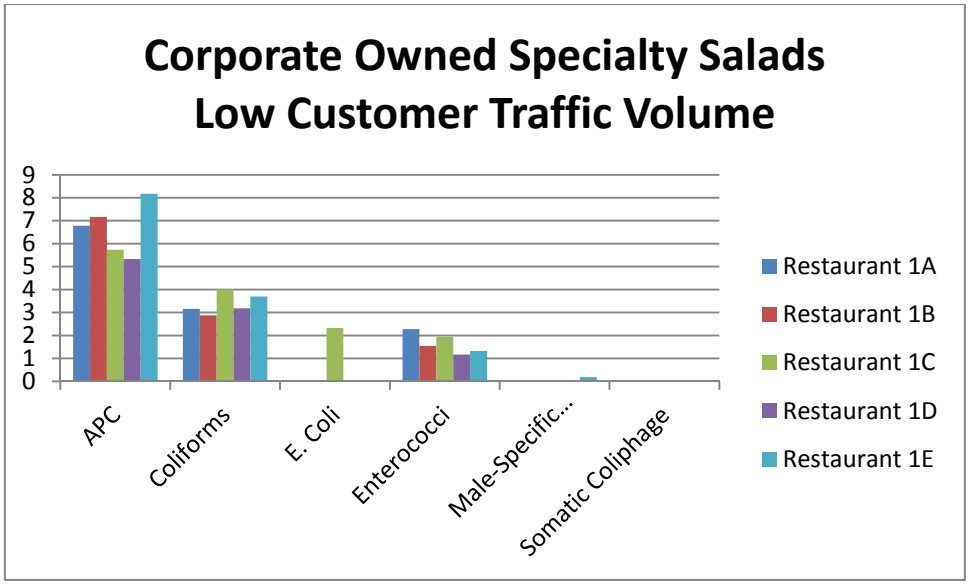
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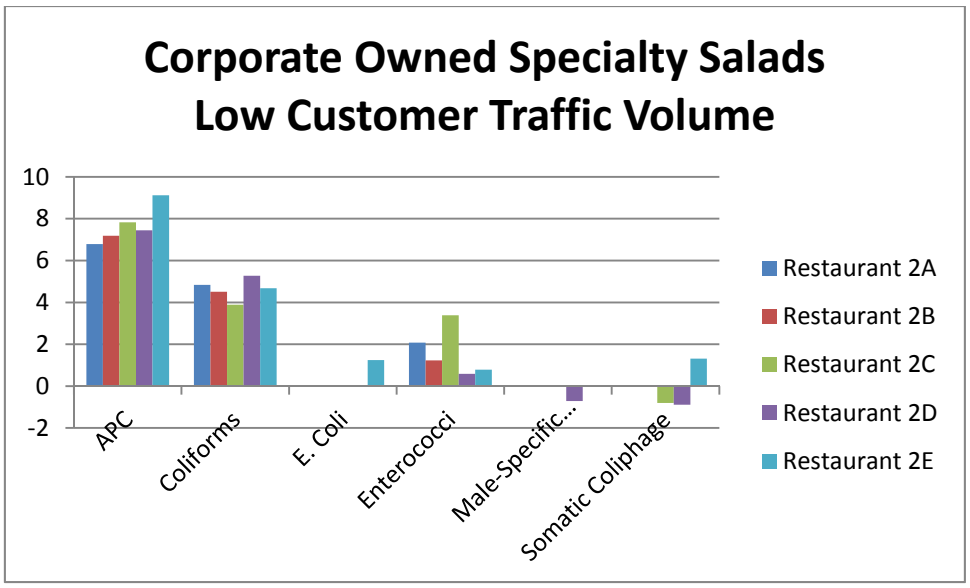
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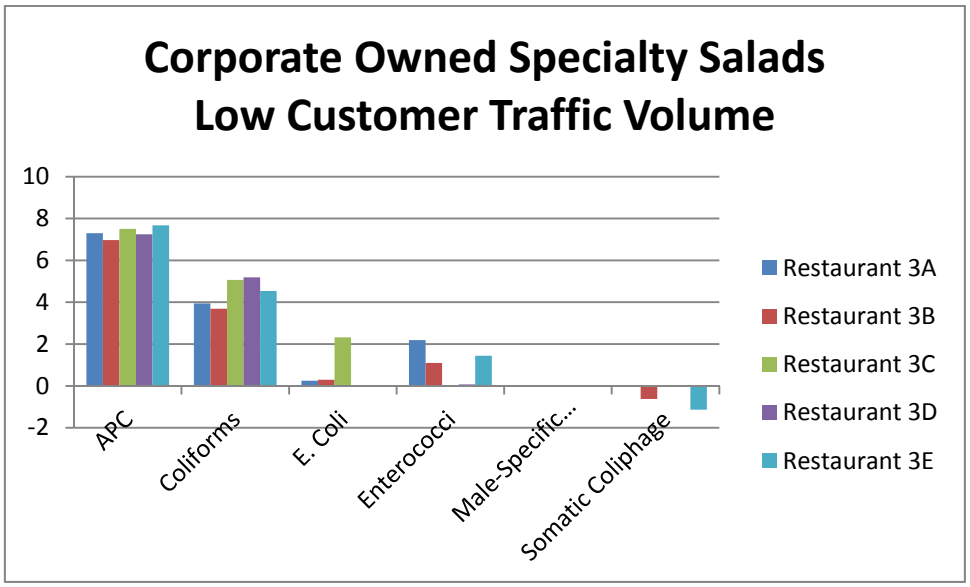
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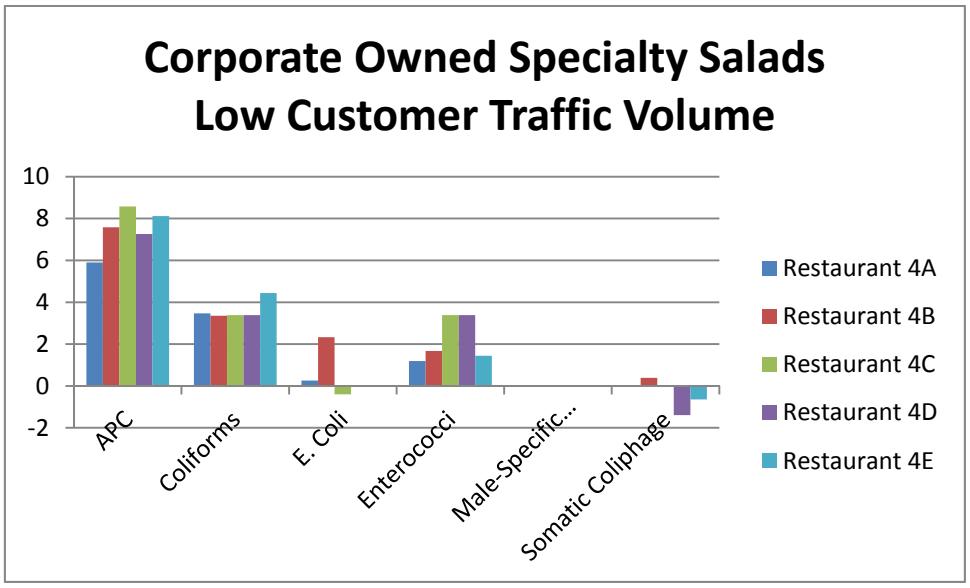
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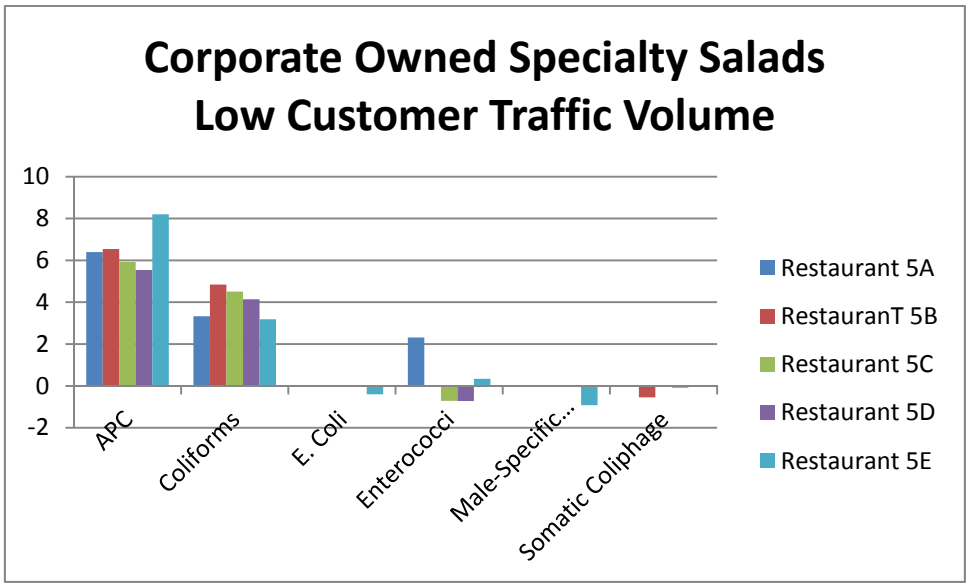
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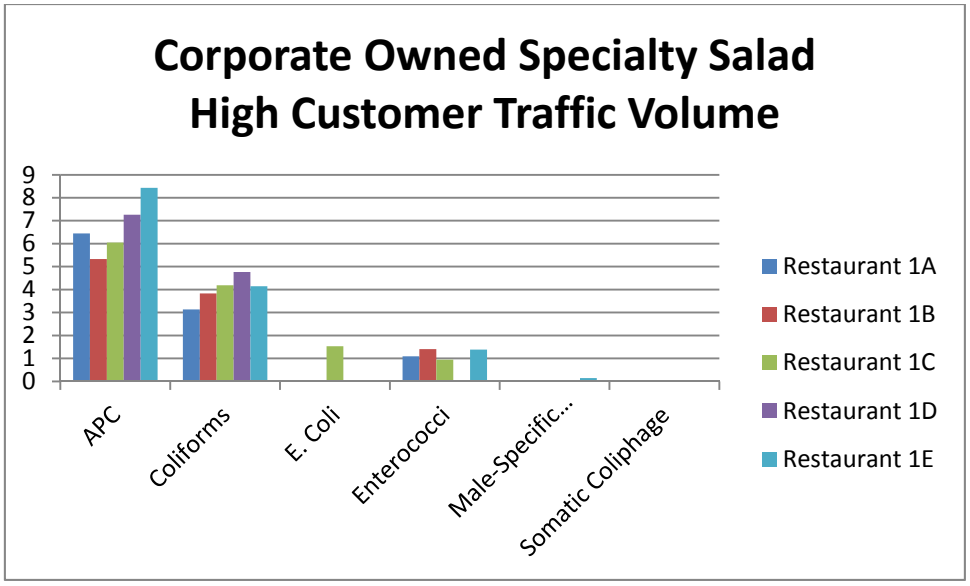
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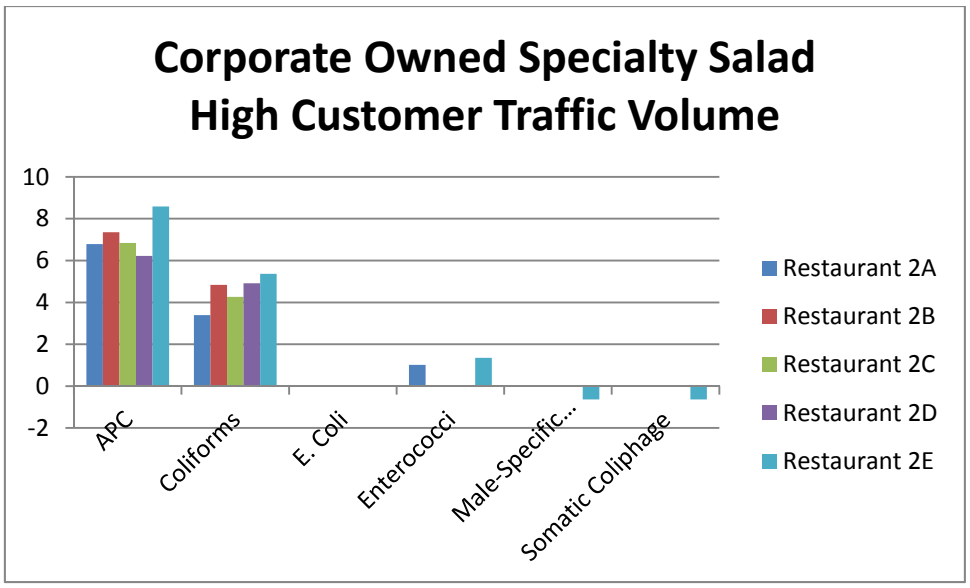
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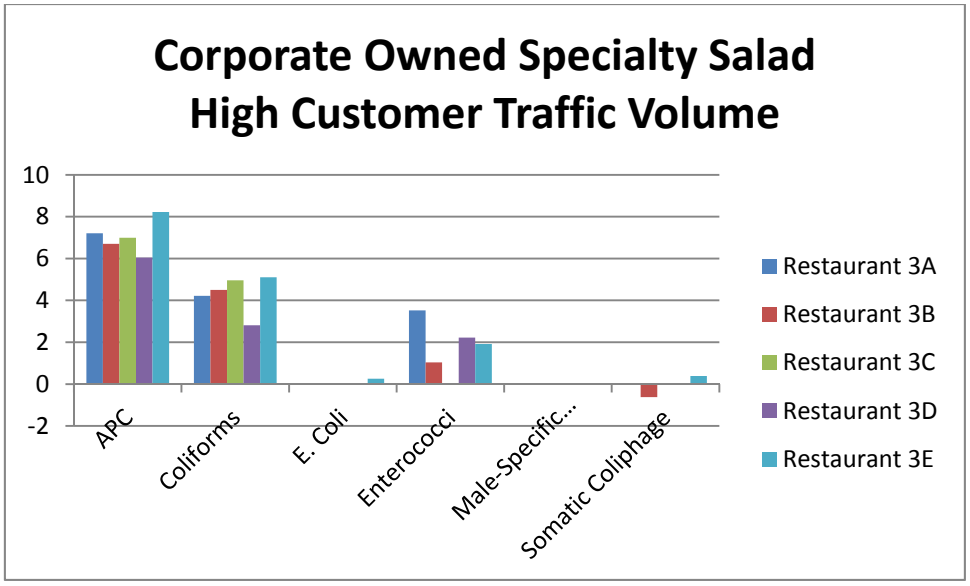
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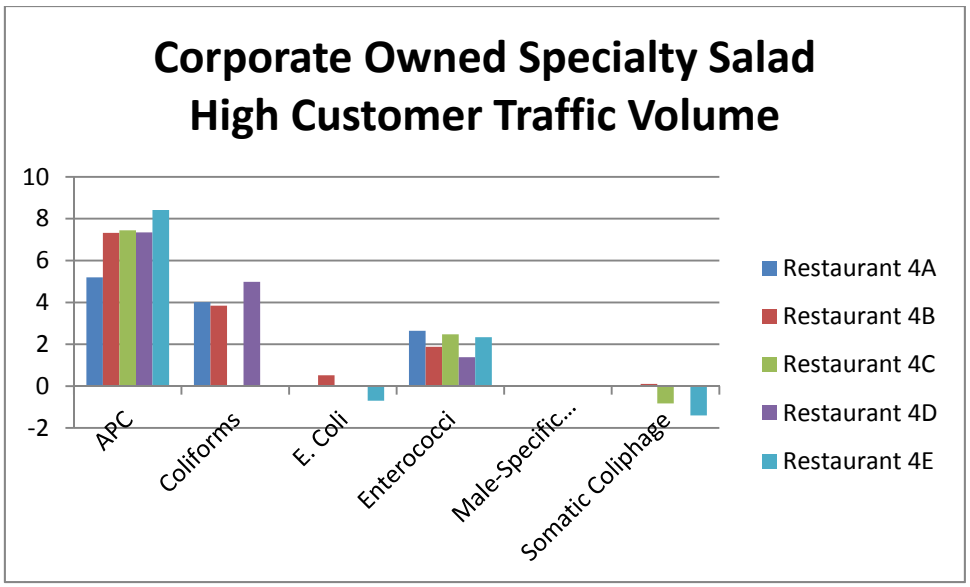
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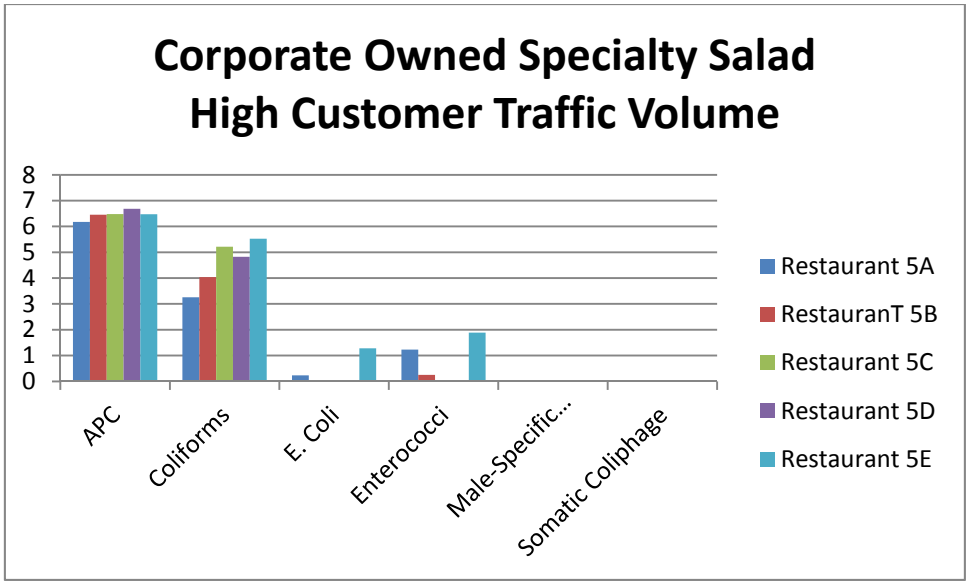
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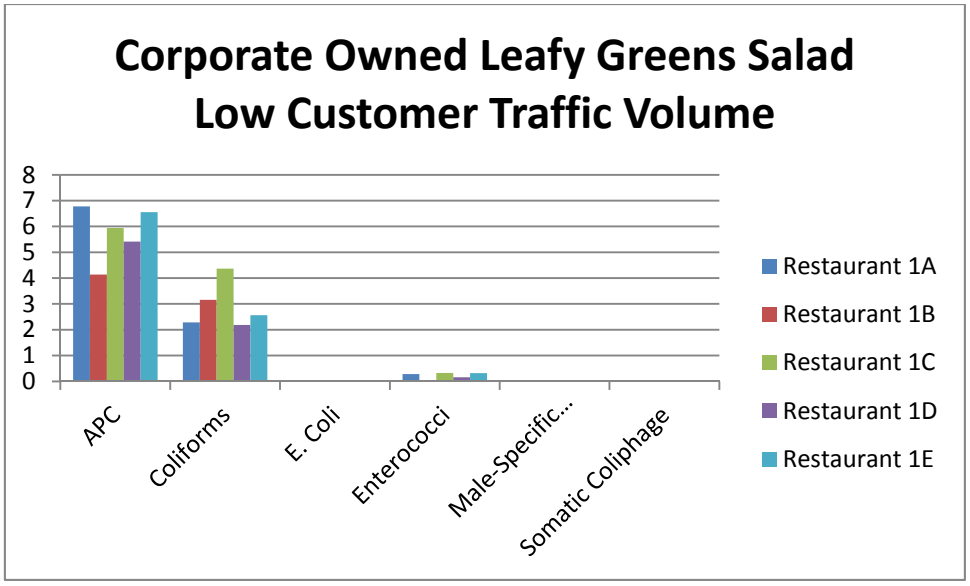
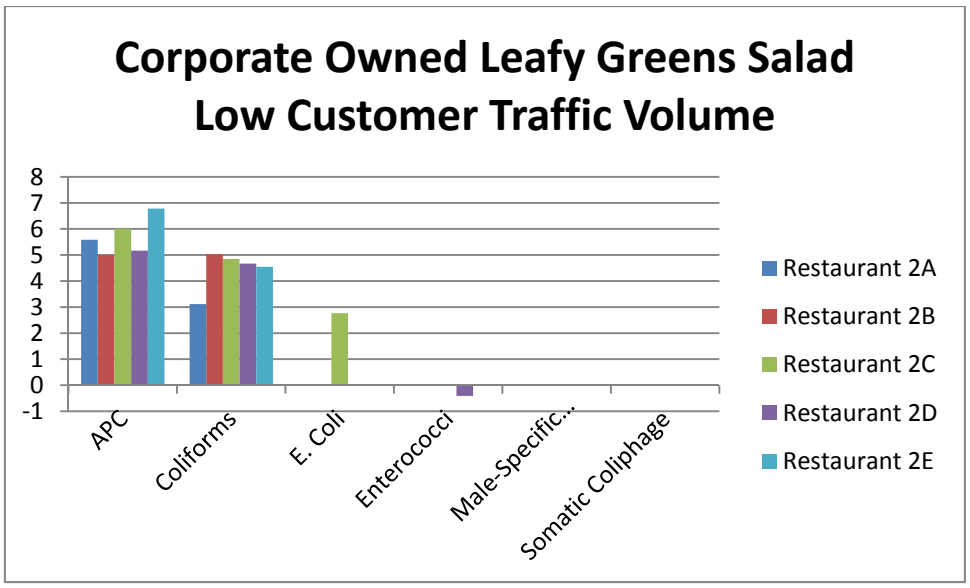
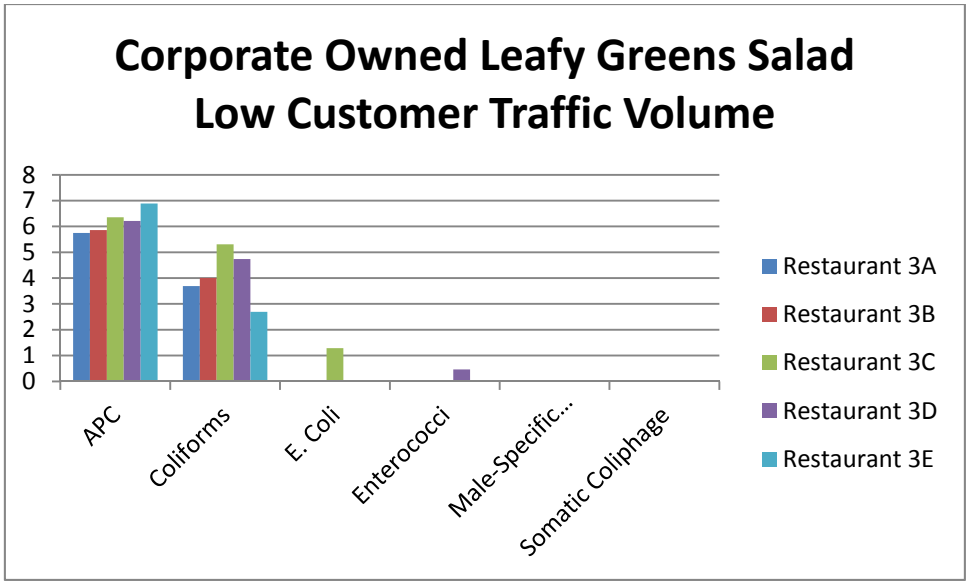
Enterococci
Male-Specific...
Somatic Coliphage
Restaurant 5A
RestauranT 5B
Restaurant 5C
Restaurant 5D
Restaurant 5E


Corporate Owned Leafy Greens Salad
Low Customer Traffic Volume
8
7
6
5
4
3
2
1
0
APC
Coliforms
E. Coli
Enterococci
Male-Specific...
Somatic Coliphage
Restaurant 1A
Restaurant 1B
Restaurant 1C
Restaurant 1D
Restaurant 1E


Corporate Owned Leafy Greens Salad
Low Customer Traffic Volume
8
7
6
5
4
3
2
1
0
-1
APC
Coliforms
E. Coli
Enterococci
Male-Specific...
Somatic Coliphage
Restaurant 2A
Restaurant 2B
Restaurant 2C
Restaurant 2D
Restaurant 2E


Corporate Owned Leafy Greens Salad
Low Customer Traffic Volume
8
7
6
5
4
3
2
1
0
APC
Coliforms
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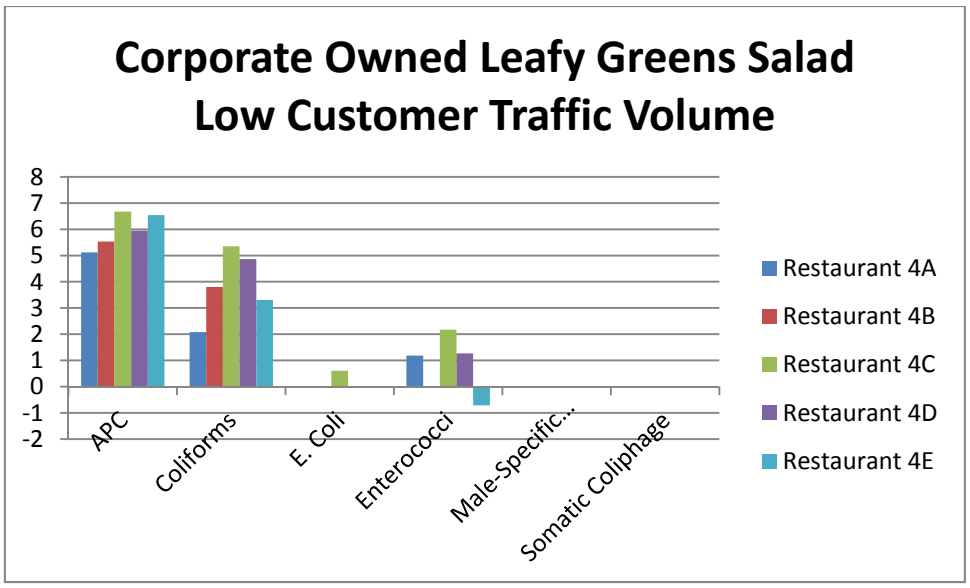
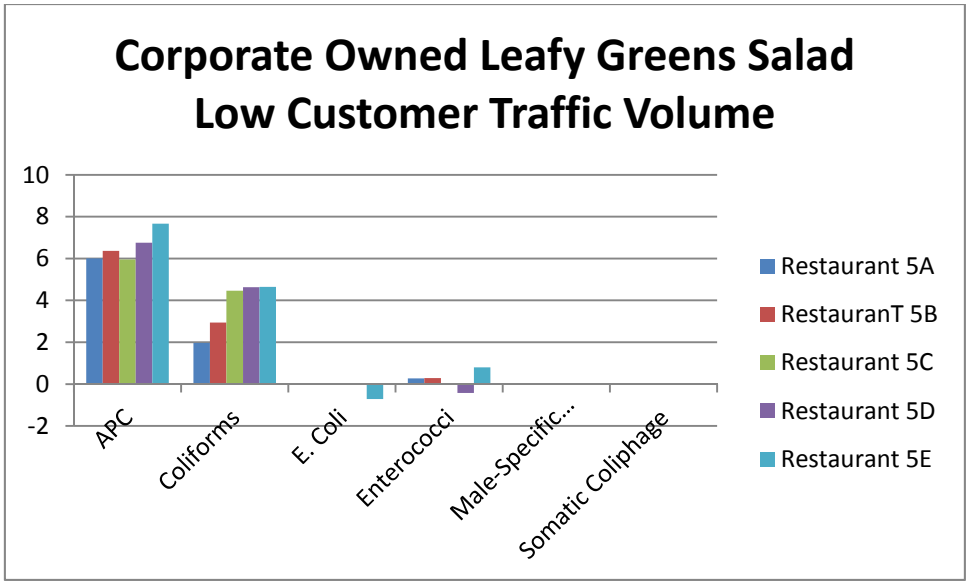
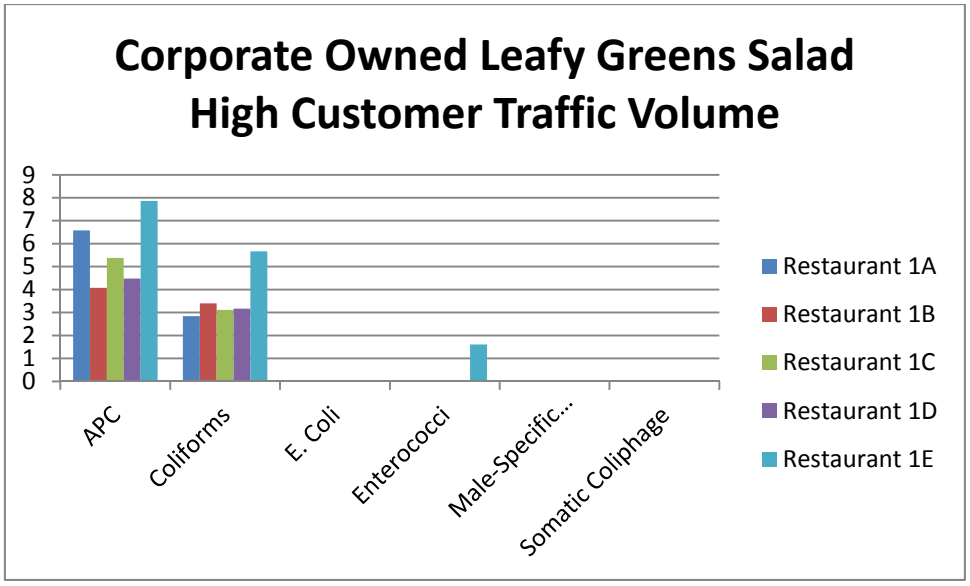
Enterococci
Male-Specific...
Somatic Coliphage
Restaurant 3A
Restaurant 3B
Restaurant 3C
Restaurant 3D
Restaurant 3E


Corporate Owned Leafy Greens Salad
Low Customer Traffic Volume
8
7
6
5
4
3
2
1
0
-1
-2
APC
Coliforms
E. Coli
Enterococci
Male-Specific...
Somatic Coliphage
Restaurant 4A
Restaurant 4B
Restaurant 4C
Restaurant 4D
Restaurant 4E


Corporate Owned Leafy Greens Salad
Low Customer Traffic Volume
10
8
6
4
2
0
-2
APC
Coliforms
E. Coli
Enterococci
Male-Specific...
Somatic Coliphage
Restaurant 5A
RestauranT 5B
Restaurant 5C
Restaurant 5D
Restaurant 5E


Corporate Owned Leafy Greens Salad
High Customer Traffic Volume
9
8
7
6
5
4
3
2
1
0
APC
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E. Coli
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Restaurant 1A
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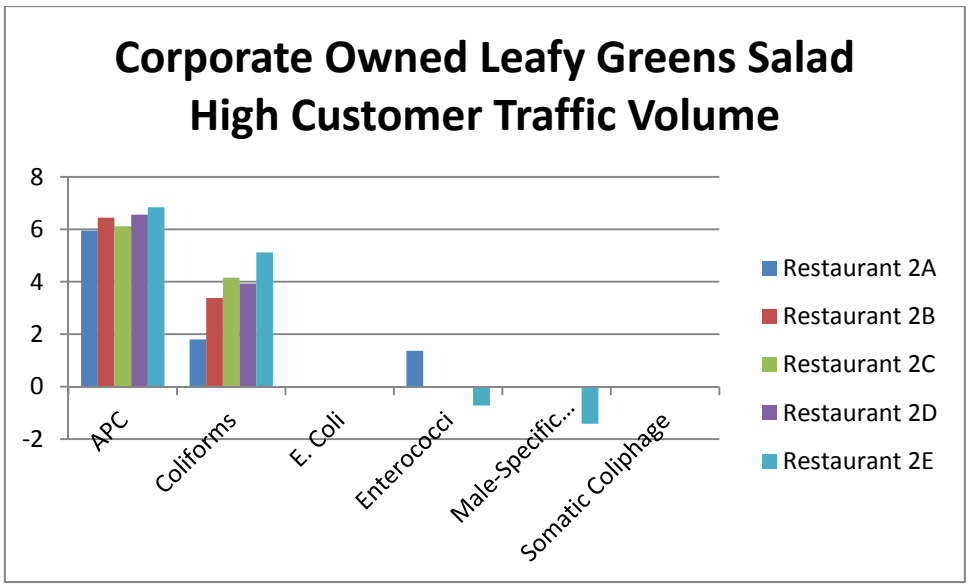
Corporate Owned Leafy Greens Salad
High Customer Traffic Volume
8
6
4
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0
-2
APC
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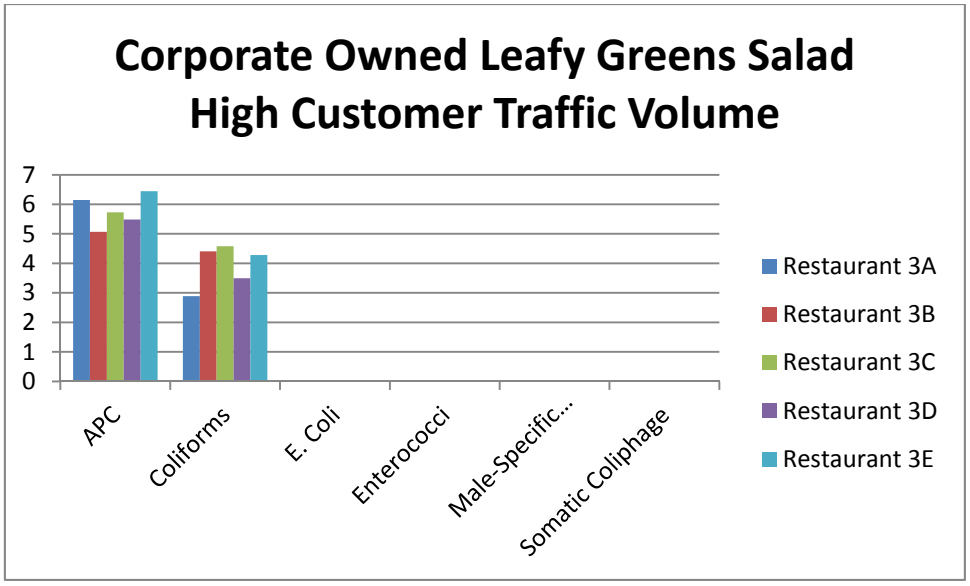
Corporate Owned Leafy Greens Salad
High Customer Traffic Volume
7
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4
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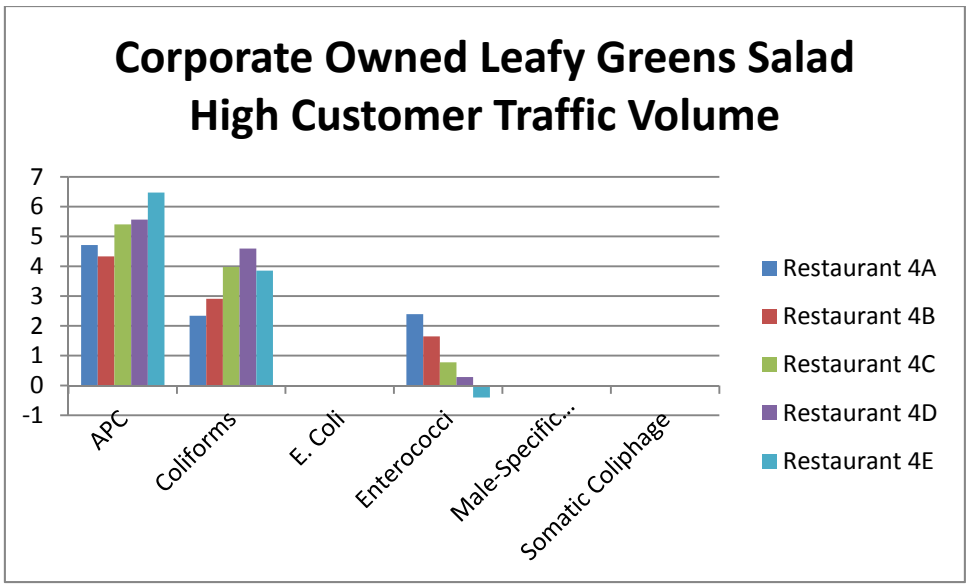
Corporate Owned Leafy Greens Salad
High Customer Traffic Volume
7
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5
4
3
2
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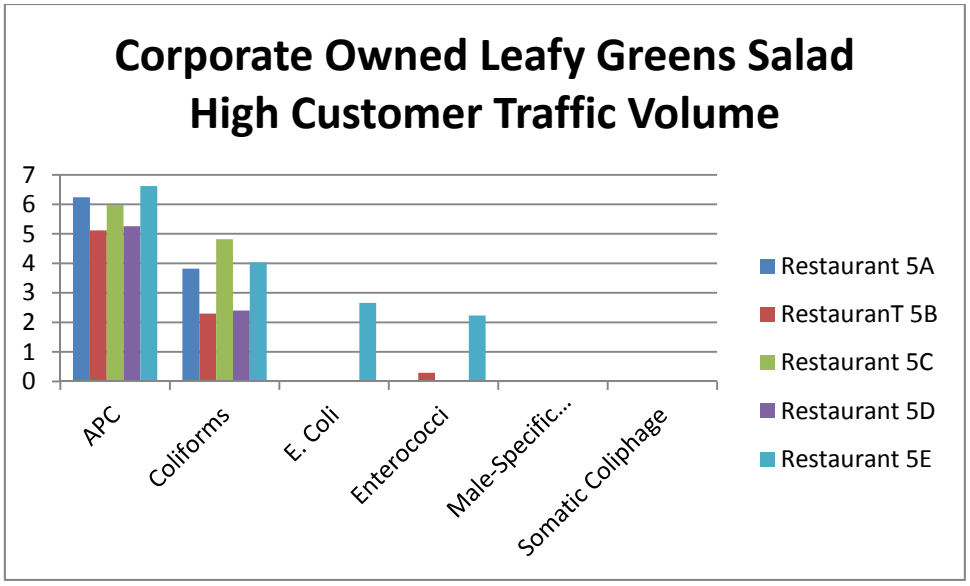
Corporate Owned Leafy Greens Salad
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7
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5
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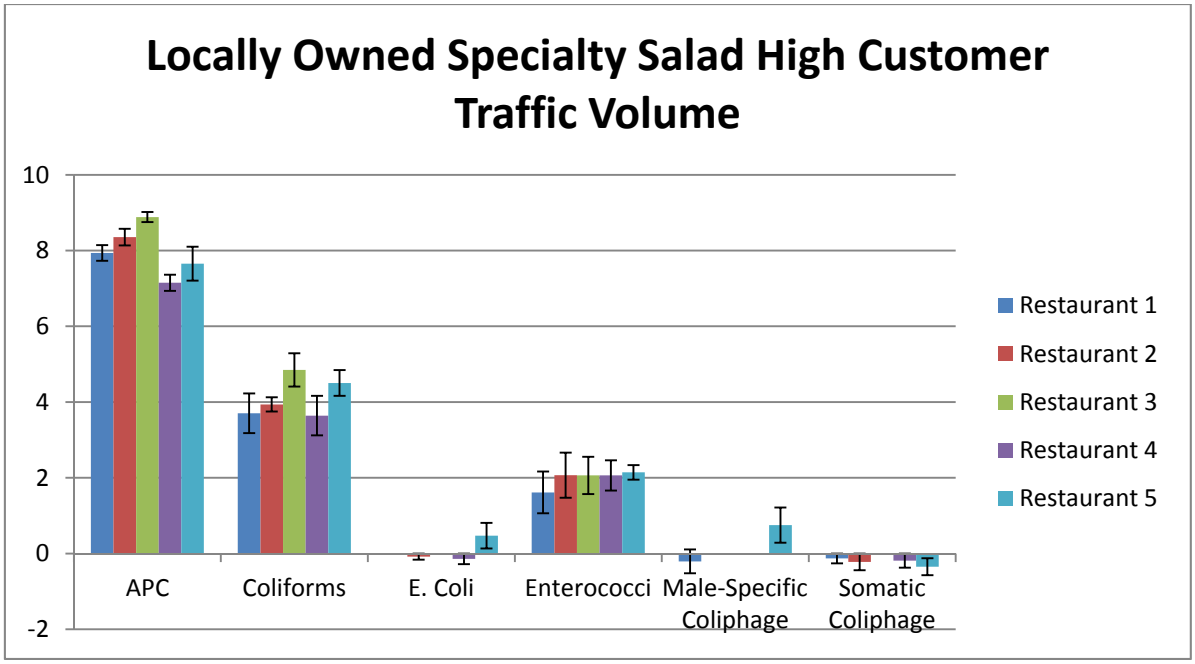
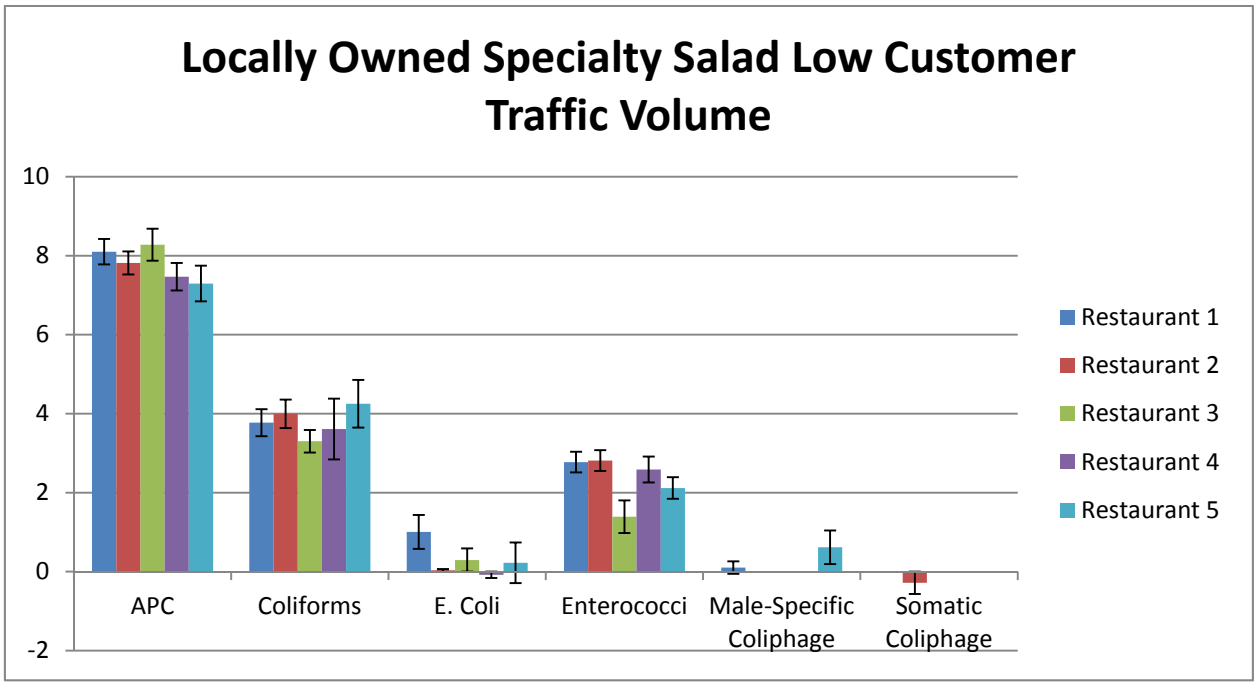

Locally Owned Specialty Salad High Customer Traffic Volume
10
8
6
4
2
0
-2
APC
Coliforms
E. Coli
Enterococci
Male-Specific Coliphage
Somatic Coliphage
Restaurant 1
Restaurant 2
Restaurant 3
Restaurant 4
Restaurant 5


Locally Owned Specialty Salad Low Customer Traffic Volume
10
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-2
APC
Coliforms
E. Coli
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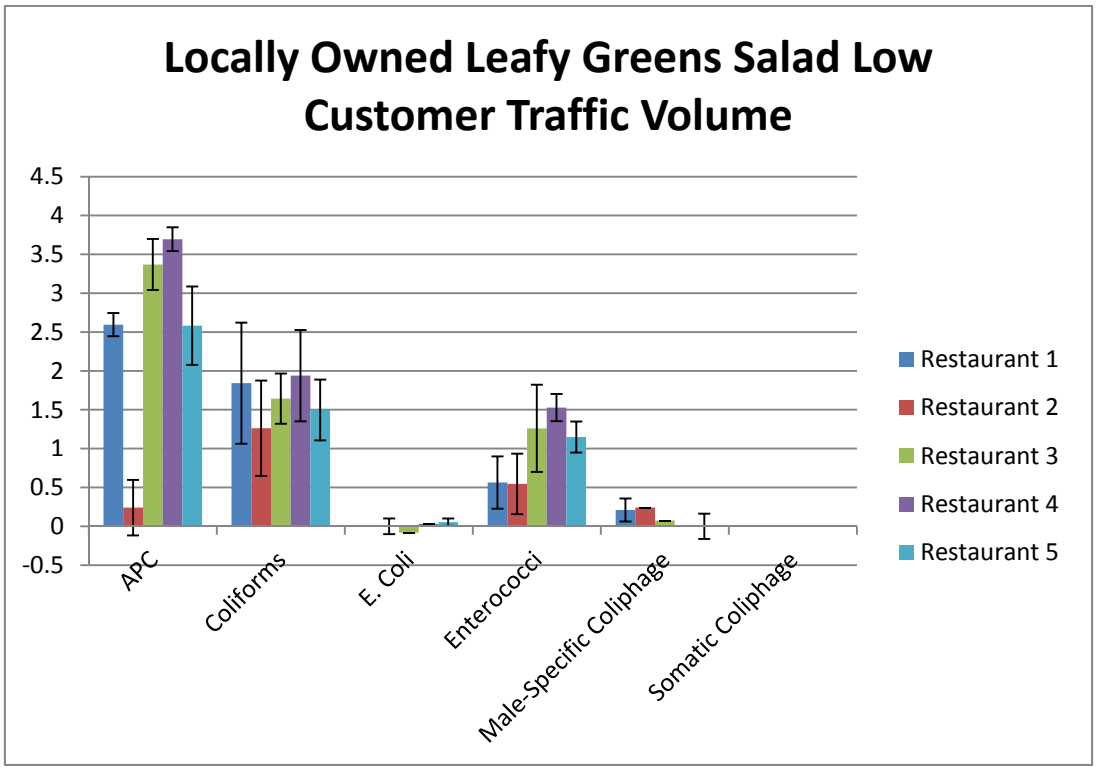
Locally Owned Leafy Greens Salad Low Customer Traffic Volume
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
APC
Coliforms
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Enterococci
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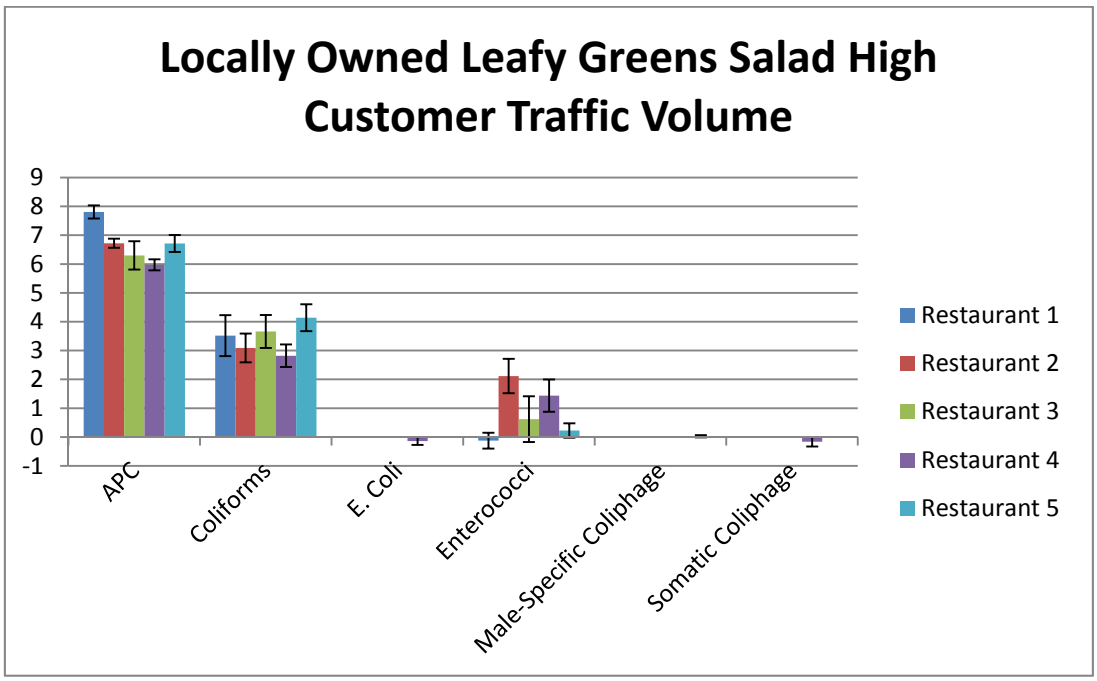
Locally Owned Leafy Greens Salad High Customer Traffic Volume
9
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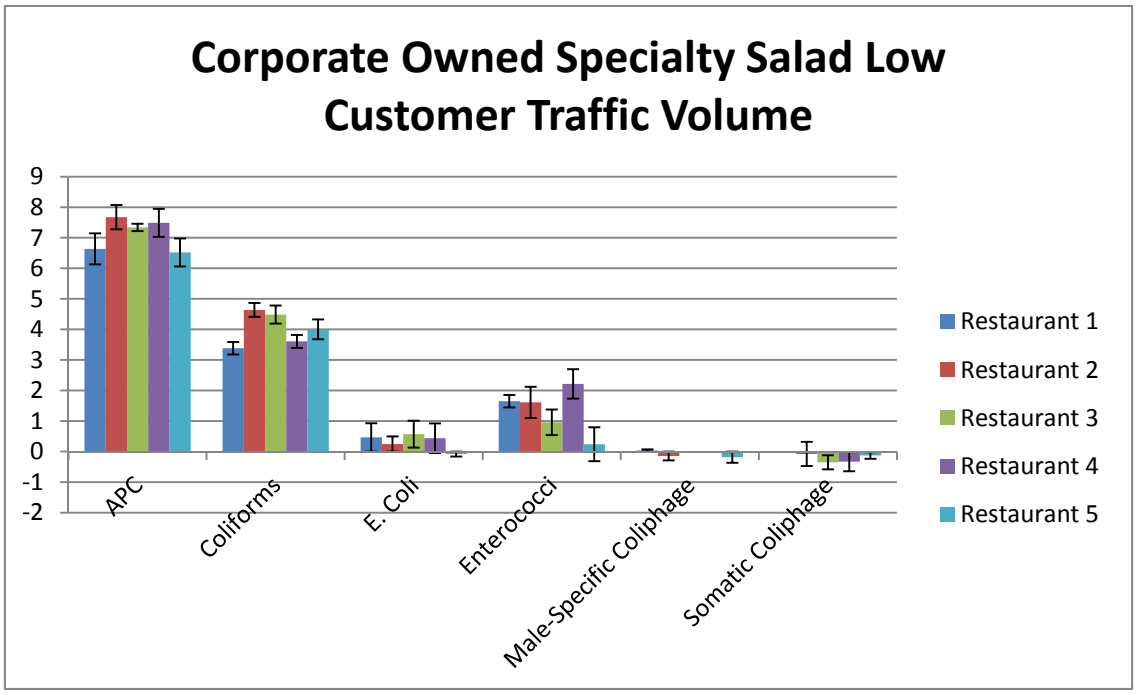
Corporate Owned Specialty Salad Low Customer Traffic Volume
9
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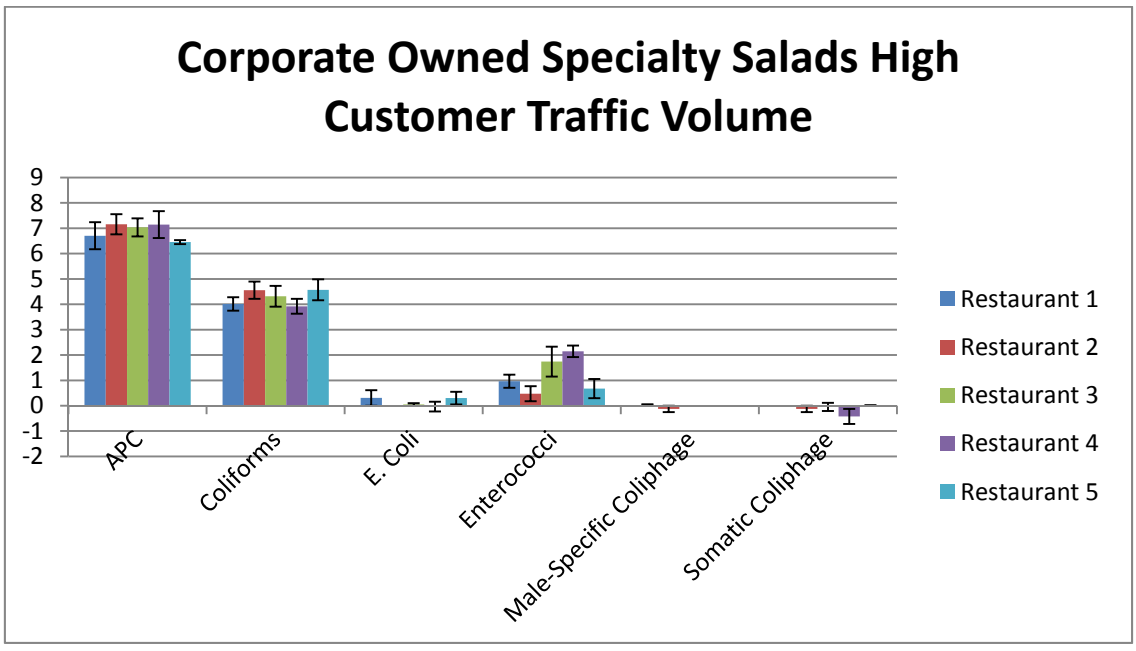
Corporate Owned Specialty Salads High Customer Traffic Volume
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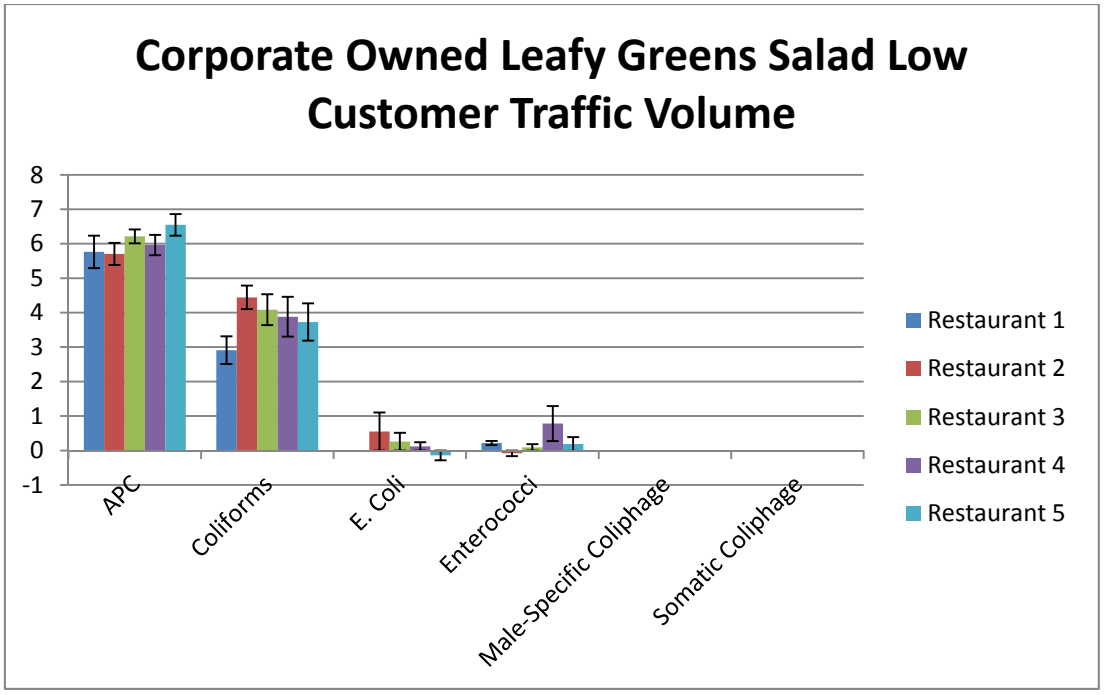
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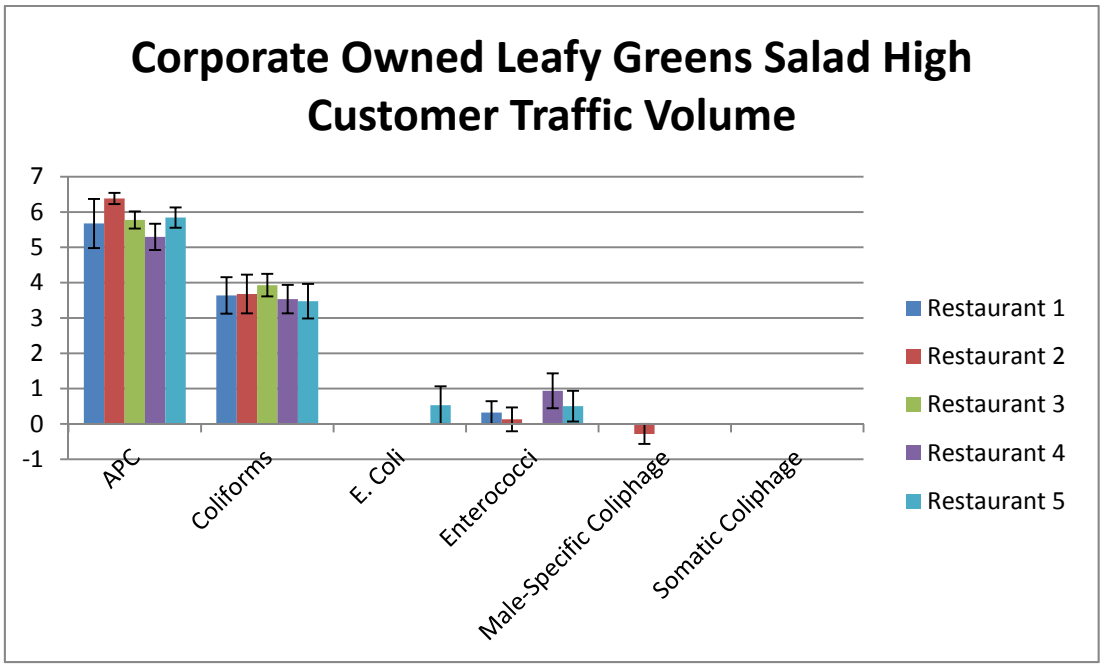
Corporate Owned Leafy Greens Salad High Customer Traffic Volume
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Restaurant 4
Restaurant 5

FRNA group	Primer	Sequence	Amplicon (bp)
Group 1	FRNA 1 F (forward)	5’ CAAACCAGCATCCGTAGCC 3’	142
Group 1	FRNA 1 R (Reverse)	5’ CTTGTTCAGCGAACTTCTTRTA 3’	
Group 2	FRNA 2 F (forward)	5’ ATGCCGTTAGGTTTAGRTGAC 3’	471
Group 2	FRNA 2 R (Reverse)	5’ GCAATHGCAACCCCAATA 3’	
Group 3	FRNA 3 F (forward)	5’ CTACTGCTGGTAATCTCTGGC 3’	795
Group 3	FRNA 3 R (Reverse)	5’ CAACRCCGTTRGTGGGATTTAC 3’	
Group 4	FRNA 4 F (forward)	5’ CTGTCCGCAGGATCTWACCA 3’	1159
Group 4	FRNA 4 R (Reverse)	5’ GGCACTGTCCTGAATCCACG 3’	