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Relationships Between Microbial Indicators on Produce, Produce Processing Equipment, Worker Handrinses and Water Used for Growing and Processing Produce on Farms in the United States

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Master of Public Health

Epidemiology

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Abstract

Relationships Between Microbial Indicators on Produce, Produce Processing Equipment, Worker Handrinses and Water Used for Growing and Processing Produce on Farms in the United States

By Rachel A. Wardlow

Foodborne pathogens, such as *Escherichia coli*, *Salmonella* and *Norovirus,* are not easy to detect in outbreak samples, but using indicator species allows for easier detection of foodborne disease risk. This study aims to assess the relationships between microbial indicator species contaminating produce, processing equipment, workers' hands and water.

This cross-sectional study examined 1,912 samples including produce (cabbage, turnip greens, cilantro and parsley), swabs of processing and packing environments, worker handrinses and various sources of water (irrigation water, processing ice and processing water) collected in the southwest U.S. between November 2000 and December 2003. Produce and swab samples were analyzed for aerobic plate count (APC), coliforms, enterococci and *Escherichia coli (E. coli)*. Water samples were analyzed for *E.coli*, fecal coliforms and somatic coliphages.

Several indicator species had significantly different $\log_{10}$ means when means were compared amongst types of produce, amongst types of swabs, and amongst types of water samples. Among produce, APC had at least 2 pairs (cilantro vs. cabbage and parsley), coliforms had 2 pairs (parsley vs. cabbage and turnip greens) and enterococci had 4 pairs (cabbage vs. turnip greens, or cilantro and parsley vs. turnip greens or cilantro) that were significantly different. Among swabs, APC had 3 pairs (turnip greens vs. cabbage or cilantro and cilantro vs. parsley) and enterococci had 5 pairs (turnip greens vs. cabbage or parsley and cilantro vs. cabbage, turnip greens or parsley) that were significantly different. Among water, *E. coli* had 4 pairs (handrinse vs. ice or processing water and irrigation vs. ice or processing water) fecal coliforms had 5 pairs (handrinse vs. ice, irrigation or processing water and irrigation vs. ice or processing water) and somatic coliphages had 2 pairs (irrigation vs. ice or processing water) that were significantly different. Correlation values showed that several pairs of indicator species had significant associations among produce ($r = 0.20- 0.69$) and swabs ($r = 0.20- 0.61$) and among water samples ($r = 0.70– 0.93$). Among unadjusted prevalence odds ratios, coliforms were most frequently a significant exposure (OR= 2.11- 16.97) compared to other indicator species. Among linear regression models, APC and *E. coli* were the most frequently significant predictors compared to other indicator species. Among adjusted prevalence odds ratios, all indicator species were found to be significant predictors when combined in produce models. Among adjusted odds ratios from swab models, coliforms had significant odds of being present when enterococci or *E. coli* were present, but enterococci and *E. coli* had significant odds of being present when coliforms were present. In summary, among produce samples, swabs of harvesting and processing equipment, various types of water that contact produce and worker handrinses there were significant relationships among microbial indicator species.

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Literature Review

Prevalence of Foodborne Illness in U.S. Related to Vegetables

Several types of vegetables contaminated with microbial pathogens, including leafy greens, vine-stalk vegetables, seeded vegetables and row crop vegetables, have been implicated in recent foodborne disease outbreaks in the U.S. In a study examining foodborne disease occurrence from 1998-2008, leafy vegetables caused 8% of foodborne disease outbreaks (1) and 13% of outbreak-associated illnesses, while 10% of outbreak-associated illness was caused by vine-stalk vegetables. As an example, leafy vegetables contaminated with shiga toxin-producing *Escherichia coli* (*E. coli*) caused close to 300 outbreak-associated hospitalizations and about 5 deaths from 1998- 2008 (1). During that time, *Salmonella* contaminated vine-stalk vegetables cause about 3,000 outbreak-associated illnesses. Reports for 2012 show that seeded vegetables and vegetable row crops caused 26 outbreaks (14%) and 583 cases of illness (14%) (2). While the U.S. is an advanced nation by many standards, foodborne illness due to produce still plays a big part in the health of the nation.

Food Safety Policies in the U.S.

In order to decrease foodborne illness due to produce, there have been three main areas of influence on past U.S. guidelines for safe produce: Good Agricultural Practices, Hazard Analysis and Critical Control Point system, and *Codex Alimentarius*. These guidelines have used scientific knowledge to develop actions and practices that can decrease microbial contamination of produce. More recently, President Obama signed into action the Food Safety Modernization Act to further help decrease U.S. foodborne illness incidence (3).

Good Agricultural Practices (GAPs) have been the basis of produce-related interventions during production (4). These guidelines included ways to decrease microbial contamination prior to planting, during planting, during harvesting, and during post-harvesting practices. Stemming from GAPs, documents have been developed by government agencies, growers, shippers, academia, and other stakeholders to help address issues and increase implementation of guidelines (4). The main document stemming from GAPs, produced by the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) in 1998, was *Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables* (5, 6). This scientifically-based document guides voluntary inspections of produce production farms (7) and helps industries create specific guidelines based on their unique challenges (5). GAPs have been the basis of an outreach campaign to share information with the agricultural community about ways to improve the safety of food production. Working with multiple growers' associations FDA helped develop documents specific to the type of produce being produced, processed and packaged, such as *Commodity Specific Food Safety Guidelines for the Lettuce* (6). The *Voluntary Food Safety Guidelines for Fresh Produce* was published by the International Fresh Cut Produce Association and the Western Growers Association (4). Other documents include *The Quality Assurance Program of the California Strawberry Commission* and *Food Safety Begins on the Farm* by Cornell University (4). GAPs are one of the more widely dispersed models in the U.S. for decreasing contamination of produce during production (4). Some wholesale and foodservice produce buyers started requesting growers use a third-party to perform production audits to increase GAP compliance (8, 9). While many industry-specific

guidelines have been developed, they are only recommendations with no specific government agency to enforce procedures that decrease microbial contamination leading to foodborne illness (9). Voluntary inspection programs were not a guarantee to microbe-free produce, only steps producers have taken to decrease microbes on produce (9).

Hazard Analysis and Critical Control Point (HACCP) system is widely used in the restaurant and food preparation industry and efforts have been made to incorporate the system into produce production (4). Using HACCP, in order to increase microbiological safety, certain points of possible weakness in the food production system are routinely monitored for compliance to ensure food safety (10). Although HACCP is a flexible system, it has yet to be widely applied to the produce production industry (4). HACCP is difficult to implement and maintain, due to lack of identified critical control points (11) and large quantity of required records (12). Some guidelines have been developed for specific products such as sprouted seeds, shredded lettuce, and tomatoes, but scientific data is insufficient to apply HACCP to the entire produce production industry.(4)

International guidelines have influenced U.S. guidelines for safer produce production, including those from the Codex Committee on Food Hygiene from the *Codex Alimentarius*. This is a joint program of the United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO) (4). There is an emphasis on prevention of food contamination with some developing standards for fresh and fresh-cut produce (4). The FAO and WHO gave leafy green vegetables the highest priority for reducing foodborne illness among all vegetables. This multinational body of researchers concluded that leafy green vegetables should be the first priority for reducing microbial contamination because they are consumed in such a large quantity, have been

associated with many large outbreak situations, are grown and processed in a variety of manners that can increase the microbial contamination level (13). Among other high level priorities, other vegetables were included as targets to which there is a needed decrease in microbial contamination level to decrease foodborne illness (13).The *Codex Alimentarius*, in addition to the previously mentioned guidelines, have been incorporated in U.S. Food and Drug Administration (FDA) guidelines. The U.S. Department of Agriculture (USDA) used the *Codex* as part of the Hazard Analysis Critical Control Plan (HACCP) system (10). Overall, the food production system guidelines had some scientific evidence for policy and production guidelines, but lacked enforcement.

In order to better protect the health of the U.S. people, President Obama signed into law the Food Safety Modernization Act (FSMA) in January 2011 (3). Through FSMA, the FDA was given new powers allowing more action for prevention, rather than reaction to outbreak situations (14). The mandate requires the use of comprehensive, science-based preventive controls for all areas of the food supply. More collaboration will be required with state and local officials. The FDA is required to set science-based standards for safe production and harvesting of fruits and vegetables (14). The standards must take into consideration hazards that might be intentional, as well as those that are unintentional and natural. New standards must address soil additions, worker hygiene, packing, and animals in the growing and water areas (14). Beyond new methods of prevention, FDA updated its inspection and compliance (14). Import policies were changed, so that standards for imported foods are much closer to standards for U.S. grown foods (14). FDA is allowed to take a more active role in response in preventing situations including the new ability to force recall by companies with unsafe products

(14). With increased state, federal and international government partnerships, inspection will be expanded beyond FDA personnel, in order to increase the capacity of the food safety inspection system. Through FSMA, regulations have changed to decrease produce related foodborne illness in the U.S.

Under the FSMA, the Produce Safety Alliance was created in October 2010 to include collaboration between FDA, USDA Agricultural Marketing Service (AMS), and Cornell University (3). Cornell has historically helped with creation of Good Agricultural Practices (15, 16). The four main objectives of the Produce Safety Alliance are assist produce growers and packers in learning about their role in food safety; creating training and education programs to help growers, packers and inspection agencies put into practice FDA regulation; creating training and education programs to help growers, packers and inspection agencies put into practice strategies that will increase food safety and environmental protection; and serving as place to disseminate information related to produce food safety (16).

Routes of Vegetable Contamination

Vegetables, including leafy greens and herbs, have many opportunities to become contaminated with microbial pathogens, including norovirus, *Salmonella*, and *E. coli* O157:H7, and microbial pathogen indicator species, including coliforms, *E. coli*, and enterococci, during the growth and harvest periods (8, 17). There are many points along the production and processing pathways that can contaminate vegetables, including the growing field and surrounding areas, water that might contact the vegetables, additions or treatments to the soil, equipment used for harvest, people that are harvesting vegetables, and environmental or climate issues that may be favorable for microbial growth (18).

For decreasing contamination during these points during growth and harvest, assessments should be performed prior to planting and immediately preceding harvest (19). Before planting and harvesting, the growing areas and surrounding environment should be checked for possible routes of contamination. These possible routes of contamination include presence of animals, sources of possible human pathogens, surrounding land that might lead to runoff water contamination, and current or past flooding (18). Nearby wildlife and livestock may have access to production areas and these animals can be carriers of human pathogens. Proximity to urban areas can influence microbial content of rain water runoff (19). Rain water can increase the microbial contamination of surface waters and splash soil onto leafy greens during production. Flood waters can often contain animal and human pathogens. If leafy greens are subject to flooding, there are no known methods to sanitize them for human consumption (18). Flooding might occur during production, harvest, storage or distribution.

Water can contaminate leafy greens directly, contaminate soil which subsequently contaminates leafy greens, or contaminate equipment used for harvesting or processing leafy greens (18). Irrigation systems are one component of direct or indirect contamination. Water can lead to different levels of contamination based on its source, so regular microbial testing should be performed (19). During harvest, water may be applied to leafy greens to help maintain their crispness, so its quality needs to be tested regularly (5). Use of untreated human or animal waste in the water systems can cause contamination of leafy greens during production. Guidelines indicate that if water testing at any of these stages shows higher than ideal levels of microbes, that the leafy greens not be harvested for human use (18).

Additions or treatments to the soil are common in the production of vegetables, but can cause microbial contamination. Manure or compost, common additions, may not use production methods adequate to kill human or animal microbes within weeks or months (18). Incorporation of heat treatment in manure and compost procedures can decrease the pathogens present (18). Other soil treatments that are possible sources of microbial contamination include fish emulsion and blood or fish meal. Once pathogens are present, they may persist for more than 4 months, even if at low levels (18). Pathogen survival also depends on soil type, environmental humidity, and sunlight present (18).

Mechanical surface contact during harvesting and processing is another possible point of contamination for leafy greens (5). Equipment should be cleaned and sanitized, inspected daily, stored properly when not being used, and used only as indicated (18). Records should document these procedures and be available for long-term recall use if needed. Placing harvested greens on soil can lead to cross-contamination of equipment surfaces and research has shown that cut greens allow pathogens to easily attach (18). Care should be taken to prevent mechanical surface contact with soil, soil amendments, animals, contaminated water, or contaminated hands.

People helping with harvesting or processing may also contaminate leafy greens with microbial pathogens (5). Since leafy greens are handled frequently during harvesting or processing, there are multiple opportunities for hands contaminated by fecal material, contaminated water, or soil to pass on pathogens (18). Even if workers do not show signs of gastrointestinal illness, they are still capable of shedding pathogens that might contaminate leafy greens (18). Workers with open cuts or other lesions should not handle produce without using proper personal protective equipment. Toilet usage, proper

placement, and hand washing stations can help decrease pathogen contamination of leafy greens and soil (18).

Environmental conditions may favor the growth of pathogenic microbes. Cool, humid weather makes it easier for pathogens to remain present (18). Dry weather, while decreasing pathogen levels present, may require the usage of irrigation water, thus increase the likelihood of contamination through that route. Blowing dust might carry pathogens from the surrounding environment. Leafy greens with a high level of soil contamination should be subjected to cleaning or possibly not harvested (18).

Pathogens and Indicator Species Relationships

Pathogens implicated in vegetable foodborne illness include, but are not limited to, *Escherichia coli, Salmonella* and *Norovirus* (20). While these microorganisms have been shown to cause illness, they are not always easy to detect in samples from outbreaks. There may be limitations to their detection due to cost, technology available and logistics (21). Detection of microbial pathogens or microbial pathogen indicator species is mostly limited to known organisms when using testing methods. Molecular testing methods only allow very small quantities of samples to be tested (21). Cultivation and identification is difficult because of very low numbers of fecal pathogens and their tendency to appear briefly and intermittently in ill animals or humans (22). Pathogen detection and identification is difficult in produce foodborne related outbreaks.

Use of microbial indicators for pathogens allow for easier detection of possible risk for foodborne illness. The World Health Organization (WHO) defines an indicator organism as one that provides information about pathogenic microorganisms (21). Leclerc gives a more detailed definition that an indicator organism is one that when

detected at a given level would show there is high probability that pathogen(s) are present, but usually at lower levels (23). Bonde first decided criteria for an ideal pathogen indicator species (21). He created this list that an ideal indicator can be found when there is a pathogen; can be found at an adequate level when pathogens are in great enough quantity to cause harm; should be found in greater quantity than the pathogen; is more hardy than the pathogen so it can be found; easy to culture; easy to accurately identify at least the genus; easy to find in a sample where pathogens are found; can be cultured despite presence or absence of other organisms in the sample (21). Building on the definition of an ideal indicator, other qualities may be needed depending on the specific situation, such as similar survival and transport ability as the pathogen, specificity to allow fecal contamination source tracking, and allows more rapid testing results (21). Indicator species absence does not guarantee that pathogens are absent and when indicators are found they do not always indicate a definite risk to the public, but their presence measured over a long period can be a good indicator of potential and relative risk (24). A meta-analysis by Wu showed that no single indicator organism has been shown to be correlated with pathogens (24), so several indicator organisms have been suggested in the scientific community.

Aerobic, or heterotrophic, plate count (APC) is a broad microbiological test to show bacterial contamination. Also known as the standard plate count, this method of testing shows aerobic and facultative anaerobic bacteria that are capable of growth from a sample (25). Many genera of bacteria are included in this testing procedure, including most of the following indicator groups or organisms (25).

Total coliforms are an indicator of fecal contamination. They are one of the most studied indicators, since they are part of drinking water regulations (24). This is a large group of bacteria including, but not limited to *E. coli*, *Enterobacter*, *Klebsiella*, and *Citrobacter* (24). Since they inhabit warm-blooded animal and human intestinal tracts, they occur in high quantity and can be found in water, even when diluted, if feces are present (26). Coliforms can replicate in the environment and are common inhabitants of biofilms, so they might be present without fecal contamination(24). This indicator is not very specific (26), so other indicators are needed in conjunction with total coliform measurements.

E. coli are an indicator of fecal contamination, including pathogenic *E. coli* O157:H7. *E. coli* was first suggested as a water quality indicator organism in 1892 (23). *E.coli* are found in intestinal tracts of humans and warm-blooded animals (27), so contamination indicates fecal presence, rather than environmental contamination (26). Most strains of *E. coli* are human bowel commensal organisms (28), but absence of *E. coli* counts have been shown to have significant predictive value for the absence of enteropathogens (29). While *E. coli* allows for simple and specific testing of microbial contamination (30), *E. coli* is not a sufficient indicator organism for viral pathogens, though, as its survival in the environment is shorter than viruses (26).

Enterococcus has become an indicator species for pathogenic fecal contamination (24, 31), including *Salmonella*. They meet several of the qualities of an ideal indicator, namely they do not reproduce outside the intestines, they survive a long time, they can still be easily detected after dilution because of their high numbers in the feces, and they are easy to detect and count in samples (26). While enterococci can be found nearly

everywhere, they are mostly found in the intestines of humans and animals (32). Enterococci more specifically indicate human fecal contamination, rather than animal contamination (24).

Coliphages have been associated with the presence of pathogens in the environment (24). Wu showed in a meta-analysis that coliphages had significant correlation with enteric viruses (24). Coliphages can be found in animal feces (28), have a similar size and shape to human intestinal viruses (24) and have shown high environmental resistance (24, 28). In some instances, coliphages have become indicators of animal viruses present in samples (33). While they have become good indicators of possible enteric virus contamination, studies have shown coliphages may be absent in presence of enteric viruses (31) Because virus particles are smaller in size than bacteria, viruses may travel faster in and out of samples, for example samples of soil in an aquifer (30). A study by Lucena indicated that while no enteroviruses were found in samples of aquifers in Argentina, Colombia, France and Spain, but bacteriophages were found (34). Often the coliphages used as indicators are not unique to human feces and can also be found in animal feces (29). Coliphages are not host-specific and may be associated with other bacteria, such as other coliforms or Enterobacteriaceae (30, 35).

Relationships Among Indicator Species

Research has shown mixed significance and directionality to relationships between indicator species, as described below. Some studies found statistically significant relationships among some indicator species, but also suggested that a relationship did not necessarily exist in all environments and exposures examined. Some of the influences on relationships among indicator species include absence or presence of

agriculture areas nearby and temperature of water. Despite research showing significant relationships among some indicators, not all studies showed that indicators had relationships to one another. Following are brief summaries of research describing relationships between different indicator species, including *E. coli*, enterococci, somatic coliphages, coliforms, and aerobic plate count (APC).

A study from Kinzelman examined the relationship between *E. coli* and enterococci in water samples. Examination of water from Lake Michigan recreational areas showed that *E. coli* and enterococci were not equal indicators of water quality, so that they were not interchangeable indicators (36). Using enterococci as a water quality indicator would have led to recreational areas being closed more frequently, compared to use of *E. coli* for water quality. Some agencies routinely use enterococci for marine water quality testing, but far fewer use enterococci for freshwater recreational water testing (36). Combining the facts that *E. coli* has been shown to have a direct correlation to gastroenteritis among swimmers and some fecal organisms can be found in the environment, despite lack of recent fecal contamination, has led some agencies to use *E. coli* over enterococci as a recreational freshwater quality indicator. While *E. coli* and enterococci might have occurred sometimes in the same recreational water sample, they cannot be used interchangeably to determine the quality of the water sample.

Lucena examined water from multiple countries to examine relationships between *E. coli*, somatic coliphages, and enterococci as indicators that were identified in groundwater samples. When examining groundwater samples from Spain, France, Argentina and Colombia a significant difference ($p < 0.01$) was found between percentages of detected *E. coli* and somatic coliphage, leading Lucena to conclude that

including coliphages in testing adds significant information when testing for enteric bacteria (34). While *E. coli* and somatic coliphages only occurred simultaneously in 1.7% of the samples, 63.3% samples were negative for both. Fecal enterococci and somatic coliphages occurred simultaneously in 17.0% of samples, but 47.8% samples were negative for both. When examining *E. coli* and fecal enterococci, 5.7% of samples were positive for both simultaneously, but 67.7% of samples were negative for both bacteria. Discussion of the results states that manners of enteric bacteria reaching groundwater sources depends on characteristics of the soil, infiltration rates of the aquifer, degree of soil saturation, soil temperature, nature of the fecal material and bacterial types being considered (34). In this study, *E. coli* and somatic coliphages and *E. coli* and enterococci had a relationship that more reliably correlated to absence, rather than presence of any indicator species.

Economou found mixed results between microbial indicator species when testing environmental water samples from Greece. Samples of river and coastal waters from Greece from a study by Economou (2013) showed significant correlation between *E. coli* and both total and fecal coliforms ($r = 0.54$, $r = 0.79$) (37). In the same study, Enterococcus was shown to have non-significant correlations with both total and fecal coliforms and *E. coli* (37).

McQuaig also found mixed results, similar to Economou, between microbial indicator species when testing U.S. environmental water samples. McQuaig (2006) examined Florida environmental waters for human fecal pollution. No significant correlation ($r = 0.015$ to 0.143) was shown between fecal coliforms and *Enterococcus*

faecium esp gene (38). The study did show a weak but significant relationship ($p < 0.05$) between *E. coli* and *Enterococcus faecium* esp gene detection (38).

Several microbial indicator species were found to have positive relationships to one another when environmental water from Canada agriculture areas was examined, which contradicted McQuaig. When examining surface waters in agricultural areas in Ontario, Canada, Wilkes (2009) found fecal and total coliforms had a moderately strong correlation ($r = 0.79$) (39). *E. coli* had a moderately strong correlation with both total ($r = 0.75$) and fecal ($r = 0.82$) coliforms (39). *Enterococcus* had a moderately strong correlation with total coliforms ($r = 0.75$), fecal coliforms ($r = 0.74$) and *E. coli* ($r = 0.79$) (39). Environmental water from agricultural areas showed that microbial indicator species had different relationships, as compared to non-agriculture water samples in the previously mentioned studies.

While exposure to agriculture is important to influence the presence of microbial indicator species in water samples, the temperature of the water was shown to be another important factor that influences the relationships between indicator species. A study by Jurzik of microbiological indicators among surface water samples showed some significant correlations among indicator species. Testing of surface water samples from $\geq$ 10°C and < 10°C was performed. *E. coli* and total coliforms had a significant moderately strong correlation ($p < 0.05$, $r = 0.88$) at $\geq$ 10°C and a very strong correlation ($r = 0.97$) at < 10°C, for an average of $r = 0.95$ (40). Overall, *E. coli* and total coliforms showed a stable relationship between the two temperatures (40). *E. coli* had a significant moderately strong correlation ($p < 0.05$, $r = 0.80$) with intestinal enterococci at $\geq$ 10°C, a significant moderate correlation ($p < 0.05$, $r = 0.44$) at < 10°C, and an overall significant

moderate correlation ($p < 0.05$, $r = 0.47$) (40). Total coliforms and intestinal enterococci had a moderately strong correlation ($p < 0.05$, $r = 0.74$) at $\geq$ 10°C, a significant moderate correlation ($p < 0.05$, $r = 0.44$) at <10°C, and an overall moderate correlation of $r = 0.44$ (40). Some indicator species relationships were confirmed in this study, adjusting for different temperatures of the water samples.

When examining public water supply samples, study results have been contradictory on the existence of relationships among indicator species. Edberg found low correlation leading to no predictable relationship between total heterotrophic and total coliform bacteria among public water supply samples (25). Contradictory to the Edberg study, a study by Horman found significant correlation when comparing total coliforms, fecal coliforms and *E. coli* to one another among samples of public supplied water (29).

Contributing to public water supply quality, rainwater runoff was examined to determine if relationships existed between microbial indicators. In a study by Ahmed, examining indicator species found in rainwater runoff from rooftops, *E. coli* and enterococci had a significant but moderate correlation ($p < 0.001$, $r = 0.50$) (41). While samples from rainwater collection tanks showed that 72% of samples were positive for both *E. coli* and enterococci, the levels were highly variable across samples (41). It was hypothesized rainwater contamination might be due to bird, mammal or insect fecal contamination or environmental contamination sample deposit on rooftops (41). Previous research mentioned by Ahmed indicates enterococci can be found in soil and plants in the study area, in addition to the previously mentioned fecal contamination routes. Previous studies by Ahmed showed that enterococci can survive longer in water tanks, compared

to *E. coli*. A water source can become contaminated through many routes and rainwater runoff can be a contributing factor to microbial contamination, which can be measured by using microbial indicator species.

More recently, research has been performed to find relationships between indicator species, so as to decrease foodborne illness among consumers ingesting various types of food, including produce. A study of fecal coliforms and *E. coli* on various foods by Doğan-Halkman (2003) showed statistically significant high correlation ($p < 0.0001$, $r = 0.89$) between fecal coliforms and *E. coli* when examining fruits and vegetables among several types of food types (42). While higher counts of fecal coliforms were routinely cultured compared to *E. coli*, a statistically significant ($p < 0.0001$) relationship between fecal coliforms and *E. coli* was found, so that either bacteria could be used for analysis of food post-processing bacterial contamination and fecal contamination (42). In this study, 33 of 500 food samples showed contamination with fecal coliforms other than *E. coli* when *E. coli* was not present, leading to the conclusion that analysis for both fecal coliforms and *E. coli* was not needed for routine food contamination control (42). Previous research has shown that *E. coli* is the main constituent of fecal coliforms, so many have said only *E. coli* analysis is necessary, but Doğan-Halkman asserts that since other important bacteria besides *E. coli* may be found it might be important to test for both fecal coliforms and *E. coli* (42). Applying these results to food industry testing procedures, when using the standard most probable number (MPN) technique, the last step may be left off due to fecal contamination and *E. coli* have a highly correlated relationship, thus shortening the testing period by two days. Unfortunately for the food industry, the entire MPN technique would still require two days, which is a long time to

wait for fecal contamination results (42). Alternatively, if lauryl sulfate tryptose (LST) broth supplemented with 4-methylumbelliferyl-b-D-glucuronic acid (MUG) were used, *E. coli* contamination could be determined in 24 hours for positive results or 48 hours if required for negative results, thus benefitting the food industry with a quicker testing method (42).

Holvoet helped contribute to the growing body of knowledge on microbial indicator species found on food. Holvoet examined the relationships between indicator species as found in irrigation water samples and lettuce samples. A study of Belgian irrigation water and lettuce samples as performed by Holvoet showed a significant low to moderate correlation between *E. coli* and total psychotrophic aerobic plate count (TPAC) ($r=0.355$, $p<0.05$) when examining soil, lettuce and water samples (43). When examining irrigation water, significant and stronger relationships were shown between several bacterial indicators. *E. coli*, coliforms, enterococci and TPAC were significantly ($p < 0.05$) correlated to one another (43). *E. coli* was strongly correlated ($r = 0.918$) with coliforms. Similarly, a strong correlation ($r = 0.846$) was seen between *E. coli* and enterococci (43). Comparison of coliforms and enterococci showed a strong correlation ($r = 0.748$). TPAC had a moderately strong correlation ($r = 0.437$) with *E. coli*, with coliforms ($r = 0.447$) and with enterococci ($r = 0.470$). The results of Holvoet showed slightly higher correlation of *E. coli*, enterococci and coliforms ($r = 0.79$ to 0.92) among irrigation water compared to previous study by Wilkes ($r = 0.75$) and moderately higher compared to correlation for *E. coli* and coliforms ($r = 0.54$) by Economou (43). As shown by this and other research, TPAC has a low correlation with *E. coli*, enterococci and total coliforms, indicating it is not a good indicator of hygiene and fecal contamination during

production when examining environmental and lettuce samples (43). When examining irrigation water, it may be unnecessary to analyze *E. coli*, total coliforms and enterococci due to their high correlation with one another (43). Thus, *E. coli* is the preferred indicator of poor hygiene compared to coliforms, because *E. coli* has a fecal origin (43). Holvoet showed that due to the high correlation between several indicator species, preferentially *E. coli* could be chosen, instead of testing for multiple indicator species, which can be time-consuming and expensive.

As the previously reviewed studies showed, the relationships between pairs of microbial indicator species are mixed and unclear. There is some agreement among the studies with several studies suggesting that *E. coli* and coliforms are significantly correlated (29, 37, 39, 40, 42, 43). In addition, temperature has an impact on relationships, especially the strength of the relationship between *E. coli* and enterococci (40). *Enterococcus* showed some correlation with other indicators, based on whether the water sample was exposed to agriculture (39). Even when a correlation between indicator species was identified, several researchers agreed that the organisms examined contributed different information to the outcome of microbial contamination (36, 42). Many studies identified positive correlations between microbial indicator species, but some studies showed that indicator relationships could be negatively correlated. For example, *E. coli* and somatic coliphages were shown to have negative correlation, as well as *E. coli* and enterococci (34). With presently conflicting evidence and low amount of research specifically on microbial contamination of food, especially vegetables, more research is needed in this area.

Study Goals and Aims

This study aims to assess the relationships between microbial indicator species contaminating processing equipment, worker's hands, water and produce from the southwest U.S. Microbial indicator species contamination will be assessed by examining samples from worker hand rinse samples, field processing equipment swabs, irrigation water samples and produce samples collected from several field production and processing sites for bacterial and viral indicators including aerobic plate count bacteria, coliforms, *E. coli*, enterococci and somatic coliphages.

Significance

While indicator species research and testing occur in the food industry, there are still unknown details about indicator species and their relationships to one another and directly to foodborne illness. By assessing the relationships between indicator species, a better understanding might be gained. Using indicator species might increase the efficiency, increase the ease, and decrease the time spent to test food for microbial contamination that might cause foodborne illness. These improvements would be especially useful in urgent outbreak situations. Testing food for pathogens can be a timely, expensive process with costs passed on to consumers buying produce. A better understanding of indicator species, especially their relationships to one another, might help influence policies and procedures concerning decreasing microbial contamination of produce during production and processing. By gaining a better understanding of indicator species, we might be able to gain a better understanding of pathogens contaminating produce. With a better understanding of pathogens and indicator species, foodborne

illness might be decreased in the US, along with decreasing associated healthcare costs both public and private.

Methods

Population Description

This cross-sectional study examined produce samples, swabs of produce processing and packing environments, handrinse samples from workers, and various sources of water that contacted the produce. This sampling process is described in detail in previous reports from this study (11, 44, 45), thus will only be described briefly here. Samples were collected from 15 farms and 8 packing sheds in the southern United States between November 2000 and December 2003. There were 14 different types of produce collected, in total 923 samples, including arugula, broccoli, cabbage, cantaloupe, celery, green Swiss chard, cilantro, collards, dill, kale, mustard greens, parsley, spinach, and turnip greens. Produce was taken from various locations, including boxes, bins, conveyor belts, dump tanks, merry-go-rounds used to transport produce into the dump tanks, wash tanks, and rinse cycle tanks. Samples of produce were collected in two 400- 600 gram groups and then divided in to smaller 25 gram samples for microbial analysis. Environmental swabs were taken from equipment used for harvesting, processing and packing the produce, including boxes, bins, conveyor belts, dump tanks, merry-go-rounds used to transport produce into the dump tanks, wash tanks, and rinse cycle tanks. For each piece of equipment, a 10 x 10 cm area was swabbed and the swab was placed in 10 mL of letheen broth. Water samples included handrinse samples from farm workers, ice used for packing, irrigation water, and processing water.

Sample Microbial Testing Methods

Produce samples, environmental swabs and water samples were analyzed for microbiological growth (11, 44, 45). Produce samples were analyzed for microbial indicators, including total aerobic bacteria (aerobic plate count [APC]), total coliforms, enterococci, and *E. coli*. Plate culture techniques were used to measure colony forming units (CFU) per gram of sample on smaller 25 gram subsamples of produce (45). The minimum detectable level for culture of produce samples was 10 CFU/g. Swabs, similar to produce samples, were analyzed for microbial indicators, including total aerobic bacteria (aerobic plate count [APC]), total coliforms, enterococci, and *E. coli*. Plate culture techniques were used to measure CFU/100 cm^2 sample (45). The minimum detectable level for culture of the environmental swabs was 10 CFU/100 cm^2.

Water samples were collected as three to five 4 liter water samples from each farm, including collection from the irrigation system, processing water and packing ice used in the packing sheds. Water samples were collected between October 2000 and November 2003. Hand washes from workers were taken, though these samples represent water that did not directly contact the produce collected. At each farm 1-5 workers were asked to provide a sample. Each person placed their hands one at a time into large resealable plastic bags containing 500 mL of sterile phosphate buffered solution (PBS). Workers left their hands in the bag for 10 seconds each. The PBS was then poured into a sterile 1 liter screw cap polypropylene bottle, labeled, and shipped on ice for microbial culture. Water samples were analyzed for microbial indicators, including *E. coli*, fecal coliforms, and bacteriophages, more specifically somatic coliphages. Plate culture techniques were used to measure CFU/100 mL of water. The minimum detectable level

for culture of water samples was 0.45 CFU/mL for *E. coli* and coliforms, but 0.015 CFU/mL for somatic coliphages.

Data Management

Log_{10} transformation of the microbial indicator species variables was performed and a normal distribution was attained of the microbial indicator species. A value of half the minimum detectable limit for bacterial culture was assigned to continuous variable samples that were originally assigned the minimum test value. For presence/absence analysis a dichotomous variable was created for each microbial indicator species in the produce, environmental swab, and water datasets. Presence of a microbial indicator was defined as having growth greater than half the minimum detectable limit of bacterial culture.

The type of produce (arugula, broccoli, cabbage, cantaloupe, celery, green Swiss chard, cilantro, collards, dill, kale, mustard greens, parsley, spinach, and turnip greens) and location of sample collection (boxes, bins, conveyor belts, dump tanks, merry-go-rounds used to transport produce into the dump tanks, wash tanks, and rinse cycle tanks) were considered as covariates to APC, coliforms, enterococci, and *E. coli*, as they would influence the types of microbial species identified during culture. Any stratification in the produce and environmental swab datasets with less than 7 observations was deleted, in order to increase the power of the analyses. For produce samples and environmental swabs, arugula, mustard greens and spinach were combined.

The type of water sample (handrinse, ice, irrigation, and processing) was originally considered a covariate to *E. coli*, fecal coliforms, and somatic coliphages, but the handrinse variable was dropped from the models due to lack of somatic coliphage

testing. The fecal coliforms variable was dropped from the models, due to their ubiquitous presence.

Statistical Analysis

Descriptive statistics were performed on produce samples, environmental swabs, and water samples using SAS 9.4 (Cary, NC). A p value less than 0.05 was considered significant during analysis.

To assess correlation, Pearson correlations were used and associated p values were used to assess the statistical significance at a 95% confidence level. Prevalence odds ratios were calculated for the microbial indicator species dichotomous indicator variables.

Linear multivariate regression models were created using concentration of microbial indicator species, type of produce sample and location of collection for the produce and environmental swab datasets. For the water dataset, variables included concentration of microbial indicator species and type of water sample. Logistic multivariate regression models were created using the dichotomous presence/absence variables and the same covariates as linear regression models. Since APC was found on all produce samples, APC had to be removed from logistic produce models as a predictor. Among water samples, the relationship among fecal coliforms and *E. coli* was so strong, that it required fecal coliforms be removed from logistic models as a predictor. Adjusted prevalence odds ratios were calculated from the logistic models.

Results

Before assessing relationships of indicator species, APC, coliforms, enterococci, and *E. coli,* from selected types of produce and swabs, the concentrations of indicators on produce and swab samples were examined. To compare the concentrations of indicators between selected types of produce, including cabbage, turnip greens, cilantro, and parsley, and their related swabs, the $\log_{10}$ means of each indicator was compared among types of produce and log means among types of swabs. Log_{10} means of three of the four indicator species were significantly different when compared among types of produce. Log_{10} means of two of the four indicator species were significantly different when compared among types of swabs (Table 1). APC growth was present on all four produce types and present on all samples for three of the four swab types. Cilantro had a significantly greater $\log_{10}$ mean of APC growth versus cabbage and parsley. Parsley had a significantly greater $\log_{10}$ mean of coliforms versus cabbage and turnip greens. Cabbage had a significantly greater $\log_{10}$ mean of enterococci versus turnip greens and cilantro. Parsley had a significantly greater $\log_{10}$ mean of enterococci versus cilantro and turnip greens. Cilantro swabs had a significantly greater $\log_{10}$ mean of APC versus turnip greens and parsley. Cabbage swabs had a significantly greater $\log_{10}$ mean of APC versus turnip greens. Cabbage swabs had a significantly greater $\log_{10}$ mean of enterococci versus cilantro and turnip greens. Parsley swabs had a significantly greater $\log_{10}$ mean of enterococci versus cilantro and turnip greens. Cilantro swabs had a significantly greater $\log_{10}$ mean of enterococci versus turnip greens. In conclusion, indicator growth had

several significant differences of $\log_{10}$ mean growth among indicator species by type of sample and it was important to adjust for type of sample in advanced analysis.

Before assessing the relationships of indicator species, the concentrations of indicators *E. coli*, fecal coliforms and somatic coliphages from different types of water samples were examined. To compare the mean concentrations of indicators between types of water samples, including handrinses, packing ice, irrigation water, and processing water, the $\log_{10}$ means of each indicator were compared (Table 2). Handrinses had a significantly greater $\log_{10}$ means of *E. coli* versus ice and processing water. Irrigation had a significantly greater $\log_{10}$ means of *E. coli* versus ice and processing water. Handrinses had significantly greater $\log_{10}$ means of fecal coliforms versus irrigation water, processing water and ice. Irrigation water had significantly greater $\log_{10}$ means of fecal coliforms versus processing water and ice. Irrigation water had significantly greater $\log_{10}$ means of somatic coliphage versus processing water and ice. In conclusion, $\log_{10}$ means of each indicator species showed several significant differences of indicator species $\log_{10}$ mean concentration among indicator species by type of sample and it was important to adjust for type of sample in advanced analysis.

In addition to comparing the $\log_{10}$ mean concentration of an indicator species between types of produce, between types of swabs and between types of water samples, the Pearson correlation r value can be calculated and used to further describe the relationship between two indicator species. To assess the strength of correlation between pairs of indicator species, Pearson correlations were calculated. Based on a guide from Quinnipiac University (46), the Pearson r value was used to indicate very strong, strong, moderate, or weak correlation (Figure 1). Among cabbage produce samples, most

indicator pairs had significant, weakly positive correlation, but one pair (enterococci versus coliforms) had a significant, moderately positive correlation. Among turnip green produce samples, most indicator pairs had significant, weakly positive correlation, but again one pair (enterococci versus coliforms) had a significant, strongly positive correlation. Among cilantro produce samples, most indicator pairs had significant, moderately positive correlation, but some (*E. coli* versus APC and coliforms) had significant, weakly positive correlation. Among parsley produce samples, most pairs had a significant, weakly positive correlation, but one pair (APC versus enterococci) had a significant, strong positive correlation. Among cabbage swab samples, most indicator pairs had a significant, weakly positive correlation, but two pairs (enterococci versus APC and *E. coli*) had a significant, moderately positive correlation. Among turnip green swab samples, APC had a significant, weakly positive correlation with coliforms. Among cilantro swab samples, coliforms versus *E. coli* had a significant, weakly positive correlation, but APC had a significant, strongly positive correlation with enterococci. Among parsley swab samples, APC had a significant, weakly positive correlation with coliforms and a similar correlation was seen between enterococci and *E. coli*. Among parsley swab samples, APC had a significant, moderately positive correlation with enterococci and a similar correlation was seen between coliforms and *E. coli*. In conclusion, there were many correlations among pairs of indicators and most correlations were weakly positive to moderately positive among the produce and swab samples. Indicator species for produce had more statistically significant correlations compared to indicator species for swabs. The few significant, strongly positive correlations involved enterococci as one part of the pair of indicators being compared.

The Pearson r value was calculated between pairs of indicator species and used to further describe the relationship between indicator species by types of water samples. To assess the strength of correlation between pairs of indicator species, Pearson correlations were calculated. Using a guide from Quinnipiac University (46), the r value was used to indicate very strong, strong, moderate, or weak correlation (Figure 2). Among handrinses and processing water, fecal coliforms and *E.coli* had a significant strongly positive correlation. Among ice and irrigation water, there was a significant very strongly positive correlation between coliforms and *E. coli*. Among processing water samples, somatic coliphages and *E. coli* had a significant, weakly positive correlation, but among irrigation water samples, a significant weakly negative correlation was observed. In conclusion, among handrinse samples, there was a wide variety of strength of correlations ranging from weakly negative to very strongly positive correlation.

Beyond assessing correlation between indicator species, prevalence odds ratios between pairs of indicator species were calculated to provide a better description of relationships, in terms of direction and magnitude. To further assess the association of indicator species based on absence or presence of growth, prevalence odds ratios were calculated for indicator species absence versus presence on produce, swab, and water samples based on type of sample (Table 3). Among cabbage, there were significant odds of having coliforms if *E. coli* were present. Among turnip greens, there were significant odds of having coliforms if enterococci were present. Among cilantro, there were significant odds of having *E. coli* if enterococci were present. Among cilantro, there were significant odds of having coliforms if enterococci or *E. coli* were present. Among parsley, there were significant odds of having coliforms if enterococci were present.

Among cabbage swabs, there were significant odds of having coliforms if enterococci or *E. coli* were present. Among cilantro swabs, there were significant odds of having coliforms present if enterococci were present. Among processing water, there were significant odds of having somatic coliphages present if *E. coli* were present. Comparing the relationships of indicator species to one another using prevalence odds ratios showed that several indicator species had statistically significant relationships, but there were more significant relationships among the produce samples, than among the swab samples.

The relationships between indicator species by type of sample had been assessed, but incorporating adjustment for the other indicator species, type of sample and in some cases location of collection, would provide a more complex description of the relationships. The relationships between indicator species were assessed (Table 4) using linear models adjusted for other indicator species, type of sample and location of collection (produce and swabs) or other indicator species and type of sample (water). When APC was the outcome in a produce model, coliforms and enterococci were significant predictors in the model ($\hat{\beta}$= 0.10- 0.32). When coliforms was the outcome in a produce model, APC, enterococci and *E. coli* were significant predictors in the model ($\hat{\beta}$ = 0.20- 0.23). When enterococci was the outcome in a produce model, APC, coliforms and *E. coli* were significant predictors in the model ($\hat{\beta}$ = 0.17- 0.61). When *E. coli* was the outcome in a produce model, coliforms and enterococci were significant predictors in the model ($\hat{\beta}$ = 0.08- 0.20). When APC was the outcome in a swab model, coliforms and enterococci were significant predictors in the model ($\hat{\beta}$ = 0.23- 0.55). When coliforms or enterococci were the outcomes in swab models, APC and *E. coli* were significant predictors in the model ($\hat{\beta}$= 0.04- 0.49). When *E. coli* was the outcome in a swab model,

coliforms and enterococci were significant predictors in the model ($\hat{\beta}$ = 0.07- 0.20). Among water models, *E. coli* and somatic coliphage were not significant predictors when the other was the outcome. In conclusion, in most produce and swab models, at least two predictor variables were significant. Coliforms and enterococci were most frequently significant predictors among produce and swab linear models with a single indicator species as the outcome and adjusting for other indicator species, type of sample and location of collection (produce and swabs).

The odds ratios among pairs of indicators by type of sample were calculated previously, but odds ratios for indicator species presence adjusted for other indicator species, type of sample and location of collection (produce and swabs) or other indicator species and type of sample (water) would demonstrate if including other covariates might better describe the association between indicator species. To further assess the association of indicator species based on absence versus presence of growth, prevalence odds ratios were calculated using logistic models for produce and swab samples, adjusting for other indicator species, type of produce and location of collection (Table 5). Water sample prevalence odds ratios were adjusted for type of water samples. Among produce, there were significant odds of having coliforms if enterococci or *E. coli* were present Among produce, there were significant odds of having enterococci if coliforms or *E. coli* were present. Among produce, there were significant odds of having *E. coli* if coliforms or enterococci were present. Among swabs, there were significant odds of having coliforms if enterococci or *E. coli* were present. Among swabs, there were significant odds of having enterococci if coliforms were present. Among swabs, there were significant odds of *E. coli* being present if coliforms were present. For swabs, APC was absent from only

one sample, so estimates for APC were not stable. There were no significant adjusted odds ratios for water samples. Handrinses were removed from logistic models because testing was not performed for somatic coliphages. Overall, among both produce and swabs, most indicators were significantly more likely to present than absent if another indicator species was present, adjusting for other indicator species, type of produce and location of collection.

Discussion

The purpose of this study was to assess the relationships between microbial indicator species from swabs of processing equipment, worker hand rinses, irrigation water samples, and produce harvested in the southwestern United States. Relationships among indicator species were assessed using several methods. Comparing $\log_{10}$ indicator species means among types of produce, among types of swabs, and among types of water samples, it was shown that several indicator species had significantly different $\log_{10}$ means. Using correlation r values and p values, it was shown that several pairs of indicator species had significant associations among produce, swab and water samples. Comparing the relationships of indicator species to one another using prevalence odds ratios, linear regression models and logistic regression models showed that several indicator species have statistically significant relationships. In conclusion, these various types of calculations showed that overall some statistically significant relationships existed between indicator species when comparing among types of produce, among types of swabs and among types of water samples.

When comparing $\log_{10}$ indicator species mean concentrations among types of produce, among types of swabs, and among types of water samples, it was shown that several types of produce, swabs and water samples had significantly different $\log_{10}$ means concentrations of for a given indicator species. A single microbial indicator species might be present in varying concentrations on produce, swabs and water based on type of produce, because each variety of produce has different exposures and possible routes of contamination during growth, harvest and processing. As mentioned previously in this

study, there are many different ways for produce to become contaminated with microorganisms. Some of these possible routes of contamination include the soil from the field and surrounding areas where leafy greens and herbs are grown, water that might contact the leafy greens and herbs, additions or treatments to the soil, equipment used for harvesting, people that are harvesting vegetables, and environmental or climate issues that may be favorable for microbial growth (12, 18). Each type of produce is exposed to possible contaminants based on specific methods used for growing, harvesting and processing that are most beneficial for that variety. Thus, leafy greens, such as cabbage or turnip greens, compared to herbs, such as cilantro or parsley, undergo different processes from production through processing. For this study, the possible routes of harvesting and processing described in subsequent sentences were documented in previous publications (47). For cabbage in this study, there are many steps between growing and processing. During the growth phase, cabbages are grown in a field with close soil contact. In addition, irrigation water, possibly contaminated with indicator species, might be necessary or rain showers might cause soil or animal feces to splatter onto leaves. Also, soil additions might be another possible route of contamination, since they often contain some form of animal feces. When the cabbages are ready to harvest there is another separate process with possible exposure points. Cabbages are harvested and placed in trailers where they are transported to a processing shed. They go on a conveyor belt where employees remove outer leaves then pack them in a box. The boxes are stacked on a palette and go through a hydro vacuum before resting in a cold room (47). All of these different steps expose cabbage to various microorganisms along the way. Cilantro and parsley have different paths from growth through processing by virtue of having different

requirements to adequately prepare the produce for sale and consumption. For these herbs, they are grown in a field, with similar possible routes of contamination as cabbage such as soil, irrigation water, rain water and soil additions. After herbs have been harvested they go to the processing shed where they are sent through a rinse cycle using a merry-go-round. The herbs are placed on a conveyor belt that allows them to be placed in boxes for shipping (47). Due to these widespread variations of microorganism exposure, using only one method of harvesting and processing or applying only one method of microorganism contamination prevention is not realistic (19). In conclusion, by virtue of each type of produce requiring different growth, harvesting and processing steps, they are possibly exposed to varying types and concentrations of indicator species contamination.

When using correlation values, it was shown that several pairs of indicator species had significant associations. Some of the reason for strong correlation might be due to the way these indicator organisms are classified as microorganisms. As discussed previously, historically, indicator organisms were partially selected based on their ease of growth on culture and ease of identification, among other qualities (21). Ease of growth might have caused selection of some indicators which are a collection of many microorganisms, rather than a single genus. This is the case for several of these indicator species. Aerobic plate count (APC) is a broad microbiological test to show bacterial contamination. This method of testing can culture aerobic and facultative anaerobic bacteria from a sample (25, 48). Coliforms have a similar widespread inclusion of many genera of bacteria in the definition of this indicator organism (25). Coliforms include the genera of *Escherichia, Citrobacter, Enterobacter,* and *Klebsiella* (49). While the indicator species of *E. coli* is a narrow description, it is a member of several other indicator organisms. Enterococci are

typically not a member of the previously described indicators, but they do originate similarly from fecal contamination (24, 32). Thus, there is biological plausibility in finding enterococci when finding these other indicator organisms that also originate from fecal contamination. Among water samples, microorganism classification might contribute to significant correlation values, as well, because the indicator species *E. coli* is similarly a member of the fecal coliforms. The indicator species fecal coliforms are a slightly more restrictive category of coliforms, but contains many genera of bacteria, including *Escherichia* and some *Klebsiella, Enterobacter, and Citrobacter* (49). The indicator species somatic coliphages infect *E. coli* bacteria (35), thus the two organisms could be expected to be found simultaneously on samples that support their growth. The type of media used in culture techniques might influence the identification of *E. coli* and somatic coliphages (35). In conclusion, use of indicator species that contain many genera of bacteria means that correlation of cultured growth could be high.

Comparing the relationships of indicator species to one another using prevalence odds ratios, linear regression models and logistic regression models showed that several indicator species have statistically significant relationships. There did not appear to be a trend for concentration or presence of a single indicator species among different types of produce, swabs or water samples, nor was a pattern seen when examining the presence of a single indicator species between types of models. A single pattern for concentration or presence of indicator species was not observed, possibly because exposures could have been time and/or geographically dependent. If all turnip greens, for example, were collected at one time and location where the indicator species contamination levels were high, but cabbage samples were collected over several visits or farms, the indicator

species contamination levels might have become on average a lower quantity. Indicator species concentration and presence might have been geographically dependent, because as one study showed, the soil and plants in the study area can influence the indicator species levels (41). Geographical and/or time dependence could influence indicator species concentration and presence, because as other studies suggest, exposure to contaminated soil or contaminated soil additions, especially animal manures or slurries, can increase the indicator species levels (45, 50), thus might change among several types of produce if they were collected at different times or more likely at different farms. Among water samples indicator species concentration might exhibit geographical and/or time dependence, because as one study suggested the amount of bacteria in groundwater, therefore possibly irrigation water from a well, was influenced by characteristics of the soil, soil temperature, nature of microorganisms being considered (34). Other possible points of contamination that might be time or geographically dependent include irrigation water quality. A study of lettuce and irrigation water showed that *E. coli* and APC had a significant low to moderate correlation when examining soil, lettuce and water samples (43). Studies have shown animal feces could be spread through irrigation splashing soil up on plants (51) or rain water runoff, which could contain both *E. coli* and enterococci (41). If irrigation water comes from surface water, it has been shown that soil, soil additions or sewage overflow can contaminate surface water, which can lead to contaminated crops (50). Irrigation and processing water might have varying concentrations of indicators, depending on time of day and month samples were collected, because one study showed that temperature has been shown to have an effect on concentration of E.coli and coliforms in water (40). Temperature has been shown to

have an effect on APC and enterococci with both showing an increase in growth for every increase of one degree Celsius (52). In addition to environmental points of contamination, if workers' hands were contaminated by soil, soil amendments or fecal matter, indicator species contamination could have occurred (53), but could be different over time or geography.

Strengths and Limitations

This study had both strengths and limitations. One strength of this study was the increased applicability of these results, due to examination of microbial indicator species on several types of produce, from leafy greens to herbs. Another strength of this study is by incorporating a variety of produce and several collection locations, many types of production processes are included, thus making these results applicable to wide variety of production systems. One limitation of this study, despite the variety of produce used in this study, was the cross-sectional study design, so the direction of relationship (cause and effect) and risk cannot be assessed. Another limitation of this study is the low quantity of water samples, thus limiting the power of the calculations performed.

Implications

As shown previously, research on indicator species has shown mixed relationships, ranging from positive correlation, to no relationship, to negative correlation being demonstrated. Several of these analyses showed there are statistically significant associations among indicator species from produce, environmental swabs from produce production equipment and water sample when considering type of sample. Statistically significant odds ratios, some with positive, high magnitude away from the null, indicate

these microorganisms might be interchangeable when performing microbial testing on produce samples such as the ones included in this study.

Since positive, statistically significant associations were exhibited among several indicator organisms, this information could contribute to development of more efficient laboratory testing procedures. This might include decreasing the number of cultures performed to test produce for food safety standards. Using this information, more analysis might be performed to determine if these samples used in predictive modeling would provide more information to decrease produce microbial contamination.

Ultimately, finding significant associations among produce microbial indicator species could be useful in creation of public policy and produce industry guidelines to decrease foodborne illness. Since leafy greens and herbs produced in a small region of the country might be consumed by the public throughout the country, foodborne illness can quickly become a widespread problem throughout the country.

Conclusions

In conclusion, this study was performed to assess the relationships between microbial indicator species from processing equipment, worker hand rinses, irrigation water samples, and produce harvested in the southwestern United States. It was shown that $\log_{10}$ means were significantly different among indicator species in different types of produce, different types of swabs, and different types of water samples. Using correlation values, it was shown that several pairs of indicator species had significant associations. Comparing the relationships of indicator species to one another using prevalence odds ratios, linear regression models and logistic regression models it was shown that several indicator species have statistically significant relationships. Overall, among produce

samples, swabs of harvesting and processing equipment, various types of water that contact produce and worker handrinses there were significant relationships shown among microbial indicator species including aerobic plate count (APC), coliforms, enterococci, and *Escherichia coli* when calculated as $\log_{10}$ means, correlation, linear parameters and unadjusted and adjusted odds ratios.

Future Directions

While this study did show some significant relationships among indicator species, more indicator species research is needed on a wider variety of produce samples. Including more types of produce would include more possible points of contamination based upon their unique processes required from growth to processing. In addition, more indicator species research needs to be performed over a wider geographical area in the U.S., which would provide a better idea if these results are applicable only to produce grown in the southwest U.S. or apply to produce grown anywhere in the country. More research is needed on water samples, especially as this sample size was small. Including somatic coliphage research in future handrinse samples would help describe the relationship this indicator species has to others. More studies are needed with prospective design, so that other calculations such as risk could be calculated for produce.

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Tables

Table 1. Microbial Indicators from Produce and Swabs of Produce Processing Equipment by Type of Sample

		APC			Coliforms			Enterococci			*E. coli*		
Sample Type	**n**	**% with growth**	**Log_{10} Mean¥**	**SD**	**% with growth**	**Log_{10} Mean¥**	**SD**	**% with growth**	**Log_{10} Mean¥**	**SD**	**% with growth**	**Log_{10} Mean¥**	**SD**
Produce													
Cabbage	124	100	13.87^{a}	1.51	66	3.99^{a}	2.09	94	$7.35^{a,b}$	2.36	20	2.22	1.41
Turnip Greens	33	100	13.56	1.67	61	3.51^{b}	2.22	61	$3.93^{a,c}$	2.39	0	0.92	0.00
Cilantro	235	100	$14.71^{a,b}$	2.09	68	4.64	2.77	71	$5.24^{b,d}$	3.06	23	2.41	1.75
Parsley	150	100	13.87^{b}	2.22	80	$5.44^{a,b}$	2.72	89	$6.88^{c,d}$	2.89	11	1.98	1.18
Swab													
Cabbage	109	100	10.04^{a}	2.03	50	2.91	1.52	87	$5.57^{a,b}$	2.48	15	2.04	1.15
Turnip Greens	33	100	$7.53^{a,b}$	1.91	18	2.00	0.88	12	$1.86^{a,c,d}$	0.73	0	1.61	0.00
Cilantro	141	99	$10.32^{b,c}$	3.52	43	2.88	1.76	55	$3.84^{b,c,e}$	2.71	3	1.67	0.44
Parsley	72	100	8.94^{c}	2.79	22	2.39	1.73	69	$5.09^{d,e}$	3.06	8	1.93	1.33

¥ Produce log_{10} mean in CFU/gram of sample. Swab log_{10} mean in CFU/10^2 cm. Among columns of indicator species for produce or columns of indicator species for swabs, superscript letters indicate pairs of log_{10} means are significantly different ($p < 0.05$) from one another. Values in produce column or swab column without a superscript letter are not significantly different.

Table 2. Microbial Indicators from Water by Type of Sample

Sample Type	*E. coli* n	*E. coli* % with growth	*E. coli* Log_{10} Mean¥	*E. coli* SD	Fecal Coliforms n	Fecal Coliforms % with growth	Fecal Coliforms Log_{10} Mean¥	Fecal Coliforms SD	Somatic Coliphage n	Somatic Coliphage % with growth	Somatic Coliphage Log_{10} Mean¥	Somatic Coliphage SD
Handrinse	293	61	$3.05^{a,b}$	4.41	298	84	$5.18^{a,b,c}$	4.26	0	---	---	---
Ice	62	19	$-0.47^{a,c}$	2.42	63	21	$0.004^{a,d}$	3.21	62	16	-4.30^{a}	1.42
Irrigation	46	87	$2.80^{c,d}$	2.39	49	96	$3.51^{b,d,e}$	2.43	49	63	$-1.92^{a,b}$	2.70
Processing Water	82	21	$-0.64^{b,d}$	1.87	83	37	$0.34^{c,e}$	2.89	80	25	-3.71^{b}	2.35

¥ Water log_{10} mean in CFU/100mL. Among columns of indicator species, superscript letters indicate pairs of log_{10} means are significantly different ($p < 0.05$) from one another. Values in column without a superscript letter are not significantly different.

Table 3. Microbial Indicator Prevalence Odds Ratios for Produce, Swabs, and Water by Type of Sample

Sample Type	n	Outcome	Exposure	POR	95% CI
Produce					
Cabbage	124	Enterococci	Coliforms	1.50	0.32, 7.04
		E. coli	Coliforms	16.97*	2.21, 130.50
Turnip Greens	33	Enterococci	Coliforms	4.80*	1.06, 21.67
Cilantro	235	Enterococci	*E. coli*	7.03*	2.43, 20.35
		Enterococci	Coliforms	5.20*	2.83, 9.55
		E. coli	Coliforms	3.03*	1.40, 6.58
Parsley	150	Enterococci	Coliforms	4.49*	1.56, 12.90
		E. coli	Coliforms	0.72	0.22, 2.42
Swab					
Cabbage	109	Enterococci	Coliforms	4.25*	1.11, 16.21
		E. coli	Coliforms	3.64*	1.09, 12.13
Turnip Greens	33	Enterococci	Coliforms	1.60	0.14, 18.72
Cilantro	141	Enterococci	*E. coli*	2.48	0.25, 24.44
		Enterococci	Coliforms	2.11*	1.06, 4.18
		E. coli	Coliforms	4.09	0.41, 40.28
Parsley	72	Enterococci	Coliforms	0.96	0.29, 3.19
		E. coli	Coliforms	1.86	0.31, 11.20
Water					
Ice	61	*E. coli*	Somatic Coliphage	1.365	0.24, 7.68
Irrigation Water	46	*E. coli*	Somatic Coliphage	0.271	0.02, 2.53
Processing Water	79	*E. coli*	Somatic Coliphage	4.727*	1.46, 15.32

***** p-value < 0.05

Table 4. Model Parameters of Concentrations of Indicator Species Adjusted± for Produce, Swab and Water

		Model Outcomes									
		Produce (n=813)				**Swabs (n=424)**				**Water (n=186)**	
Model Predictors		APC	Coliforms	Enterococci	*E. coli*	APC	Coliforms	Enterococci	*E. coli*	*E. coli*	Somatic Coliphage
APC	$\hat{\beta}$	---	0.23*	0.61*	0.02	---	0.11*	0.04*	-0.02	---	---
	SE	---	0.05	0.04	0.03	---	0.03	0.04	0.02	---	---
Coliforms	$\hat{\beta}$	0.10*	---	0.17*	0.08 *	0.23*	---	0.07	0.20*	---	---
	SE	0.02	---	0.03	0.02	0.07	---	0.07	0.03	---	---
Enterococci	$\hat{\beta}$	0.32*	0.20*	---	0.20 *	0.55*	0.02	---	0.07*	---	---
	SE	0.02	0.04	---	0.02	0.04	0.04	---	0.02	---	---
E. coli	$\hat{\beta}$	0.02	0.21*	0.42*	---	-0.12	0.49*	0.10*	---	---	0.06
	SE	0.04	0.05	0.05	---	0.11	0.07	0.10	---	---	0.07
Somatic Coliphage	$\hat{\beta}$	---	---	---	---	---	---	---	---	0.07	---
	SE	---	---	---	---	---	---	---	---	0.08	---

* $p < 0.05$

± produce and swabs adjusted for other indicator species, type of produce and location of collection; water adjusted for type of sample.

--- indicates value not calculated.

Table 5. Presence versus Absence of Indicator Species Adjusted± Odds Ratios for Produce, Swab and Water Model Outcomes

		Produce (n=813)			Swabs (n=424)			Water (n=186)	
Model Predictors		Coliforms	Enterococci	*E. coli*	Coliforms	Enterococci	*E. coli*	*E. coli*	Somatic Coliphage
Coliforms	OR	---	3.35*	2.31*	---	2.31*	3.64*	---	---
	95% CI	---	2.11, 5.32	1.26, 4.22	---	1.37, 3.91	1.51, 8.80	---	---
Enterococci	OR	3.36*	---	6.72*	2.29*	---	4.00	---	---
	95% CI	2.15, 5.25	---	2.30, 19.62	1.35, 3.88	---	0.85, 18.72	---	---
E. coli	OR	2.32*	6.03*	---	4.61*	4.36	---	---	1.87
	95% CI	1.27, 4.23	2.08, 17.51	---	1.89, 11.21	0.92, 20.61	---	---	0.80, 4.35
Somatic	OR	---	---	---	---	---	---	1.87	---
Coliphage	95% CI	---	---	---	---	---	---	0.80, 4.35	---

*p< 0.05

± produce and swabs adjusted for other indicator species, type of produce and location of collection; water adjusted for type of sample.

--- indicates value not calculated.

Figures

Sample Type	Indicator Species	APC	Coliforms	Enterococci	*E. coli*
Produce					
Cabbage	APC		0.34	0.34	0.22
n=124	Coliforms	0.34		0.43	0.38
	Enterococci	0.34	0.43		0.39
	E. coli	0.22	0.38	0.39	
Turnip Greens	APC		0.26	0.39	
n=33	Coliforms	0.26		0.63	
	Enterococci	0.39	0.63		
	E. coli				
Cilantro	APC		0.45	0.47	0.34
n=235	Coliforms	0.45		0.41	0.23
	Enterococci	0.47	0.41		0.53
	E. coli	0.34	0.23	0.53	
Parsley	APC		0.33	0.69	0.30
n=150	Coliforms	0.33		0.35	0.15
	Enterococci	0.69	0.35		0.39
	E. coli	0.30	0.15	0.39	
Swabs					
Cabbage	APC		0.23	0.48	0.22
n=109	Coliforms	0.23		0.22	0.20
	Enterococci	0.48	0.22		0.44
	E. coli	0.22	0.20	0.44	
Turnip Greens	APC		0.20	0.40	
n=33	Coliforms	0.20		0.05	
	Enterococci	0.40	0.05		
	E. coli				
Cilantro	APC		0.11	0.61	0.06
n=141	Coliforms	0.11		0.07	0.26
	Enterococci	0.61	0.07		0.16
	E. coli	0.06	0.26	0.16	
Parsley	APC		0.21	0.53	0.07
n=72	Coliforms	0.21		0.17	0.49
	Enterococci	0.53	0.17		0.25
	E. coli	0.07	0.49	0.25	

	$r < 0.20$; $p > 0.05$
	$r = 0.20$ to 0.39; $p < 0.05$; weakly positive correlation
	$r = 0.40$ to 0.59; $p < 0.05$; moderately positive correlation
	$r = 0.60$ to 0.79; $p < 0.05$; strongly positive correlation
	$r > 0.79$; $p < 0.05$; very strongly positive correlation

Figure 1. Microbial Indicator Species Correlations from Produce and Swabs by Type of Sample. There is a table header for sample type and another header for indicator species. The column under sample type lists the various types of produce and swab samples (cabbage, turnip greens, cilantro or parsley). The column with indicator species is cross-referenced with an indicator species in the top row to locate a Pearson correlation value where r is significant if $p < 0.05$. The legend shows that colors (blue shades) indicate statistically significant positive correlations.

Sample Type	Indicator Species	*E. coli*	Fecal Coliforms	Somatic Coliphage
Handrinse	*E. coli*		0.80	
n=298	Fecal Coliforms	0.80		
	Somatic Coliphage			
Ice	*E. coli*		0.93 n=62	0.17 n=61
n=63	Fecal Coliforms	0.93 n=62		0.08
	Somatic Coliphage	0.17 n=61	0.08	
Irrigation	*E. coli*		0.93	-0.17
n=49	Fecal Coliforms	0.93		-0.20
	Somatic Coliphage	-0.17	-0.20	
Processing Water	*E. coli*		0.70	0.20 n=79
n=81	Fecal Coliforms	0.70		0.14 n=80
	Somatic Coliphage	0.20 n=79	0.14 n=80	

r > 0.79; p < 0.05; very strongly positive correlation

r = 0.60 to 0.79; p < 0.05; strongly positive correlation

r = 0.40 to 0.59; p < 0.05; moderately positive correlation

r = 0.20 to 0.39; p < 0.05; weakly positive correlation

r = -0.19 to 0.19; p > 0.05; no correlation

r = -0.20 to -0.39; p < 0.05; weakly negative correlation

r=-0.40 to -0.59; p < 0.05; moderately negative correlation

r = -0.60 to -0.79; p < 0.05; strongly negative correlation

Figure 2: Microbial Indicator Species Correlations from Water by Type of Sample. There is a table header for sample type and another header for indicator species. The column under sample type lists the various types of water samples (handrinse, ice, irrigation or processing water). The column with indicator species is cross-referenced with an indicator species in the top row to locate a Pearson correlation value where r is significant if $p < 0.05$. The legend shows that colors indicate statistically significant positive (blue shades) or negative (red shades) correlations.

Appendix A: Additional Tables

Table 6. Associations of Microbial Indicator Species (Presence versus Absence) from Produce and Swabs by Type of Sample

Sample Type	n	Indicator Species	APC	Coliforms	Enterococci	*E. coli*
Produce						
Cabbage	124	APC		a	a	a
		Coliforms	a		0.27	12.37*
		Enterococci	a	0.27		1.86
		E. coli	a	12.37*	1.86	
Turnip Greens	33	APC		a	a	a
		Coliforms	a		4.27*	ND
		Enterococci	a	4.27*		ND
		E. coli	a	ND	ND	
Cilantro	235	APC		a	a	a
		Coliforms	a		30.54*	8.34*
		Enterococci	a	30.54*		16.32*
		E. coli	a	8.34*	16.32*	
Parsley	150	APC		a	a	a
		Coliforms	a		8.72*	0.28
		Enterococci	a	8.72*		2.27
		E. coli	a	0.28	2.27	
Swabs						
Cabbage	109	APC		a	a	a
		Coliforms	a		5.03*	4.82*
		Enterococci	a	5.03*		2.74
		E. coli	a	4.82*	2.74	
Turnip Greens	33	APC		a	a	a
		Coliforms	a		0.14	ND
		Enterococci	a	0.14		ND
		E. coli	a	ND	ND	
Cilantro	141	APC		0.76	1.24	0.03
		Coliforms	0.76		4.54*	1.68
		Enterococci	1.24	4.54*		0.64
		E. coli	0.03	1.68	0.64	
Parsley	72	APC		a	a	a
		Coliforms	a		0.01	0.46
		Enterococci	a	0.01		2.84
		E. coli	a	0.46	2.84	

***** p-value < 0.05

a: all samples had growth of APC

ND: no growth detected on culture

Table 7. Associations of Microbial Indicator Species (Presence versus Absence) from Water by Type of Sample

Sample Type	n	Indicator Species	*E. coli*	Fecal Coliforms	Somatic Coliphage
Handrinse	293	*E. coli*		86.62*	
		Coliforms	86.62*		
		Somatic coliphage			
Ice	62	*E. coli*		55.18*	0.12
		Coliforms	55.18*		0.003
		Somatic Coliphage	0.12	0.003	
Irrigation	46	*E. coli*		36.59*	1.43
		Coliforms	36.59*		0.66 (n=49)
		Somatic Coliphage	1.43	0.66 (n=49)	
Processing Water	82	*E. coli*		36.72*	7.30* (n=79)
		Coliforms	36.72*		0.87 (n=80)
		Somatic Coliphage	7.30* (n=79)	0.87 (n=80)	

***** p-value < 0.05