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The Effect of Produce Type, Season, and Postharvest Handling on Microbial Quality of Fresh Produce Collected Near the U.S.-Mexico Border

By

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Master of Public Health

Global Epidemiology

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By

Elizabeth Adam

B.S., University of Georgia – Athens, 2009

Faculty Thesis Advisor: Juan Leon, PhD, MPH

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Abstract

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By Elizabeth Adam

Food quality has important implications to human health, and fresh produce is becoming increasingly recognized as a vehicle for pathogen transmission to humans. Since produce is often eaten raw and pathogens may persist after washing, it is essential to identify potential routes of contamination in the production environment in order to establish preventative measures. The study goals were to evaluate the effects of produce type, season, and packing shed step on produce microbial concentrations and to assess how statistical treatment of samples below the microbial assay's limit of detection would affect results. Produce samples were collected from farms and packing sheds near the U.S.-Mexico border (n=727) and processed by enumerative methods for *E. coli*, Enterococcus, total aerobic bacteria (APC), and total coliforms. Linear regression and maximum likelihood estimation for left-censored data (Tobit regression) were compared. Cantaloupe, mustard greens, cabbage, fall and spring sampling, and most packing steps were significantly and positively correlated with microbial concentrations on produce. Both regression methods produced estimates of similar direction and significance, but beta estimates from the Linear models were underestimated and the standard deviations were too small. In summary, produce type, season, and packing shed step were significantly associated with microbial concentrations on produce. Additionally, the Tobit regression produced more accurate results compared to the Linear regression. This investigation highlights several potential routes of produce contamination in the production environment and demonstrates the need to account for left-censored data in the analysis of microbial datasets.

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INTRODUCTION

The burden of foodborne illness, due to the consumption of contaminated produce, is an increasing concern on a global and national level. From 1998-2007, 684 outbreaks accounting for over 26,000 cases of illness were linked to contaminated produce [1]. Moreover, produce was associated with the largest number of foodborne illnesses during this period compared to other food commodities (e.g. poultry, beef) [1]. A wide variety of produce and pathogens have been implicated in high-profile outbreaks. Several examples include *E. coli* O157:H7 linked to ready-to-eat bagged spinach [2], *Salmonella enterica* serovar Saintpaul traced to jalapeño and serrano peppers [3, 4], *Salmonella enterica* serovar Poona linked to cantaloupe [5], *Cyclospora cayetanensis* traced to raspberries [6], and Hepatitis A linked to green onions [7]. Recently, an outbreak of *Listeria monocytogenes* linked to contaminated cantaloupe was responsible for 30 deaths across 28 states [8]. These examples, among others, highlight the need for continued food safety efforts and novel approaches to counteract the increasing burden of disease stemming from the consumption of contaminated fruits and vegetables.

Contamination in the Production Environment

Despite the fact that there are numerous established risk factors present throughout the farm-to-fork chain, the production environment lacks sufficient preventative measures and mitigating practices (reviewed in [9, 10]). At the farm level, contaminated irrigation water, stormwater runoff, animal intrusion, and field application of feces have been linked to outbreaks [11], (reviewed in [9]). During post-harvest

production, contaminated shed surfaces, worker hygiene, and improper cooling have all been implicated as microbial hazards [12, 13]. There is a need to identify high-risk steps early in the supply chain because produce is often eaten raw, without a step to kill pathogens, and washing contaminated produce has not been proven to effectively remove pathogens (reviewed in [14, 15]).

Limitations of Outbreak Studies

While outbreak studies have established several risk factors for produce safety, they do not provide sufficient evidence to establish critical control points in daily production practices because of time constraints and the retrospective nature of these studies. Outbreak studies and subsequent media coverage have been shown to increase consumer awareness and knowledge about food safety [16], but a limitation is that they often do not identify the source of contamination within a short enough timeframe to prevent illnesses. Due to the retrospective nature of outbreak investigations, the implicated harvest is often finished by the time the investigation has been initiated (reviewed in [9]), making it difficult for researchers to identify the source of fecal contamination. For example, during the 2003 Hepatitis A outbreak due to the consumption of contaminated green onions, researchers were able to trace contamination to two farms in Mexico, but were not able to locate the specific point of contamination within the production process [7]. Conversely, researchers were able to trace the 2008 outbreak strain of *Salmonella enterica* serovar Saintpaul to agricultural water on a Mexican farm, but the outbreak had spread across 43 states and resulted in 1,500 reported cases of illness [3]. Although trace-back investigations play an essential role in reducing

the burden of foodborne illness, the food safety field lacks prospective studies that can improve prevention and mitigation practices by evaluating produce, farm, and packing shed features during daily production activities. Identifying these factors in the production environment that impair produce microbial quality will help identify potential access points for future interventions.

Past and Current Studies

To address this need, we conducted two cross-sectional field epidemiological studies in farms and packing sheds along the U.S.-Mexico border [17-19]. We found that produce samples taken from packing sheds were more contaminated than those taken from the fields. Also, the microbial indicator concentrations on cantaloupe changed significantly throughout the production process, although microbial concentrations on herbs and leafy greens did not. Generic *E. coli* concentrations on cantaloupe increased significantly throughout the packing process, and Enterococcus concentrations increased between the conveyor belt (after washing) and the packing box [18, 19]. Further analysis showed that produce type, country of origin, season of collection, and post-harvest processing were significant predictors of *E. coli* prevalence on produce [17]. In this current study, as well as our past studies, we chose to asses produce quality through quantifying indicator concentrations rather than by testing for the presence of pathogens because pathogens are often found in low levels and are assumed to be focally distributed in the environment, making them difficult to locate [17-20]. Additionally, because the scope of this study involves determining potential contamination points in the production process rather than locating the source of a specific pathogen (e.g. as in an outbreak

study), indicators were sufficient in exposing vulnerabilities in the supply chain. In addition to building on our past work, there is a need to address new statistical challenges, including the treatment of samples that are below the microbial assay's limit of detection (LOD).

Statistical Background

It is important to account for samples that are below the microbial assay's LOD in order to optimally make use of the full dataset and to avoid biased results. The microbial assay's inherent LOD prevents us from quantifying the exact numeric estimates of the portion of the dataset that is below the LOD, although these samples still reflect information about the measurement (i.e. a value below the LOD indicates a low microbial concentration even though it cannot be quantified) (reviewed in [21, 22]). Data values that are below the LOD are referred to as left-censored. There are various methods for addressing left-censored data observations including, substituting imputed numbers that follow the microbial concentration distribution of the samples, setting the left-censored data points to half of the LOD value or equal to the LOD (reviewed in [22-24]). These approaches are convenient, commonly practiced, and do not require researchers to learn alternative modeling approaches. However, they yield biased results, and the bias increases as the proportion of left-censored data points increases (reviewed in [22]). A simulation study found that the approach of using imputed values from the distribution to represent left-censored data observations distorted the variance by producing overly narrow confidence intervals and thus increased the potential for Type I error when 30% or more of data were left-censored [22]. When more than 5-10% of the measurements

were left-censored, assignment of the left-censored data observations to half of the LOD value led to biased results [22]. Using the substitution of single (e.g. LOD/2) or imputed values method for left-censored data produces standard deviations that are too small because the censored data points are moved towards the center of the distribution (reviewed in [25]). Consequently, the overly narrow confidence intervals of the estimates may increase the likelihood of incorrectly finding a risk factor to be significant (Type I error) (reviewed in [22]). An alternative to substituting values for the left-censored data observations is to perform a Tobit regression, which uses the maximum likelihood estimation (MLE) method to model the distribution of the censored data. The benefits of the MLE method are that it provides estimates closer to the true population values, produces smaller confidence intervals than other processes, and allows convenient hypothesis testing with likelihood ratio tests (reviewed in [24]). Although the benefits of Tobit regression for microbial data have been demonstrated in simulation studies, there has been limited application to the field of food safety. To the best of our knowledge, this method has not been used in produce studies, but has been implemented for studies on Campylobacter in poultry and *Listeria monocytogenes* in smoked fish [21, 25]. Therefore, there is a need to evaluate the application of Tobit regression for modeling risk factors of fruit and vegetable contamination.

Project Goals

In our analysis, we aimed to evaluate the relationship between the produce-associated factors: produce type, season of sample collection, post-harvest processing step and microbial concentrations on produce. A secondary goal was to compare standard

modeling procedures used in food safety studies (Linear, Logistic) to Tobit regression and to evaluate the impact of model choice on study results. Our findings can contribute to the field of food safety through identifying crops, seasons, and packing shed steps that should be targeted for prevention and mitigation practices in order to prevent potential fecal contamination. Our statistical investigation can provide practical instruction to food safety experts on the most appropriate and accurate analysis methods.

METHODS

Produce Sample Collection

From November 2000 to December 2003, we collected 14 types of leafy greens and produce from 15 farms and 8 packing sheds on the U.S. side of the U.S.-Mexico border. Produce sampling methods have been described thoroughly in our previous studies [18, 19]. We limited this analysis to the 13 produce types that were both grown and packaged on the U.S. side of the border (arugula, cabbage, cantaloupe, celery, Swiss chard, cilantro, collard greens, dill, kale, mustard greens, parsley, spinach, and turnip greens). A total of 767 produce samples were collected from the field and at various steps in the packing shed, including the harvesting bin, the wash tank, the turntable, the rinse cycle, the conveyor belt, and the final packing box. The harvesting bin was used to move samples from the field to the packing shed, and the turntable was used to move leafy greens through the rinse cycle.

Microbial Quality

The post-harvest processes and therefore sampling locations varied by produce type. At any unique sampling location, or time, duplicate samples of 400 to 600g of produce were collected. The samples were packed on ice, shipped overnight to North Carolina State University, and processed for microbial indicators (APC, total coliforms, total Enterococcus, and total *E. coli*) using enumerative methods within 24 hours of sample collection. Microbial methods have been described more thoroughly in our

previous studies [18, 19]. The limit of detection of the microbial assays was 10 cfu/g produce.

Statistical Analyses

Microbial indicator data were analyzed using SAS 9.2 (SAS Institute Inc., Cary, N.C.) at an alpha level of 0.05. Indicator concentrations (APC, total coliforms, total Enterococcus, and total *E. coli*) were normalized using a $\log_{10}$ transformation; however, *E. coli* distributions remained positively skewed. Therefore, non-parametric methods were used for *E. coli* analysis. Season of produce collection was defined as fall (September, October, and November), winter (December, January, and February), and spring (March, April, and May). There were no samples collected in the summer. Kruskal-Wallis tests with Tukey's tests for multiple comparisons were used to determine if there were significant indicator concentration differences by season and by packing shed step. We evaluated several modeling approaches to best determine significant predictors of fecal indicator concentrations on produce, and found that the preferred regression method varied by indicator type. For all models, we checked model assumptions and used manual forward and backwards selection processes [26].

We used multivariate predictive Linear regression models to evaluate the effects of produce type, season of sample collection, and processing location on APC, total coliforms, and Enterococcus concentrations ($\log_{10}$ cfu/g). Key assumptions were that produce type, season, and processing location were independent, and that the outcome variable, $\log_{10}$ cfu/g of each specific indicator, was normally distributed. For the

categorical predictors, the referent group was the category that provided the most stable estimate. The output beta estimate represents the change in $\log_{10}$ cfu/g due to one factor (e.g. produce type), adjusted for the other variables in the model. However, some total coliform and Enterococcus concentrations were below the limit of detection of the microbial assay (10 cfu/g). For the Linear models, we assigned a value (5 cfu/g) halfway between 0 and the detection limit (10 cfu/g) to avoid over or under-representing the sample concentration [27]. In addition to the Linear regression models with an assigned value of 5 cfu/g to samples below the limit of detection, we constructed Tobit regression models, with a left-censoring limit (10 cfu/g) (reviewed in [22, 23]). The Tobit regression method is preferred to the Linear method because the Linear approach likely leads to biased estimates and variances since often a single value replaces all measurements below the limit of detection, and the distribution of the data is not considered (reviewed in [22]). The Tobit regression, on the other hand, better represents the data by considering the shape of the full distribution [21]. The Tobit regression model was constructed using the maximum likelihood estimation (MLE) method, which adjusts for the number of samples below the limit of detection when calculating and comparing the means of multiple groups (reviewed in [23]). Key assumptions are that the residual of the $\log_{10}$ microbial concentrations were normally distributed, and that crops for which all samples are above or below the limit of detection were excluded from analysis (reviewed in [23]). The output beta estimates represent the marginal effects of the underlying relationship between the $\log_{10}$ microbial concentrations and the predictors.

As mentioned previously, *E. coli* concentrations were not normally distributed, even after $\log_{10}$ transformation. We used a multivariate, predictive logistic regression

model to evaluate the effect of produce type, season of sample collection, and processing location on $\log_{10}$ *E. coli* concentrations. *E. coli* was treated as a binary variable, with samples above the limit of detection (5 cfu/g = 0.70 $\log_{10}$ cfu/g) coded as "1", and samples equal to the limit of detection coded as "0". To meet a key assumption of logistic regression, we excluded crops for which all samples were above or below the limit of detection. The output odds ratio estimates the effect of one factor (e.g. produce type) controlling for the other variables in the model. In addition to the logistic regression model, we also constructed a general Linear model and a Tobit model for comparison. Although these models do not meet the assumption of normality of the dependent variable, we wanted to compare general trends of the estimates' magnitude, direction, and significance across models.

RESULTS

<u>Descriptive Statistics</u>

To describe the distribution of microbial indicators (APC, Enterococcus, total coliforms, and *E. coli*) for the various produce types, we determined indicator prevalence, mean microbial concentrations, and standard deviations (Table 1). Microbial concentrations varied across indicators, but overall, cabbage, cantaloupe, cilantro, and mustard greens showed higher indicator concentrations compared to celery, collards, and kale. The highest *E. coli* and APC concentrations were found on cantaloupe samples, the highest Enterococcus concentrations were found on mustard greens and cantaloupe, and the highest total coliform concentrations were found on arugula and cantaloupe. APC was the most prevalent indicator, with all produce samples showing detectable concentrations, and generic *E. coli* was the least prevalent, with 6 out of 13 crop types having detectable concentrations. Of the 6 crop types with detectable *E. coli*, cabbage, cantaloupe, and cilantro had the highest prevalence of positive samples. All cantaloupe and mustard greens and 98% of cabbage were positive for Enterococcus. Additionally, all arugula and 95% of dill samples were positive for total coliforms. Overall, indicator prevalence and concentrations varied by produce type, but high-risk crops including cabbage, cantaloupe, cilantro, and mustard greens appeared to be consistent across indicators.

To determine whether produce total indicator levels (APC, Enterococcus, total coliform, and *E. coli*) varied by season, for each indicator, we evaluated concentration differences by season (Figure 1). For all indicators, samples from the spring and fall had significantly higher indicator levels than those collected in the winter. Samples with the highest levels of APC, total coliforms, and *E. coli* were collected in the fall, and samples

with the highest levels of Enterococcus were collected in the spring. In summary, produce indicator levels varied by season.

To determine whether total indicator levels varied by post-harvest processing location, for each indicator, we evaluated the significance of concentration differences by processing location (Figure 2). Considering all indicators, samples from the rinse cycle, conveyor belt, and box had microbial indicator concentrations that were consistently, significantly higher than those taken from the field. Additionally, compared to other processing steps, only samples from the harvesting bin and final packing box had significantly higher *E. coli* indicator levels compared to samples from the field. In summary, post-harvest processing location appeared to influence indicator concentrations on produce.

Predictors of APC, Enterococcus, and Total Coliform Concentrations on Produce

To evaluate the adjusted association between indicator concentrations (APC, Enterococcus, and total coliform) and produce-associated factors (crop type, post-harvest processing step, and season), we used multivariate regression models (Table 2). As described in the methods, we also compared whether there was a difference in the analyses between defining the limit of detection as a single specific value (Linear regression), as we and others have previously done, or specifying a left-censoring limit (Tobit regression) [17, 21]. Tobit regression uses the MLE method to adjust for sample concentrations that are below the limit of detection (reviewed in [22]). Applying a left-censoring limit in a Tobit regression is a statistically more efficient and accurate

approach to model microbial datasets that contain samples whose exact value cannot be quantified and so a range must be used (e.g. below the limit of detection) (reviewed in [23]). In our case, there was no exact value for indicator concentrations below the limit of detection of 1.0 $\log_{10}$ cfu/g (Table 2).

Across all three indicators (APC, Enterococcus, and total coliforms), celery, collards, and turnip greens were associated with significantly lower indicator concentrations than parsley, while cantaloupe was significantly associated with higher indicator concentrations than parsley, controlling for season and processing location. Cantaloupe, cilantro, and mustard greens were positively correlated with APC concentrations compared to parsley, while celery, collards, dill, and turnip greens were negatively correlated. For Enterococcus, cabbage, cantaloupe, and mustard greens were positively correlated with microbial concentrations compared to parsley, while celery, cilantro, collards, spinach, and turnip greens were negatively associated. Arugula and cantaloupe were positively correlated with total coliform concentrations compared to parsley, while cabbage, celery, cilantro, collards, spinach, and turnip greens were negatively correlated. Similarly, across all indicators and using both analysis methods, the post-field processing steps appeared to contribute to significantly increased microbial indicator concentrations, compared to field. Additionally, fall and spring harvest appeared to be significantly and positively associated with microbial indicator concentrations compared to winter harvest. Overall, produce type, post-harvest processing step, and season appeared to be significantly associated with APC, Enterococcus, and total colifom concentrations on produce.

Both the Linear and Tobit regression methods produced estimates of varying magnitude and variance, but not significance and direction. In both the Enterococcus and total coliform models, the estimates from the Linear models were underestimated compared to the Tobit regression model. This underestimation of effect was likely due to the approach of specifying a single value for all data points below the limit of detection without considering the true distribution of the data (reviewed in [25]). Additionally, the standard errors of the estimates from the Linear models were smaller than those from the Tobit models, demonstrating an additional bias of the use Linear models for censored datasets, which fail to account for all of the variability in the data. However, because the overall significance and direction of the estimates remained unchanged, the differences in the two methods did not influence the overall inferences of our results. In summary, the Linear regression method produced biased and underestimated standard errors and estimates; however, the estimates from the Linear models had the same significance and direction as estimates from the Tobit regression.

Predictors of *E. coli* Presence and Concentrations on Produce

To determine the association between *E. coli* contamination and produce-associated factors (crop type, post-harvest processing step, and season), we created multivariate, adjusted Linear, Tobit, and Logistic regression models. We chose to model *E. coli* on produce differently than the other indicators because its distribution did not meet the normality assumption of the Linear and Tobit regression methods, even after log-transformation. Therefore, we decided to use Logistic regression, which allowed *E. coli* to be modeled as a binary variable indicating presence or absence on a sample. We

also compared the three methods to evaluate whether there was a difference in the analyses between defining the limit of detection as a particular value (Linear and Logistic regression) and specifying a left-censoring limit (Tobit regression) to account for unobserved, left-censored data (Table 3). Cabbage and cantaloupe were significantly more likely to be contaminated compared to parsley. The samples from the harvesting bin had the highest chance of *E. coli* contamination compared to the field, although samples from the final packing box, conveyor belt, and turntable were also significantly associated with a higher probability of *E. coli* prevalence compared to the field. Fall sample collection was significantly associated with *E. coli* presence compared to winter harvest. In summary, crop type, post-harvest processing step, and fall season were significantly associated with *E. coli* presence on produce.

Similar to the Linear and Tobit regression results for the other indicators (Enterococus, total coliforms), the Linear regression estimates were biased towards the null and showed decreased variability compared to the Tobit regression estimates. Although the directionality of all estimates remained the same between the two models, the significance of some estimates varied between models. The significance of the estimates also differed slightly from the Logistic model. It is highly probable that the estimates from both the Linear and Tobit models were unstable due to the non-normal distribution of *E. coli,* which violates key model assumptions. Thus, the estimates from the Logistic model should be considered when drawing inferences about the *E. coli* results. In summary, while the Tobit method provides a convenient means to analyze censored data, failure to meet model assumptions (e.g. normality of the dependent variable) may yield biased results.

DISCUSSION

The primary goal of this study was to evaluate the relationship between microbial indicator concentrations (APC, total coliforms, *E. coli*, Enterococcus) and produce-associated factors: produce type, season of produce collection, and post-harvest processing step. A secondary goal was to compare two approaches to analyze microbial datasets that contain samples below the microbial assay's limit of detection: standard Linear and Tobit regression models. We found produce type (e.g. cabbage, cantaloupe, and mustard greens), season (e.g. fall and spring), and most post-harvest processing steps to be significant predictors of microbial indicator concentrations. Lastly, the standard Linear and Tobit regression models produced estimates of different magnitudes and variances, but similar significance and direction.

Predictors of Produce Microbial Quality

Even after adjusting for multiple factors and processing steps, produce type was a significant predictor of microbial indicator concentrations on produce. We found that cantaloupe was significantly and positively associated with microbial indicator concentrations compared to parsley for all indicators (APC, total coliforms, *E. coli*, Enterococcus). Mustard greens were positively associated with microbial indicator concentrations for indicators APC, Enterococcus, and *E. coli* compared to parsley. Additionally, arugula was positively correlated with total coliform concentrations. Other leafy greens and herbs showed mixed results, compared to parsley. Cabbage was positively correlated with *E. coli* and Enterococcus concentrations, and negatively associated with total coliform concentrations. Cilantro was positively associated with

APC concentrations and negatively associated with total coliform and Enterococcus concentrations. Still other leafy greens and herbs were negatively associated with microbial indicators compared to parsley (celery, collards, dill, spinach, turnip greens) (Tables 2-3).

Although there is a gap in knowledge about what biological mechanisms increase the susceptibility of certain fresh produce items to increased microbial concentrations, numerous factors may affect microbial concentrations on produce, including overall plant health, the presence of competitive microflora, the sites of microbial attachment, and produce surface characteristics. Plant damage, including bacterial soft rot and fungal decay are known risk factors for pathogen contamination of produce [28], (reviewed in [29]). A study comparing fungal-rotted fresh produce to healthy produce found that the diseased produce had almost a third more Salmonella incidence than the healthy fruits and vegetables [28]. On the other hand, plant-associated microflora may inhibit pathogens by producing antimicrobial compounds and by competing for nutrients, iron, and colonization sites (reviewed in [30, 31]). Additionally, the site of microbe attachment helps determine survival. While the produce’s surface provides a harsh environment that is subject to rapid fluctuations in temperature, UV light, and moisture (reviewed in [31]), pathogens that attach to plant roots can feed off of the nutrients that the plant creates and persist longer in the environment [32, 33]. In addition to pores and cut surfaces that can shelter microbes, biofilms and waxy cuticles have been shown to promote strong pathogen attachment (reviewed in [29]). A study on the *in vitro* attachment of 24 Listeria strains to cabbage observed that Listeria cells preferred to cluster around tissue that was damaged by small abrasions or tears. Researchers also found that the number of attached

cells and the strength of cell binding increased with time exposed to the cabbage tissue. In fact, after 24 hours, over 80% of the cells remained attached following multiple washings [34]. For cantaloupes, the rough, netted rind favors pathogen attachment and complicates sanitizing [35]. A study that investigated the attachment of 16 Salmonella strains to cantaloupe rinds found that washing with water did not significantly reduce pathogen concentrations [35]. Mustard greens have not been studied extensively, but pathogen attachment to leafy greens has been investigated [36, 37]. For leafy greens, the site of pathogen attachment on the leaf is an important factor in survival. A study on the attachment of *E. coli* O157:H7 to lettuce leaves found that pathogen cells tended to gather inside the stomata and were able to penetrate the leaf's surface through cut edges. Researchers found that after chlorine treatment, the *E. coli* O157:H7 cells that were on the leaf's surface died, the cells that had gathered in the stomata were marginally viable, and cells that had penetrated the leaf's surface through the cut edges were viable [37]. This study indicates potential survival mechanisms for pathogens on leafy greens during harsh external conditions. Since our study methods involved homogenizing the produce samples, we may have detected internalized microbes on mustard greens, cabbage, and arugula. We did observe differences in results among the leafy greens vegetables, with some showing positive correlations to indicators and some showing negative associations, which may be due to additional produce-specific factors. A study on the attachment of 5 Salmonella serovars to cabbage, iceburg, and romaine lettuce found that Salmonella attachment to romaine lettuce was significantly greater than attachment to iceburg lettuce and cabbage, even under controlled lab conditions [36], indicating that unknown produce-specific factors may contribute to increased pathogen growth, even

among similar produce types. This may explain why mustard greens and cabbage had increased microbial indicator concentrations compared to other herbs and leafy greens. It is important to note that our results reflect fecal indicator concentrations rather than pathogen concentrations, and therefore, the underlying biology and mechanisms of the microbe attachment may be different. Additionally, although our findings identify several crops that are potentially at risk for contamination, the results do not explain which biological mechanisms were responsible for microbial transfer to produce.

In addition to produce type, we found that the season of sample collection was significantly associated with increased microbial indicator concentrations [17]. The highest microbial concentrations were found in samples taken in the fall, followed by those taken in the spring (Figure 1). Increased temperatures and humidity in the spring and fall may have favored microbial growth. In a study where researchers inoculated several types of leafy greens with generic *E. coli* and *E. coli* O157:H7 to measure survival in the production environment, the microbes declined at a slower rate in the summer than in the fall [38]. Another study on *E. coli* O157:H7, Listeria, and Salmonella growth in compost found that temperature, humidity, light intensity, and pathogen growth increased seasonally from the winter to summer trials [39]. Additionally, excess rainfall in the spring and fall may have led to increased stormwater runoff or water system overflows, leading to field and reservoir pollution [40].

We also found that the post-harvest processing steps were associated with microbial indicator concentrations. For APC, total coliforms, and Enterococcus, all packing shed steps were significantly associated with increased microbial concentrations compared to the field, adjusting for produce type and season (Table 2). For *E. coli*,

samples from the bin, turntable, conveyor belt, and packing box were significantly more likely to be contaminated compared to the field (Table 3). Although we were not able to identify the exact mechanism of microbial transfer to produce using our study design, numerous studies have implicated equipment surfaces, wash tanks, and worker hands [13, 41, 42]. A study on microbial contamination of Satsuma mandarin fruit found that surface microbe counts increased after harvesting and sorting. Additionally, researchers isolated *Bacillus cereus* from sorting equipment, the harvest basket, worker gloves, and fruit sampled after sorting, although the pathogen was not detected on fruit sampled directly from the orchard [42]. A study on potential routes of cantaloupe contamination in packing sheds found that low wash tank temperatures resulted in no significant reduction in microbe counts. Additionally, researchers found increased microbial counts on melons after removal from the wash tank, indicating that later stages in the packing shed contributed to increased microbial counts [41]. Another study that investigated pathogen contamination at cantaloupe farms and packing sheds found that a high proportion of samples that were positive for *E. coli* were collected on transport trailers. Researchers suggested poor sanitation and cross-contamination from flies as potential mechanisms of contamination. Additionally, researchers isolated 3 Salmonella serovars from a cooler surface, which they hypothesized may have been due to the presence of birds or other animals [43]. These studies highlight the numerous potential routes of contamination in the post-harvest environment.

Comparison of Linear and Tobit Regression Methods

Lastly, the Tobit and Linear models produced estimates of different magnitudes and variances, but similar significance and direction. Therefore, our overall conclusions that produce type, season of collection, and processing location were significantly associated with produce microbial concentrations did not change based on analysis method, but the Tobit regression provided more accurate measures. The estimates from the Linear regression models were biased towards the null, and therefore underestimated the effects of the risk factors on the microbial concentrations on produce. Additionally, the standard deviations from the Linear regression models were too small and did not account for the variability in the data. Based on the literature (reviewed in [22-25]) and our findings, we recommend use of the Tobit analysis method over the Linear method for microbial datasets that contain values below the assay's limit of detection.

Strengths and Limitations

Our study had both strengths and limitations. One potential limitation is that we assumed homogeneous sampling across farms while it is possible that samples from the same farm or packing shed may have had similar characteristics or a "clustering effect" that was not considered. However, our analysis reflects actual field conditions, and those inferences can be applied directly to developing preventative measures or locating potential mitigation points in the production environment. Another potential limitation is that we chose to model microbial indicators instead of pathogens because we expected low pathogen prevalence. In our previous analyses, we found that among 864 produce samples, 3 cantaloupe samples were positive for *Salmonella enterica* serovar Montevideo (2.3% among 132 cantaloupes, 0.3% among all produce) and 3 domestic cabbage

samples were positive for *Listeria monocytogenes* (7.0% among 43 cabbages, 0.3% among all produce). No samples were positive for *E. coli* O157:H7 or Shigella [18, 19]. Although the presence of microbial indicators may not indicate pathogen contamination, we were able to determine vulnerabilities within the production environment by identifying several produce-associated factors that were associated with microbial concentrations on produce. However, our study design did not allow us to identify the biological mechanisms of microbial transfer to produce. Nevertheless, we implemented the use of novel Tobit regression methods for produce microbial analysis to demonstrate the effectiveness and accuracy of this statistical tool in produce safety studies.

Conclusions

In summary, we found that produce type, season of sample collection, and packing shed processing step were all significant predictors of fecal indicator concentrations (APC, total coliforms, Enterococcus, *E. coli*) on fresh fruits and vegetables. We also found that the Tobit and Linear regression models provided estimates with similar direction and significance but different magnitudes and variances. This analysis contributes to the field of food safety through increasing content knowledge on potential preventative measures in the production environment. Identifying produce type, season, and packing shed step as potential predictors of produce contamination reaffirms results from our past analyses. This suggests that our findings are reliable, and that these produce-associated factors should be considered when designing interventions to prevent or control microbial contamination of produce. We also showed that performing standard Linear regression on microbial datasets with samples below the limit

of detection led to biased results. In addition to demonstrating the need to incorporate left-censored data in the analysis of microbial datasets, this study may provide a guide for such analyses.

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TABLES

Table 1. Indicator concentrations for all produce items (n=767)*

Crop[ǂ]	n	*E. coli* log$_{10}$ cfu/g Mean ± SD	*E. coli* Prevalence %	Enterococcus log$_{10}$ cfu/g Mean ± SD	Enterococcus Prevalence %	Coliforms log$_{10}$ cfu/g Mean ± SD	Coliforms Prevalence %	APC[¥] log$_{10}$ cfu/g Mean ± SD
Arugula	15	1.0 ± 0.00	0	2.3 ± 1.13	60.0	3.4 ± 1.23	100.0	5.8 ± 0.52
Cabbage	58	1.3 ± 0.60	29.3	3.3 ± 1.00	98.3	1.7 ± 0.72	58.6	5.7 ± 0.65
Cantaloupe	126	1.5 ± 0.98	24.6	4.1 ± 1.03	100.0	3.1 ± 1.22	90.5	6.6 ± 0.67
Celery	44	1.0 ± 0.23	2.3	1.1 ± 0.36	18.2	1.1 ± 0.23	22.7	4.6 ± 0.56
Cilantro	187	1.3 ± 0.72	24.6	2.4 ± 1.28	69.0	2.2 ± 1.15	66.8	6.4 ± 0.94
Collards	27	1.0 ± 0.00	0	1.2 ± 0.39	37.0	1.2 ± 0.49	22.2	4.4 ± 0.86
Dill	21	1.0 ± 0.00	0	3.1 ± 0.98	95.2	2.4 ± 1.00	95.2	5.2 ± 0.64
Kale	9	1.0 ± 0.00	0	1.0 ± 0.00	0	1.4 ± 0.40	66.7	4.9 ± 0.39
Mustard Greens	70	1.3 ± 0.79	12.9	4.3 ± 1.29	100.0	2.4 ± 1.26	78.6	6.2 ± 0.96
Parsley	141	1.1 ± 0.44	11.4	3.1 ± 1.21	88.7	2.4 ± 1.10	80.9	6.0 ± 0.99
Spinach	27	1.0 ± 0.00	0	2.2 ± 0.80	77.8	1.7 ± 0.67	63.0	5.8 ± 0.82
Swiss chard	9	1.0 ± 0.00	0	1.7 ± 0.50	77.8	1.0 ± 0.00	0	5.3 ± 0.64
Turnip Greens	33	1.0 ± 0.00	0	1.8 ± 0.92	60.6	1.6 ± 0.87	60.6	5.9 ± 0.73

* Values are the mean microbial concentration of the produce type ± the standard deviation of the mean

ǂ The limit of detection is equal to 1.0 log$_{10}$ cfu/g, so produce with values of 1.0 had undetectable levels of microbial indicators

¥ APC was detectable on all produce samples

Table 2. Linear and Tobit regression models of the association between produce indicator concentrations and produce-associated factors (n=749)

	APC		Enterococcus				Coliforms			
	Linear model		Linear model		Tobit model		Linear model		Tobit model	
Parameter	**Beta**	**SE**	**Beta**	**SE**	**Beta**	**SE**	**Beta**	**SE**	**Beta**	**SE**
Intercept	5.07*	0.11	1.93*	0.15	1.75*	0.18	1.66*	0.15	1.36*	0.20
Arugula	0.16	0.20	-0.46	0.29	-0.56	0.34	1.32*	0.29	1.51*	0.35
Cabbage	-0.23	0.12	0.37*	0.17	0.41*	0.19	-0.91*	0.17	-1.16*	0.22
Cantaloupe	0.68*	0.10	1.04*	0.14	1.08*	0.16	0.56*	0.14	0.61*	0.18
Celery	-0.91*	0.14	-1.58*	0.20	-2.74*	0.32	-1.28*	0.21	-2.26*	0.32
Cilantro	0.19*	0.09	-0.94*	0.12	-1.08*	0.14	-0.42*	0.13	-0.54*	0.16
Collards	-1.60*	0.16	-1.97*	0.22	-2.56*	0.30	-1.49*	0.23	-2.42*	0.36
Dill	-0.63*	0.17	0.31	0.25	0.37	0.28	0.06	0.25	0.11	0.30
Mustard greens	0.38*	0.11	1.46*	0.16	1.52*	0.18	0.07	0.16	0.09	0.20
Spinach	0.09	0.16	-0.57*	0.23	-0.61*	0.26	-0.54*	0.23	-0.63*	0.29
Turnip greens	-0.34*	0.16	-1.52*	0.22	-1.80*	0.26	-1.13*	0.22	-1.38*	0.29
Parsley						Referent				
2. Bin	0.66*	0.10	0.95*	0.14	1.10*	0.17	0.70*	0.15	0.94*	0.19
3. Wash tank	0.55*	0.11	0.75*	0.15	0.90*	0.18	0.32*	0.15	0.44*	0.20
4. Turntable	0.56*	0.11	0.34*	0.16	0.35	0.20	0.44*	0.17	0.60*	0.22
5. Rinse cycle	0.39*	0.10	0.61*	0.14	0.71*	0.16	0.82*	0.14	1.09*	0.18
6. Conveyor belt	0.42*	0.13	0.71*	0.19	0.77*	0.21	0.95*	0.19	1.19*	0.23
7. Box	0.61*	0.08	0.61*	0.11	0.68*	0.13	0.89*	0.11	1.14*	0.14
1. Field						Referent				
Spring	0.55*	0.08	0.65*	0.11	0.73*	0.13	0.21	0.12	0.27	0.15
Fall	1.24*	0.10	1.53*	0.14	1.74*	0.17	0.71*	0.15	0.84*	0.19
Winter						Referent				

*P < 0.05

Table 3. Linear, Tobit, and Logistic regression models describing *E. coli* concentrations on produce (n=626)

	Linear model			**Tobit model**			**Logistic model**		
Parameter	**Beta**	**95% CL**		**Beta**	**95% CL**		**OR**	**95% CL**	
Intercept	0.53*	0.29	0.76	-3.61*	-4.96	-2.27	-3.65*	-4.71	-2.58
Cabbage	0.25*	0.00	0.49	1.50*	0.48	2.52	4.00*	1.68	9.49
Cantaloupe	0.47*	0.27	0.68	1.93*	1.02	2.84	4.09*	1.82	9.17
Celery	-0.01	-0.31	0.29	-1.14	-3.18	0.90	0.26	0.03	2.26
Cilantro	0.02	-0.16	0.20	0.33	-0.49	1.14	1.39	0.68	2.88
Mustard Greens	0.26*	0.03	0.49	1.40*	0.35	2.45	2.35	0.89	6.17
Parsley					Referent				
2. Bin	0.46*	0.24	0.68	2.35*	1.35	3.35	7.24*	3.02	17.35
3. Wash tank	0.11	-0.12	0.34	0.86	-0.24	1.95	2.24	0.84	5.94
4. Turntable	0.21	-0.06	0.47	1.51*	0.25	2.77	3.79*	1.24	11.57
5. Rinse cycle	0.12	-0.10	0.33	0.79	-0.20	1.78	2.13	0.87	5.20
6. Conveyor belt	0.10	-0.17	0.37	1.29*	0.22	2.35	3.65*	1.47	9.04
7. Box	0.38*	0.22	0.55	1.80*	0.99	2.61	3.97*	1.94	8.14
1. Field					Referent				
Spring	0.10	-0.09	0.28	0.38	-0.45	1.21	1.22	0.60	2.51
Fall	0.78*	0.54	1.03	2.66*	1.65	3.67	7.96*	3.46	18.36
Winter					Referent				

*P < 0.05

FIGURES

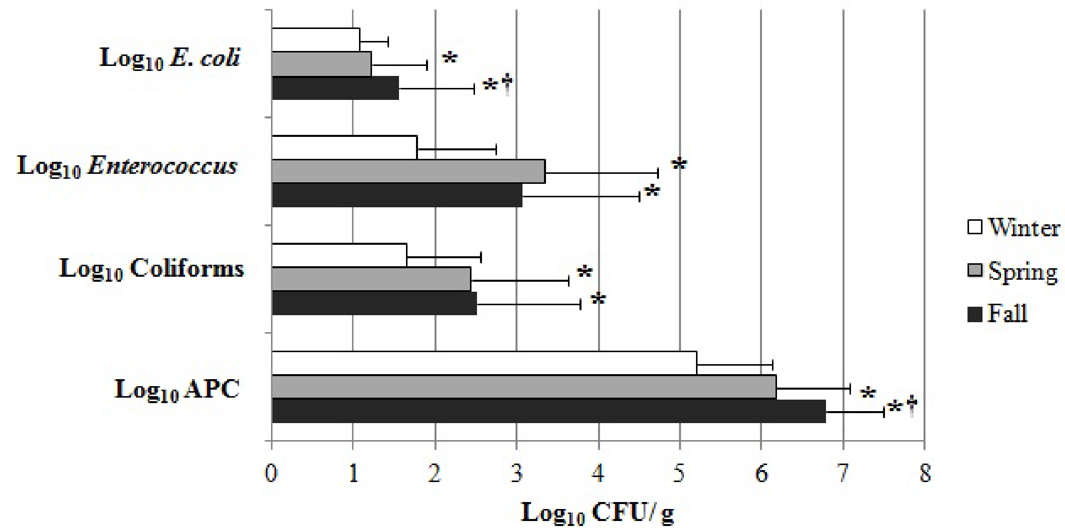

Figure 1. Produce fecal indicator concentrations vary by season. Bars represent the mean microbial concentrations for all produce, and error bars represent the standard deviation of the mean (n=727). The Kruskal-Wallis test, coupled with Tukey's multiple comparison method, was used to evaluate the significance of concentration differences by season ($\alpha = 0.05$).* $P < 0.05$ compared to winter; †$P < 0.05$ compared to spring

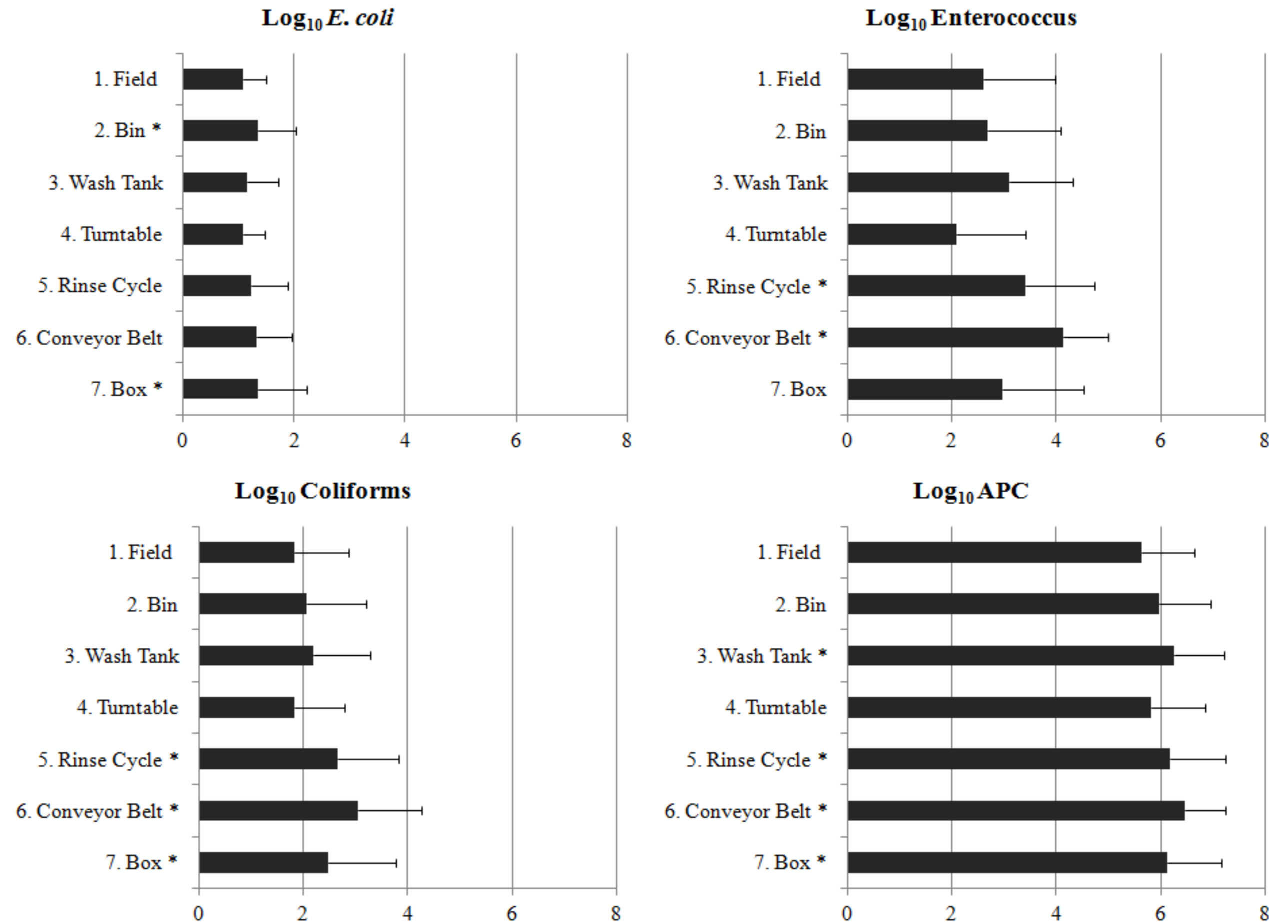

Figure 2. Produce fecal indicator concentrations vary by processing location. The bar headers number the path of produce sample collection, which represents the order of the production process, starting at the field and ending at the final packing box. Bars represent the mean microbial concentrations for all produce samples, and error bars represent the standard deviation of the mean. The limit of detection is equal to 1.0 $\log_{10}$ cfu/g, so produce with values of 1.0 had undetectable levels of microbial indicators. The Kruskal-Wallis test, coupled with Tukey's multiple comparison method, was used to evaluate the significance of concentrations differences by processing location ($\alpha = 0.05$). We only reported significant comparisons between the initial field step and other processing locations (bin, wash tank, turntable, rinse cycle, conveyor belt, packing box).$^*P < 0.05$ compared to field processing location.

APPENDIX A: IRB CLEARANCE

EMORY UNIVERSITY | Institutional Review Board

TO: Juan Leon, PhD, MPH
Principal Investigator
Global Health

DATE: June 15, 2011

RE: **Continuing Review Expedited Approval**
CR1_IRB00035460
IRB00035460
Identification and Control of Microbiological Hazards in Imported Fresh Fruits and Vegetables: A Field Epidemiological and Intervention Study in Northern Mexico

Thank you for submitting a renewal application for this protocol. The Emory IRB reviewed it by the expedited process on 06/14/2011, per 45 CFR 46.110, the Federal Register expeditable category(ies) F(7), Subpart D 46.404, and 21 CFR 56.110. This reapproval is effective from **06/29/2011** through **06/28/2012**. Thereafter, continuation of human subjects research activities requires the submission of another renewal application, which must be reviewed and approved by the IRB prior to the expiration date noted above. Please note carefully the following items with respect to this reapproval: HIPAA does not apply. A waiver of parental consent has been renewed, as well as a waiver of documentation of written/signed informed consent.

Documents renewed with this application:

- Clean Greens scientific protocolCLEAN6-16-10
- consentimientoenjuaguemanos MAR 23 2011
- Informacion-Encuesta-Productor-Manager 23 MAR 2011
- Informacion-Encuesta Manipulador 23 MAR 2011
- InformationSheet_HandRinseSampling_ver3.22.2011_CLEAN
- Oral Script for Written Consent_FarmManagerSurvey_Spanish_4.26.2011
- Oral Script for Written Consent_FarmManagerSurvey_ver4.26.2011_CLEAN
- OralScript_Hand Rinsing_ver3.22.2011_CLEAN

Any reportable events (e.g., unanticipated problems involving risk to subjects or others, noncompliance, breaches of confidentiality, HIPAA violations, protocol deviations) must be reported to the IRB according to our Policies & Procedures at www.irb.emory.edu, immediately, promptly, or periodically. Be sure to check the reporting guidance and contact us if you have questions. Terms and conditions of sponsors, if any, also apply to reporting.

Before implementing any change to this protocol (including but not limited to sample size, informed consent, study design, you must submit an amendment request and secure IRB

approval.

In future correspondence about this matter, please refer to the IRB file ID, name of the Principal Investigator, and study title. Thank you.

Sincerely,

Carol Corkran, MPH, CIP
Senior Research Protocol Analyst
This letter has been digitally signed

CC:	Bartz	Faith	Global Health
	Fabiszewski	Anna	Global Health

Emory University
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EMORY UNIVERSITY

Institutional Review Board

TO: Juan Leon, PhD, MPH
Principal Investigator

CC: Fabiszewski Anna Global Health
There are no items to display

DATE: December 9, 2010

RE: **Notification of Amendment Approval**
AM1_IRB00035460
Amendment 1 for IRB Study #IRB00035460
Identification and Control of Microbiological Hazards in Imported Fresh Fruits and Vegetables: A Field Epidemiological and Intervention Study in Northern Mexico

This is your notification that your above referenced amendment was reviewed and APPROVED by the IRB on **12/9/2010**.

Personnel Change only: Addition of Anna Fabiszweski as study Coordinator and Faith Bartz, Gaelle Gourmelon, Elizabeth Bitler and Elizabeth Adam as Emory Study Staff.

All correspondence and inquiries concerning this research study must include the IRB ID, the name of the Principal Investigator and the Study Title.

Sincerely,

Donna Thomas
Senior Office Assistant
Emory University Institutional Review Board
This letter has been digitally signed

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