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Molecular characterization of bacteria isolated from farmers market produce in Allegany County, Maryland

Abstract

Eating fruits and vegetables protects us from free radicals produced by metabolic reactions and safeguards us from cardiovascular diseases and cancer. However, there has been an increased concern about foodborne diseases tied to contaminated farmers market produce. Produce sold at open-air farmers markets has become popular among consumers because it is perceived as fresher and healthier than retail produce. Increased urbanization in the Northern Hemisphere has shifted how people shop for fresh fruits and vegetables. These small farmers are not legally required to follow the same USDA and FDA guidelines as chain companies if they meet certain rules in farmers markets, but bacteria that live on produce can contribute to foodborne outbreaks. This research explored bacterial diversity on produce collected from Allegany County, Maryland, to educate farmers in terms of minimizing cross-contamination. Three replicates of lettuce, cucumbers, strawberries, and tomatoes were collected from three farmers markets in the area, and the bacteria were identified using molecular methods, including DNA extraction, PCR, gel electrophoresis, and Illumina next-generation DNA sequencing. The alpha and beta diversities were observed using Qiime 2 with $p < 0.05$. The Faith's PD curve plot indicated sufficient sequencing depth to characterize microbial diversity. The most persistent genus in strawberries was *Klebsiella,* and the other genera isolated were Lettuce: *Enterococcus*, *Acinetobacter*, *Lysinbaccillus*, and *Trabuisiella*; Tomatoes: *Bacillus*, *Bradyrhizobium*, and Cucumber: *Klebsiella*. These results may contribute to understanding the cross-contamination of organisms during the harvesting, transport, and sale of fresh produce at farmers markets, as well as informing farmer training.

Keywords: farmers market produce, DNA extractions, and Next-generation sequencing

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Kumudini Munasinghe, Christina Richardson, Keegan Runge
Biology Department, Frostburg State University, 101 Braddock Rd. Frostburg, Maryland, USA

Correspondence: Kumudini Munasinghe, Frostburg State University, Frostburg, Maryland, USA, Tel 301-687-4299, Email kamunasinghe@frostburg.edu

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Introduction

Various chemicals have influenced the foods we collect from conventional markets via fertilizing, fumigation, preservatives, and additives to maintain their appearance and shelf life. Notice early ripened tomatoes, bananas, grapes, and pineapple to name a few produce that have chemical influences on their ripened color longer than home-grown produce. Fresh produce is recommended for everyone to eat by many health journals and biologists due to their vitamins, minerals, fiber, and antioxidant properties that can boost our healthy lifestyles. Eating colorful varieties of produce protects us from DNA damage by free radicals formed from metabolic reactions. Further, selecting healthy food over high-calorie junk food ensures protection not only from cardiovascular diseases, cancer, diabetes, and high blood pressure, but also from the speed of aging.

Farmers markets are popular for their farm-to-table atmosphere and community-based events. Products sold at open-air farmers markets are marked as fresher and healthier compared to retail produce. Increased urbanization in the Northern Hemisphere has shifted the way people shop for fresh fruits and vegetables, and they are attracted to community-centered farmers markets. These markets help small farmers reach out to more consumers, as well as lowering the carbon footprint from chain companies' shipping produce from further locations into cities. In addition, local food markets contribute substantially to the community's economic growth.[1] There are more than 8,600 U.S. farmers' markets available, which leads significantly in generating employment and gross domestic product.[2]

Farmers market regulations

The caveat is that these small farmers are not legally required to follow the same USDA and FDA guidelines as chain companies. There are state-to-state and market-to-market differences in food guidance and rules, making it difficult for farmers to navigate through the various regulatory levels.[3] Farmers may be unaware of foodborne illness risks associated with their products.[4] The FDA Food Code, the Cottage Food Laws, university cooperative extension services, and local governments elaborate various recommendations and guidelines for selling produce. However, there is a data gap in strategies established for improving food safety in farmers markets and evaluating the knowledge to understand safety risks.

According to the Wisconsin Department of Health[5] and Young et al.,[6] there were several confirmed outbreaks that occurred due to contaminated cheese, meat, fruit, and vegetables collected from farmers markets.

Foodborne illness risk factors

The key foodborne illness risk factors in farmers markets may include food from unsafe sources, inadequate cooking, inadequate temperature controls, contaminated equipment, and poor personal hygiene.[7] Farmers market safety studies have shown that practices such as a lack of handwashing, using gloves, and holding food at room temperature are reported across farmers markets, which are tied to foodborne illnesses.[8] It has been found that the risk of foodborne illness reduces after acidification and pickling, canning, and creating hypertonic environments with salt and sugar if the food is prepared

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with basic hygiene and best practices.[9–12] However, Richard et al.[13] showed that vendors at farmers markets utilize their creativity and offer value-added foods with little or no knowledge of food safety. For example, homemade beverages and ready-to-eat food have a risk of contaminated ingredients or non-hygienic preparation. It had been reported that some outbreaks of *Escherichia coli* (O157:H7) and *Salmonella* associated with guacamole and apple cider sold in farmers markets.[14] Further, cheeses made from raw milk harbor foodborne pathogens like pathogenic *E. coli*, *Salmonella*, and *Listeria monocytogenes,* and these pathogens are associated with the foodborne outbreaks in North America.[15] In addition, meat products such as pork and wild boar are tied to outbreaks of *Salmonella* in farmers markets in Canada and the US.[16,17] More than 90% of what is sold in farmers markets are fruits and vegetables, and among them, some that are generally consumed raw are considered high-risk products due to a lack of knowledge about safe food handling practices during harvesting, transport, and post-harvest handling.[4]

Among some frequently eaten raw fruits, cantaloupe, strawberries, blackberries, and tomatoes from farmers markets have been associated with outbreaks of *E. coli* O157: H7, *Salmonella*, and Hepatitis A virus.[18] It was also reported that farmers markets in Wisconsin and Alaska have had outbreaks of *Campylobacter jejune* and *Salmonella*.[5,19]

Bacterial cross-contamination and prevention

At the farmers markets, foodborne pathogens can be introduced to foods at any stage, including harvesting, transporting, and selling, to the extent pathogens persist and cause foodborne illnesses.[6,20]

The produce consumed raw and pathogens in meat, seafood, and poultry can be sources of cross-contamination via contaminated equipment, food contact surfaces, or inadvertent cross-contamination between products by leaking raw animal protein products onto fresh produce.

In integrated farms, the use of raw manure without waiting between application and harvest and the use of a water source with no microbiological testing are common practices that are often tied to cross-contamination.[20]

Some studies showed risks of higher microbial presence in farmers markets on foods compared to conventional retail stores.[21] The same study observed total coliform concentration in leafy greens, and spinach was 2 to 3 times higher at farmers markets than at supermarkets. It was also reported that raw chicken in Pennsylvania farmers markets was positive for *Campylobacter* and *Salmonella* spp.

Vendors' limited food safety knowledge regarding fresh cut fruits, sprouts, ready to eat salads, eggs, milk products, raw and undercooked meat, poultry and seafood combined with a lack of refrigeration and freezing availability at farmers markets may raise the risk of food born illnesses.[20,22,23]

Advantages of visiting farmers markets

It is a great opportunity for communities to access fresh, non-processed food directly from farmers without intermediaries. The shorter phase of reaching out to consumers made it possible to maintain the appearance of the produce. Retail markets get ethylene-treated produce for the ripening process from the large-scale producers. In addition, farmers market produce is thought to be less processed with salt and sugar.

Educate individuals involved in farming and selling

It is essential to understand the prevalence of organisms in farmers markets and how these organisms are spread through harvesting practices that involve soil and cross-contamination due to poor hygiene practices.

The primary objective of this research is to identify the most prevalent bacteria in produce at farmers markets in Allegany County, Maryland, and to educate individuals involved in farming and selling at these markets in the area. Bioinformatic analysis will be conducted to analyze and compare bacterial populations in the produce collected.

Materials and methods

Sample collection

Materials and methods were adapted from Munasinghe et al.[24] with slight modifications. Selected produce, such as lettuce, cucumbers, strawberries, and tomatoes, was collected from three Allegany County, Maryland farmers markets and transported to the microbiology lab at Frostburg State University. All the transported produce was stored at 4°C until they were used for bacterial isolations.

Isolating Bacteria

The produce was chopped into small one-centimeter cubes, and three replicates of 250g of each kind of produce were mixed with 250ml of Tryptic Soy broth and incubated overnight using the shaker incubator at 37 °C. Then, cultures made on Tryptic Soy Agar (TSA) slants were used for molecular characterization, including next-generation sequencing.

DNA extraction and purification

The DNeasy Ultraclean microbial DNA extraction kit (Qiagen, Valencia, CA) was used to extract DNA from 28 samples. About the amount of five colonies of isolated bacteria and 800µl of CD 1 solution were added into the PowerBead Pro tube and vortexed for 10 min. Then, the PowerBead Pro tubes were centrifuged at 14,000 x g for 1min at room temperature. Then, 600 µl of the supernatant from the mixture was transferred to a Microcentrifuge tube, and 200 µl of CD2 was added. The mixture was then vortexed using the same centrifuge specifications. The other reagents, CD3-CD6, were also added according to the manufacturer's protocol, and DNA was extracted from each bacterial isolate.

DNA amplification

Polymerase Chain Reaction (PCR) was performed using nucleotide oligos such as 806R reverse primer and 515S barcode primers for isolated bacteria.

The reaction mixture for the PCR

PCR was performed using the AccuPower®Taq PCR PreMix and various Illumina 16S primers. The reaction mixture was prepared according to the Earth Microbiome protocol with PCR master mix, forward primer, reverse primer, template DNA, and PCR-grade water to get a total of 25µl.

PCR reaction cycles

The thermocycler temperatures for denaturation, annealing, and extension were 95, 65 °C, and 72°C, respectively, and amplification was performed for 35 cycles. The amplicons were stored at 4°C until cleaning up the amplicons with the PCR clean-up kit (Qiagen, Valencia, CA).

DNA quality checks with the Nanodrop

When using the nanodrop spectrometer, the sample was subjected to ultraviolet light for 5 seconds and gets the readings. The dsDNA

(factor 50) assay type from the home screen was selected, and a blank using 2µl of distilled water. 2 µl of amplified and cleaned DNA was added onto the pedestal of the Nanodrop, and the DNA concentration in ng/µl and A260/A280 ratio were recorded. For best sequencing results, at least 500ng of DNA was required with the A260/A280 ratio1.8-2.0.

DNA quality checks with DNA fingerprinting

The gel electrophoresis was performed with 1% agarose under a voltage of 100V for 40min to confirm the fragment length of the V4 region of the bacterial 16S. The expected length of the fragment of bacteria was around 300-350bp.

Next-generation sequencing

After the quality control checks, all the amplicons from isolated bacteria from farmers market produce were shipped overnight to Mr. DNA (Dulles, Texas) for DNA sequencing with the Illumina MiSeq sequencing platform.

Data analysis

The data analysis was performed using Qiime 2. The significance of the analysis was defined as $p<0.05$.

Results and discussion

The Alpha and beta diversity analyses were rarefied to 2500 sequences. Alpha diversity refers to the diversity within an area. The Faith's PD curve (Figure 1) reaches a plateau at approximately 1000 sequences, indicating sufficient sequencing depth to characterize microbial diversity. The Faith's PD (phylogenetic diversity) has been used to measure evolutionary history in microbial communities isolated from farmers market produce. Beta diversity is an analysis of microbial community structure used to compare the relatedness of species collected from different sources, such as produce. A principal coordinate analysis (Figure 2) is performed to find beta diversity and visualize the relationship between microbial community samples.

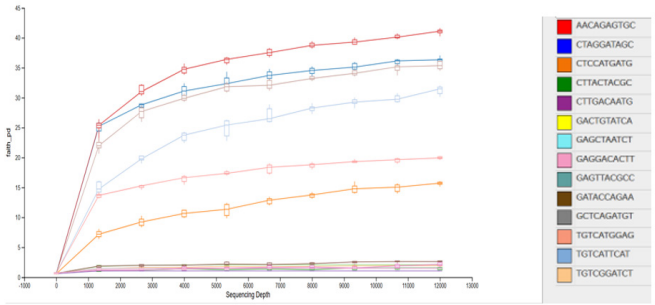

Figure 1 Faith's PD curve.

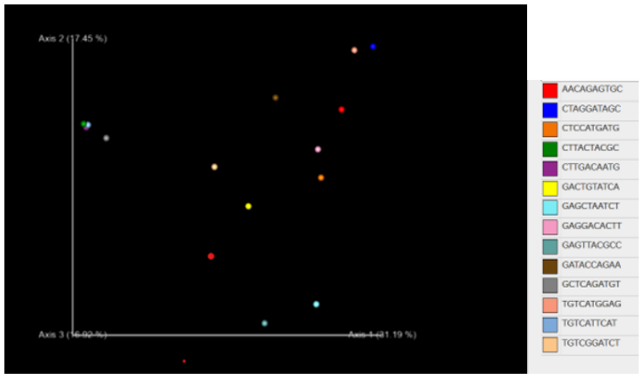

Figure 2 Bray-Curtis index.

According to the bioinformatics analysis, the most prevalent genus in strawberries was *Klebsiella*; Lettuce had *Enterococcus*, *Acinetobacter*, *Lysinbaccillus*, and *Trabuisiella*; Tomatoes had *Bacillus*, *Bradyrhizobium*, and Cucumber had *Klebsiella*.[25]

Conclusion

Three replicates of produce collected from three farmers markets in Allegany County, Maryland, were used to isolate bacteria and identify them using molecular characterization. The quality checks of DNA for next-generation sequencing were performed using Nanodrop and gel electrophoresis. Nanodrop readings of A260/A280 ranged from 1.85 to 1.87, and gel electrophoresis results had only one band per sample, around 350bp. Bacteria were identified using Metagenomics. The alpha and beta diversities were conducted using Qiime 2 with $p< 0.05$. The Faith's PD Index curve plot indicated sufficient sequencing depth to characterize microbial diversity. According to the bioinformatics analysis, the most prevalent genus in strawberries was *Klebsiella*; Lettuce had *Enterococcus*, *Acinetobacter*, *Lysinbaccillus*, and *Trabuisiella*; Tomatoes had *Bacillus*, *Bradyrhizobium*, and Cucumber had *Klebsiella*. This research is accomplished through next-generation DNA sequencing, which is a more efficient method of identifying both culturable and nonculturable bacteria. Future research is needed to show potential cross-contamination between animals and crops produced in integrated crop-livestock farms (ICLF) and backyard farms (BFs). There is a need for in-person training of farmers and workers involved in selling food products in farmers markets and determining microbial levels of pre- and post-harvest produce.

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Conflicts of interest

The authors declare no conflict of interest related to this publication.

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