REPORT

Project title: Leaf Exudate Composition Affects *Escherichia coli* O157:H7 Growth on Lettuce

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Abstract:
Despite extensive efforts to deliver safe and nutritious food, *E. coli* O157:H7 disease outbreaks associated with lettuce (especially romaine) continue to occur, which are linked to contamination on the farm. Thus, begging the question: "*does the lettuce genetics contribute to this human pathogen survival through the production chain*?" We have taken the first steps to show that, in fact, the ability of *E. coli* O157:H7 to grow on/in edible leaves varies according to the lettuce genotype. However, one can only devise mitigation strategies by understanding how exactly the plant genetics control the pathogen. One possibility is that leaf exudates contain nutrients that sustain pathogen growth, or at least survival. It is well known that different lettuces have different amounts of nutrients and chemicals such as sugars and secondary metabolites. Whether these compounds are leached to the compartments where *E. coli* O157:H7 resides (leaf surface and leaf apoplast) and this bacterium can utilize these chemicals (or be negatively affected by them) remain elusive. We have designed experiments to identify the metabolic profile of leaf exudates of diverse lettuce cultivars (Objective 1) and determine whether exudates promote bacterial growth as a factor of the lettuce type (Objective 2). The results of this research will have two immediate impacts: **(a)** identification of a new plant trait (leaf exudate composition) that can be used by breeding programs to enhance lettuce safety and **(b)** inform breeders and the industry of lettuce types that pose the highest and lowest risk of *E. coli* O157:H7 contamination based on the chemical composition of their leaf exudates, which can be source of nutrients for the bacterium. All experiments were completed; however, our data analysis is too preliminary to include in this report. The project is expected to be completed by September 2022.

Objective:
1) To determine the nutrient composition of the lettuce leaf surface and apoplast.
2) To assess *Escherichia coli* O157:H7 growth in washes of the leaf surface and apoplast.

Procedures:

Plant material and growth conditions: We selected 31 lettuce genotypes to screen for persistence of *E. coli* O157:H7 in the apoplast and on the leaf surface and determine the metabolic profiles of their exudates. These genotypes were selected based on their importance to the industry and breeding programs, as well as a representative sample of different lettuce types. The Melotto Lab at UC Davis coordinated the experimental setup with Dr. Ivan Simko's team in Salinas, CA. Seeds were planted in potting soil (Premium Growers Mix, Sun Land Garden Products), covered with sand, and wetted. Trays with seeds were kept for 48 h at 10°C in the dark to improve uniformity of germination. Afterward, the trays were maintained for two weeks in a growth room with 20°C and 16-hr/8-hr light/dark photoperiod for germination and initial growth. Established, uniform-looking plants were transplanted to 3-gallon pots (28 cm in diameter) containing 1:1 mix of potting soil and sand, fertilized with Osmocote Smart-Release Plant Food Flower & Vegetable (Scotts), and grown in a greenhouse until four true leaves on majority of plants reached circa 10 cm. After that, plants were transferred outdoor and grown in a Randomized Complete Block Design with four biological replicates. Daily temperature and relative humidity (minimum and maximum) were recorded. Mature plants were transported to UC Davis, allowed to settle in the new environment for 3-4 days and then inoculated with *E. coli* O157:H7 and sampled as described below.

Bacterial infiltration: *E. coli* O157:H7 strain 86-24 was grown in Low Salt Luria-Bertani (LSLB) medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with 50 µg/mL of streptomycin. Frozen bacterial stock was streaked onto solid LSLB medium and incubating overnight at 28°C. From this culture, a single colony was used to inoculate liquid LSLB medium, which was incubated until reaching an OD600 of 0.9 to 1. Bacterial cells were collected by centrifugation at 10,000 xg for 5 min at 20°C and suspended in sterile distilled water (DI) to obtain an inoculum concentration of 6 log CFU/ml. Fully expanded third and fourth leaves were syringe-infiltrated and dip-inoculated. Silwet L-77 at 0.03% was added to the inoculum for dip-inoculation only. Mock-inoculation of plants was used as controls.

Collection of leaf exudates and bacterium enumeration: At 1 or 7 days post-inoculation (DPI), plants had their inoculated leaves aseptically removed at the petiole and photographed for surface area calculation. To collect leaf surface exudates, leaves were washed with phosphate buffered saline (PBS) by shaking them for 10 min at 125 rpm. To collect leaf apoplast exudates, the PBS infiltration-centrifugation method was used as previously described by O'Leary et al. (2014). To estimate bacterial population size in these leaf rinses, 1 mL of these rinses was serially-diluted and plated on solid LSLB medium for CFU counts. Aliquots of these leaf rinses were stored at -20ºC.

Metabolic profile of leaf exudates: To identify the nutrients available in the leaf surface and intercellular space that support bacterial persistence, we determined the metabolome profile of the exudates. Aliquots (300 µl) of exudates were filter-sterilized and submitted to the UC Davis Metabolomics Facility (http://metabolomics.ucdavis.edu/) to perform this analysis. Briefly, metabolites were identified using gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF-MS) according to procedure described by Han and Micallef (2016). GC-TOF-MS analysis allows the identification of organic acids, amino acids, mono- and di-saccharides, among many others (up to 700 metabolites). The relative amount of these metabolites was determined

according to a standard pipeline optimized by the facility. We are in the process of analyzing the data. The experimental procedure for this analysis is depicted in **Fig. 1**.

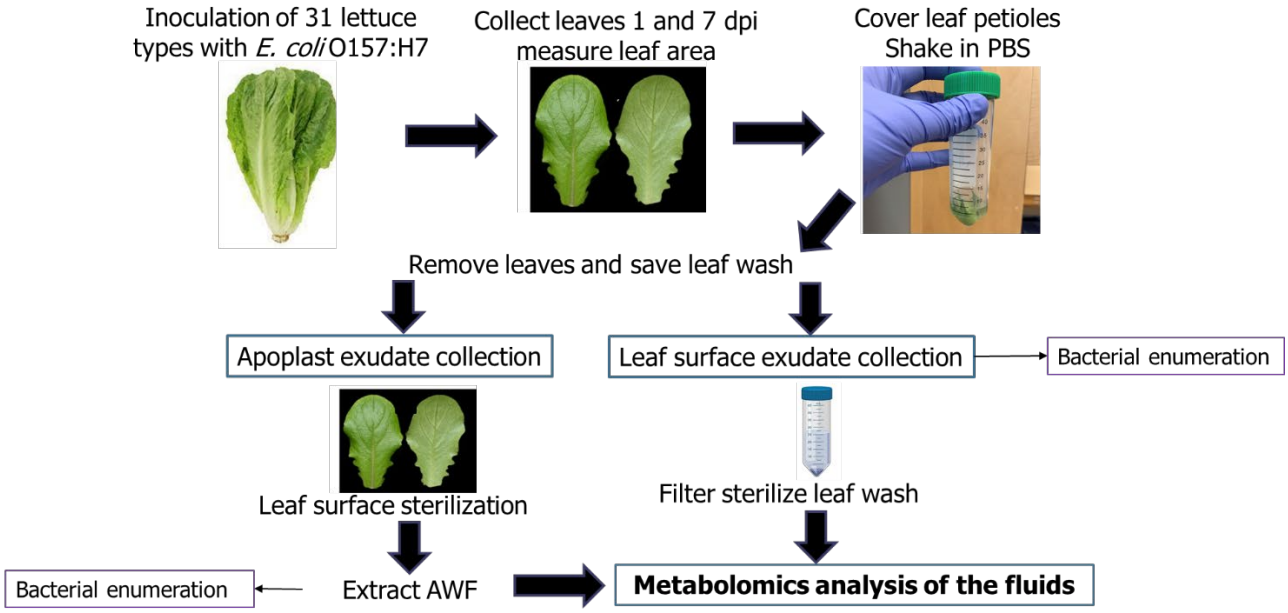

Fig 1. Flowchart illustrating the experimental procedure to determine the metabolite profile of lettuce leaf exudates.

Bacterial growth in different media: Plants were grown and syringe-infiltrated with *E. coli* O157:H7 as described above and apoplast wash fluid (AWF) was extracted as described by O'Leary et al. (2014), except that M9 salt solution was used to wash the apoplast. Bacterial growth curves were generated by adding a 5 µL of inoculum (6 log CFU/mL) to 195 µL of LSLB (positive control), M9 salts (negative control), or AWF extracted from infiltrated or non-infiltrated plants. Thus, the initial bacterial concentration was ~2.5 x 10^5 CFU/mL. These cultures were placed in each well of a 96-well microplate. Bacterial growth curves in each were obtained by growing the cultures under an orbital speed of 282 cycles per minute and double orbital continuous shake mode at 30°C. Reads were measured at OD600 every 30 minutes throughout a 24-hour period using a microplate reader (Synergy H1 Hybrid Multi-Mode Reader, Biotek, Winooski, VT, USA). We are in the process to determine the bacterial growth rates in each type of media (LSLB, M9, or AWF from different lettuce cultivars).

Experimental design and statistical analysis: Four metabolomic analysis of plant exudates, we used separate batches of plants (n=4). The number of samples for metabolic profiles were as follows: 2 types of exudate (surface and apoplast) x 2 time points (1 and 7 DPI) x 4 biological replicates x 31 lettuce cultivars = 496 samples. We are developing a pipeline for statistical analysis of this large data set.

For the bacterial growth in leaf exudates, the number of samples processed were 384, representing 31 lettuce genotypes, 2 treatments (mock and bacterium infiltration), 2 leaf exudates (surface and apoplast), 2 control media (LSLB and M9), and 3 biological replicates. We are yet to analyze this data set.

Results and Discussion:

Due to an unexpected closure of our lab during the "Omicron Wave" in the winter and the backlog at the UC Davis metabolomics facility, this project needed to be extended for a few more months. Thus far, we finished all experiments, including sampling and data collection. We are now developing pipelines for data analyses and interpretation and the project is estimated to be concluded in September 2022. Unfortunately, our analysis is too preliminary to include in this report.