CENTER for PRODUCE SAFETY

CPS 2010 RFP
FINAL PROJECT REPORT

Project Title
Non-invasive imaging approaches to evaluate potential infusion of pathogens during vacuum cooling of lettuce leaves and real time dynamics of microbes on leaf tissues as a function of moisture content

Project Period
January 1, 2011 – January 31, 2012

Principal Investigator
Nitin Nitin
Department of Food Science and Technology
University of California, Davis
Email: nnitin@ucdavis.edu
Phone: 530-752-6208

Co-Principal Investigator
N/A

Objectives
1. Develop a non-invasive imaging approach for in-situ measurement of localization of microbes in a plant matrix and characterize changes in distribution of microbes as a function of moisture conditions
2. Evaluate potential of infusion/infiltration of microbes in selected leafy green vegetables and determine the role of process control variables in controlling surface localization of microbes during a vacuum cooling process

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Abstract

Introduction: Internalization of bacteria in fresh produce is a major food safety risk factor as internalized bacteria are refractory to most washing and sanitation procedures[1-3]. Vacuum cooling has been identified as one of processing steps that can enhance internalization of microbes in fresh produce. Currently there is limited insight into what factors during vacuum cooling influence internalization of microbes?

Purpose: The aim of our study was to elucidate the risk of internalization of surface inoculated *E. coli O157:H7* upon vacuum cooling of lettuce as a function of microbial inoculation (high: 6log CFU per leaf disk) or low: 3log CFU per disk) and surface moisture and on both sides (abaxial and adaxial side) of lettuce leaves. To measure internalization of microbes in intact leafy greens multiphoton 3-d microscopy was used. This novel microscopy approach address challenges of autofluorescence and limited depth penetration to enable 3D microscopy of intact leaves.

Methods: Commercial lettuce purchased from a grocery store was washed, and the leaf surface was sprayed evenly with a solution of *E. coli O157:H7* GFP to inoculate the surface of lettuce. After vacuum cooling treatment the lettuce leaves were imaged with multiphoton microscopy. For quantitative and statistical analysis, the number of microbes associated with stomata, and infiltrated into the leaf was quantified.

Results: Results based on imaging measurements demonstrated that the vacuum cooling does not significantly increase the risk of internalization ($p>0.5$) of surface inoculated E*. coli* into an intact lettuce leaf. The imaging results also indicated that the vacuum cooling process increased the number of bacteria associated with stomata for both high moisture and low moisture conditions for samples inoculated on abaxial and adaxial surface of lettuce leaves. However, this increase in microbial association with stomata was only statistically significant for the high levels of microbial inoculation on abaxial surface under low moisture conditions.

Significance: The imaging measurements highlight that the vacuum cooling process does not significantly increase the risk of internalization of microbes.

Background:

Infiltration of microbial pathogens in fresh leafy greens significantly increases the risk of food borne illness as the infiltrated microbes cannot be effectively removed or treated using standard sanitation and washing processes[1-3]. Infiltration of microbes has been reported in both pre-harvest and post harvest processing of leafy greens[1-4]. It was recently reported that vacuum cooling of lettuce after harvesting can significantly increase the frequency of microbial infiltration in lettuce[1]. The report indicated that the microbial infiltration is mediated through stomata openings on leaf surface. Despite this report, there is lack of fundamental understanding of how vacuum cooling process can influence the infiltration of microbes?

In a vacuum cooling process, low pressure conditions results in rapid evaporation of surface moisture that provides efficient cooling of the fresh produce[5-8]. To reduce moisture loss from the product, the product may be sprayed with water during the vacuum cooling process[7, 8]. This approach is commonly used for leafy greens to reduce significant loss of product moisture during a cooling process. Increase of microbial infiltration during cooling operations is a matter of serious concern[1] as cooling of fresh produce is critical to maintain the quality of fresh produce and vacuum cooling process is a highly energy and time efficient operation that is being currently used for cooling of fresh produce. Thus, there is a critical need to both validate the reported results and also to establish fundamental understanding of physical and biological processes that may influence the potential infiltration of microbes during vacuum cooling.

One of the key challenges in establishing this fundamental understanding is to distinguish the surface inoculated and infiltrated microbes on a plant surface[1-3]. Optical microscopy such as fluorescence confocal microscopy is often used for these measurements but there are significant challenges in imaging plant leaves using conventional confocal microscopy[1]. These challenge results from heterogeneous topology of plant surface, extensive scattering of excitation light in plant matrix and significant endogenous fluorescence of plant leaves in the visible wavelengths. Surface topology of plant leaves is highly heterogeneous with micron scale variations (valleys and peaks). Furthermore, the broad endogenous fluorescence due to native chromophores such as chlorophyll and other pigments in plant leaves results in significant high background. High background fluorescence of plant leaves limits the detection of small number of fluorescently tagged microbes and also confounds the reliable detection of microbes as many of the endogenous fluorescence features in leafy greens are similar in size and morphology of microbes. Extensive scattering of visible excitation light on leafy greens further reduces the resolution of optical microscopy based detection of microbes. In summary, both the structural and compositional features of plant leaves significantly reduces the spatial resolution, sensitivity of detection using conventional confocal microscopy and introduces confounding background features.

To address this critical need, we have evaluated the use of multiphoton microscopy to detect and distinguish microbes on surface and inside of lettuce leaves. The unique advantages of multiphoton microscopy as compared to standard confocal microscopy are: (a) NIR

wavelength of excitation light in multiphoton significantly reduces the scattering and increases the depth of penetration[9-11]; and (b) non-linear combination of pulsed laser excitation sources significantly reduces the out of focus excitation as compared to conventional confocal microscopy[9-11]. This approach has a significant advantage in reducing the background autofluorescence of plant leaves and can significantly enhance the sensitivity of detection as well as reduce contributions from confounding plant microstructures. Despite significant advantages of multiphoton microscopy, this approach has not been extensively used in plant research especially to map potential changes in localization of microbes induced by post-harvest processing of leafy greens.

In this study using the multiphoton microscopy, we have evaluated the role of (a): microbial inoculation level; (b) surface moisture and (c) abaxial and adaxial surfaces in understanding the potential infiltration of microbes induced by vacuum cooling process. These parameters are selected to represent various structural, physical and biological parameters that can significantly influence the potential infiltration of microbes in lettuce leaves. These parameters are well known to influence the efficiency of a related but distinct bioprocess of vacuum infusion of microbes in plants from aqueous solutions.

In summary, the result of this study demonstrates the application of multiphoton microscopy to comprehensively evaluate the potential of vacuum cooling induced infiltration of microbes in lettuce leaves. Using this imaging approach, this study provides comprehensively evaluation of the key physical, structural and biological process parameters that can influence vacuum cooling induced infiltration of microbes. Thus, the results of this study demonstrate the potential risk of internalization of microbes in leafy greens during vacuum cooling and establish the significance of selected key parameters in influencing the potential internalization during vacuum cooling process.

Research Methods

Development of fluorescently strain of non-pathogenic E.coli -0157-H7: Escherichia coli O157:H7 GFPlux with deleted pathogenicity genes was used in all experiments. E. coli O157:H7 with deleted pathogenicity genes was a gift from Dr. Maria Marco (University of California, Davis) and was transformed with pAKgfplux1 plasmid (Addgene plasmid 14083) using standard procedures. Briefly, pAKgfplux1 was extracted from its E. coli DH5α host using the PureLink Quick Plasmid Miniprep Kit (Invitrogen). After extraction, E. coli O157:H7 was made competent by SSCS Solution (BioPioneer, Inc.) and transformed through heat shock. The transformed cells were then incubated with LB for one hour and plated on 100μg/mL Ampicillin LB agar plates. After overnight incubation at 37°C, resistant colonies were checked for fluorescence and luminescence. After confirmation, aliquots of E. coli O157:H7 GFPlux was then mixed with 50% glycerol and stored at -70°C. Before all experiments, E. coli O157:H7 GFPlux was inoculated into 50mL of LB broth with 100μg/mL Ampicillin and grown overnight.

Culture of microbes: An overnight culture of E. coli O157:H7 GFPlux, at approximately 10^8 CFU/mL, was used in all experiments. For high concentration conditions, the overnight culture was used unmodified, and for low concentration conditions, the culture was diluted to 10^6 CFU/mL with 1X PBS Buffer. Approximately 10mL of culture was loaded into a consumer sprayer before use on lettuce leaves.

Surface Inoculation of microbes on Lettuce Leaves: Lettuce was purchased from a local supermarket. The outer whorl of the lettuce was discarded, and the rest of the leaves were separated from the head before washing in water. After washing, the lettuce leaves were dried and separated. E. coli was inoculated by spraying 5 times evenly on the chosen surface of the lettuce leaf. Selected leaves for the dry condition were placed in a laminar air flow to speed drying. Once dry, the leaves were weighed and bagged. Wet condition leaves were immediately weighed and bagged to prevent evaporation of surface moisture.

Enumeration of number of microbes on plant surface: Enumeration of E. coli O157:H7 GFPlux on the surface of lettuce leaves was accomplished using standard plate counting protocols. After inoculation on the surface, 1 cm diameter disks were cut from the leaf and placed in 1.5mL tubes with 1mL of 1X PBS buffer. The leaves were then vortexed at medium speed to wash the bacteria from the surface. After 2 minutes of washing, the leaf was removed, and the supernatant was plated on 100μg/mL Ampicillin LB agar plates and incubated overnight at 37°C. Colony counts were reported as CFU/disk.

Design of Experiment: The factors examined in addition to vacuum cooling, the effect of wet or dry surface, high (10^6 CFU/disk) or low (10^3 CFU/disk) concentration, and abaxial or adaxial side of the leaf. The set up is summarized in Table 1. Each set includes a treatment with vacuum cooling and a control without vacuum cooling.

Set up for vacuum cooling process: Leaves were placed in a single layer on a platform in the vacuum cooling unit. A needle thermocouple was placed in one of the leaves and connected to readout on the outside. After preparations to read temperature were made, the leaves were vacuum cooled at 27mmHg for an average of (5-7 minutes) to an average temperature of 6-8 (°C). After reaching the average temperature, the vacuum cooling unit was shut off for one minute, and then the vacuum was released slowly over several minutes.

Preparation of samples for microscopy: For microscopy analysis, the leaves were cut and mounted to slides. Briefly, two 1 cm diameter disks were cut with a cork borer from each leaf at points away from the main veins. All disks were cut from similar positions in the leaves. Each leaf disk was placed, inoculated side up, on a microscope slide. A cover slip was mounted with tape one either side to prevent movement of the leaf disk.

Multiphoton imaging set up: A Zeiss LSM 510 upright microscope with a titanium sapphire laser tuned to 880nm was used in all imaging experiments. A 700/488nm filter with a 500-550nm filter for GFP signal was used to image the leaves. All images were taken with a 40x DIC oil objective. For each leaf disk, 2 sets of image stacks were acquired. Each stack image began above the leaf surface and ended 20-50 microns below the surface.

Image analysis and quantification: Each image stack was evaluated for stomata number, microbe association with the stomata, and stomata association. Each stomata that fit the parameters of association or infiltration was counted as one. Stomata association is defined as a bacterium cell within the boundaries of the guard cells of the stomata. Infiltration is defined as the cell penetrating the leaf below the level of the surface. After quantification, the data was analyzed.

Statistical analysis: The data was analyzed through ANOVA models with the R Statistical Software. The models used were:

$$Stomata\ Association = Vacuum\ Treatment + Surface\ Condition + Leaf\ Side$$

$$Stomata\ Infiltration = Vacuum\ Treatment + Surface\ Condition + Leaf\ Side$$

Significant values ($p<0.05$) are marked.

Results:

(a) **Develop a non-invasive imaging approach for in-situ measurement of localization of microbes in a plant matrix**

Multiphoton imaging of microbes on surface and inside of lettuce leaves: To enable detection of infiltration of microbes in lettuce leaves induced by the vacuum cooling process, it is essential that the selected imaging approach can detect both the surface dispersed and the internalized microbes in individual lettuce leaves. **Figures 1 (a and c)** shows the z- stack images acquired using a multiphoton microscopy to map spatial distribution of GFP expressing microbes on surface and inside of lettuce leaves respectively. To represent the distribution of microbes along the depth of the microbes, the individual z- stack images were combined to generate a projection image. **Figures 1(b and d)** shows the z- projection of surface dispersed and infused microbes in lettuce leaves. To disperse microbes on surface of lettuce leaves, the microbes were sprayed using the procedure outlined in the materials and method section. To infiltrate the microbes in lettuce leaves, the microbes were infused into lettuce leaves using a vacuum infusion process. It is important to note that the vacuum infusion process is significantly different from the vacuum cooling process although both processes are based on using vacuum pressure. In vacuum infusion process, the microbes are dispersed in the aqueous solution and the aqueous solution is infused into lettuce leaves using a rapid release of vacuum pressure. In a vacuum cooling process, leaves are not submerged in an aqueous solution and the vacuum pressure levels are an order of magnitude higher than the vacuum pressure used for vacuum infusion process. The results demonstrate that multiphoton imaging can detect distribution of microbes both the surface and inside of lettuce leaves without any significant contributions from plant autofluorescence. Furthermore, the results also show that multiphoton imaging can image microbes at depth levels greater than 80 microns (spanning the full depth of lettuce leaves). In summary, using multi-photon imaging we can detect distribution of microbes both on the surface and inside of lettuce leaves.

Comparison of Multiphoton and Confocal Microscopy for Imaging Microbes on Plant Surface: In a previous published vacuum cooling study and in vacuum infusion literature, internalization of microbes through stomata has been reported. To evaluate the increase in internalization of microbes using vacuum cooling process, it is essential to detect stomata on intact plant leaves and image microbes both on the surface and inside of stomata. Figure 2 (a-d) compares the fluorescence and the overlay of DIC and fluorescence images of plant leaf surface acquired using confocal and multiphoton microscopy respectively. Comparison between the fluorescence images from confocal and multiphoton (Figures 2 (a-c)) measurement based on an intensity line scan through a selected region of microbes on surface of lettuce leaf highlight the improvement in spatial resolution with multiphoton microscopy as compared to confocal microscopy. The improved spatial resolution is critical in mapping localization of

microbes on a spatially varying topology of a plant surface. Figures 2(b-d) compares the overlay of fluorescence and DIC images acquired using NIR excitation in multiphoton imaging as compared to visible excitation in confocal imaging. The results of this comparison clearly illustrate the improvement in detection of stomata on plant surface using NIR multiphoton excitation as compared to visible excitation in confocal microscopy (the stomata are marked with arrows on the image). The improvement in the DIC image results from decreased scattering of NIR excitation light as compared to visible excitation in confocal microscopy. In summary, the results clearly demonstrate that multiphoton imaging has significantly improved spatial resolution to detect stomata in plant tissue and microbial distribution as compared to confocal imaging. In a previous report, the stomata on lettuce leaves were detected based on plant autofluorescence signal that often overlaps with the fluorescent signal from GFP expressing microbes. This can significantly limit the sensitivity to detect microbial association with stomata as well as often lead to confounding measurement. Use of multiphoton microscopy addresses this limitation as illustrated in the results of Figure 2.

Objective 2: Evaluate potential of infiltration during vacuum cooling

Design of experiment: **Table 1** outlines the design of experiment to assess potential infiltration of microbes during vacuum cooling. With this design of experiments, the impact of both wet and dry conditions on infiltration of microbes was measured at two level of microbial inoculation of both adaxial and abaxial surfaces of lettuce leaves. To quantify localization of microbes as a function of the selected variables in Table 1, the experimental approach outlined in **Figure 3** was used for both adaxial and abaxial surfaces. Acquisition of multiple z- stack images (10-z stack images per imaging sample) for both adaxial and abaxial surface of lettuce leaves was used. The imaging results were quantified to measure association of microbes with stomata and internalization of microbes in lettuce leaves using the methods outlined in the materials and methods section. Association of microbes with stomata was quantified based on localization of microbes within the structural boundary of stomata as illustrated in **Figure 4 (a).** This boundary was defined based on the DIC white light images of multiphoton microscopy. **Figure 4(b)** illustrates the imaging approach to characterize internalization of microbes in stomata. The figure shows a z- stack that illustrate the localization of microbes at different depth levels within stomata. To evaluate the significance of the selected variables in influencing localization of microbes on surface of lettuce leaves, the statistical analysis (outlined in the materials and method) section was conducted.

Abaxial Surface: This section describes the results based on inoculation of microbes on surface of lettuce leaves for high moisture and low moisture conditions.

Figures 5 and 6 shows the representative micrographs and quantification of imaging data to illustrate the potential infiltration of microbes inoculated on abaxial surface of lettuce leaves maintained under high and low moisture conditions. These results clearly demonstrate that no significant increase in internalization (defined by localization of microbes inside stomata and under stomata-penetration into leaf) of microbes was observed under the experimental conditions selected for this study. The quantitative comparison of vacuum treated sample as compared to control does indicate a slight increase in association of inoculated microbes with stomata for vacuum cooled samples under both high and low moisture conditions. Based on

statistical analysis, this increase in association of microbes with stomata with vacuum cooling was not significant ($p>0.5$) for high moisture conditions and significant ($p<0.5$) in case of low moisture conditions.

Adaxial Surface: The same design of experiment as outlined in **Figure 3** was used for measuring the potential infiltration of microbes through the adaxial surface. **Figures 7 and 8** shows the representative micrographs and quantification of imaging data to illustrate the potential infiltration of microbes inoculated on surface of lettuce leaves maintained under wet and dry conditions. These results clearly demonstrate that no significant increase in internalization (defined by localization of microbes inside stomata and under stomata-penetration into leaf) of microbes was observed under the experimental conditions selected for this study. These results are similar to the results obtained on abaxial surface. It is also important to note that the total number of stomata on adaxial surface is significantly less (approximately 30 -40 % less than the similar measurements on abaxial surface). Similar to the results on abaxial surface, the results on adaxial surface also show an increase in number of stomata associated with microbes in vacuum cooled samples as compared to control samples under both dry and wet surface conditions. Based on statistical analysis, this increase in association of microbes with stomata was not significant ($p>0.5$).

Low Inoculum Levels: In addition to high inoculum (6 log CFU), similar experimental measurements were also conducted using low inoculum level of microbes (3 log CFU) on surface of lettuce leaves. The results of these measurements demonstrate similar trend as observed with high inoculum levels on both adaxial and abaxial surfaces although the absolute number of stomata associated with microbes decreases significantly in case low inoculum levels. In summary, the results with local inoculation level demonstrate no significant increase in infiltration of microbes with vacuum cooling.

Outcomes and Accomplishments

- Demonstrated novel application of multiphoton imaging to measure distribution of microbes both on surface and inside of intact lettuce leaves
- Multiphoton imaging results demonstrate significant improvement in spatial resolution and definition of plant structure as compared to standard confocal imaging
- Evaluated the potential of microbial infiltrated during vacuum cooling as a function of surface moisture on both abaxial and adaxial side of lettuce leaves using both high and low inoculum levels

Summary of Findings and Recommendations

- Results of multiphoton imaging highlight the significant advantages in spatial mapping of the distribution of microbes on surface and inside of intact lettuce leaves as compared to confocal microscopy.

- Results of microscopic measurements demonstrate no significant increase in infiltration of microbes during vacuum cooling process. These results were validated using fresh lettuce from green house as well as lettuce leaves from the market.
- Results also highlight a slight increase in association of microbes with stomata with vacuum cooling as compared to control lettuce samples. However, statistical analysis of the data highlight that the increase was not significant for all conditions except high inoculation level of microbes on lettuce leaves (6 log CFU/ leaf disk) under low moisture conditions.
- Further studies are needed to quantify if dispersion of microbes during vacuum cooling process is responsible for the increase observed in association of stomata with microbes. In addition to direct inoculation of microbes on surface of lettuce leaves as used in this study, further analysis may also include analysis of pre-formed biofilms on the surface of produce to mimic the environmental conditions.
- Further studies are required to compare these measurements based on attenuated strain of E. coli 0157:H7 with the measurements using pathogenic strains. These measurements may also include following the fate of microbes over an extended period of time after vacuum cooling process. This is particularly important for the population of microbes that show an increase in association with stomata following vacuum cooling process.

APPENDICES

Publications and Presentations (required)

An abstract has been submitted for a poster presentation at the IAFP Annual Meeting in Rhode Island and the manuscript for publication in a peer-reviewed journal.

Budget Summary: Total budget awarded was $45008. This budget supported a graduate research assistant for one year (including tuition and fringe benefits) and the material and supplies. The total awarded amount was expended.

Tables and Figures (optional) Tables and figures are attached above.

Suggestions to CPS (optional)

Table 1: Experimental Design of the Vacuum Cooling Study

	Abaxial	Adaxial
6log CFU	Wet/Dry	Wet/Dry
3log CFU	Wet/Dry	Wet/Dry

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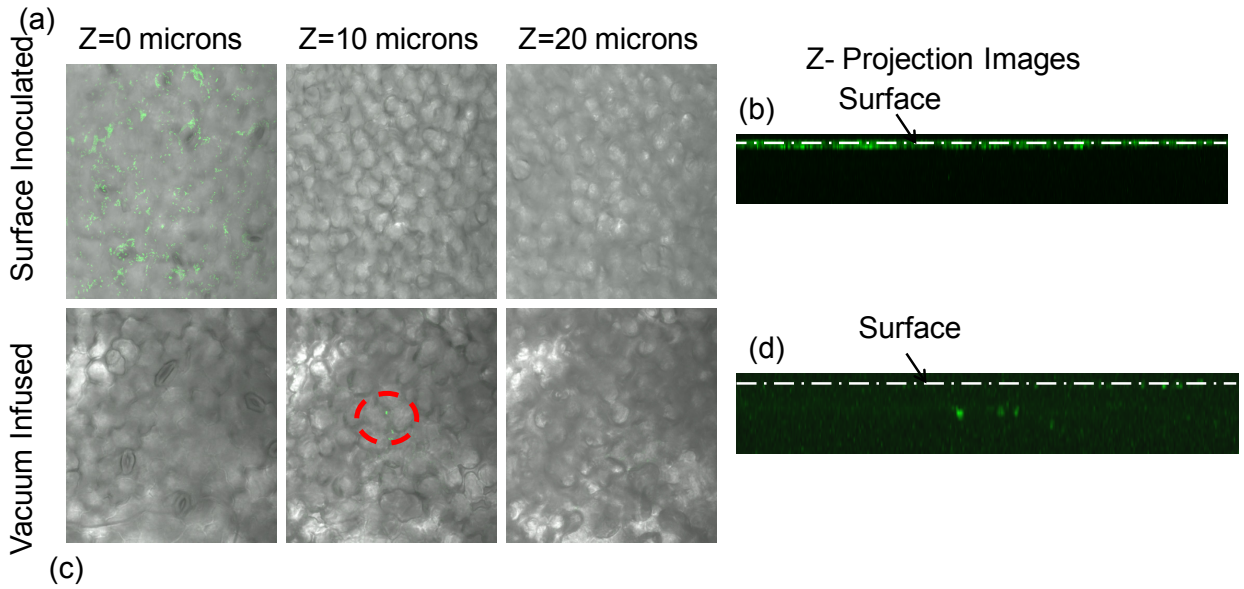

Figure 1: Imaging of surface inoculated and infiltrated microbes in intact lettuce leaves using multiphoton imaging.

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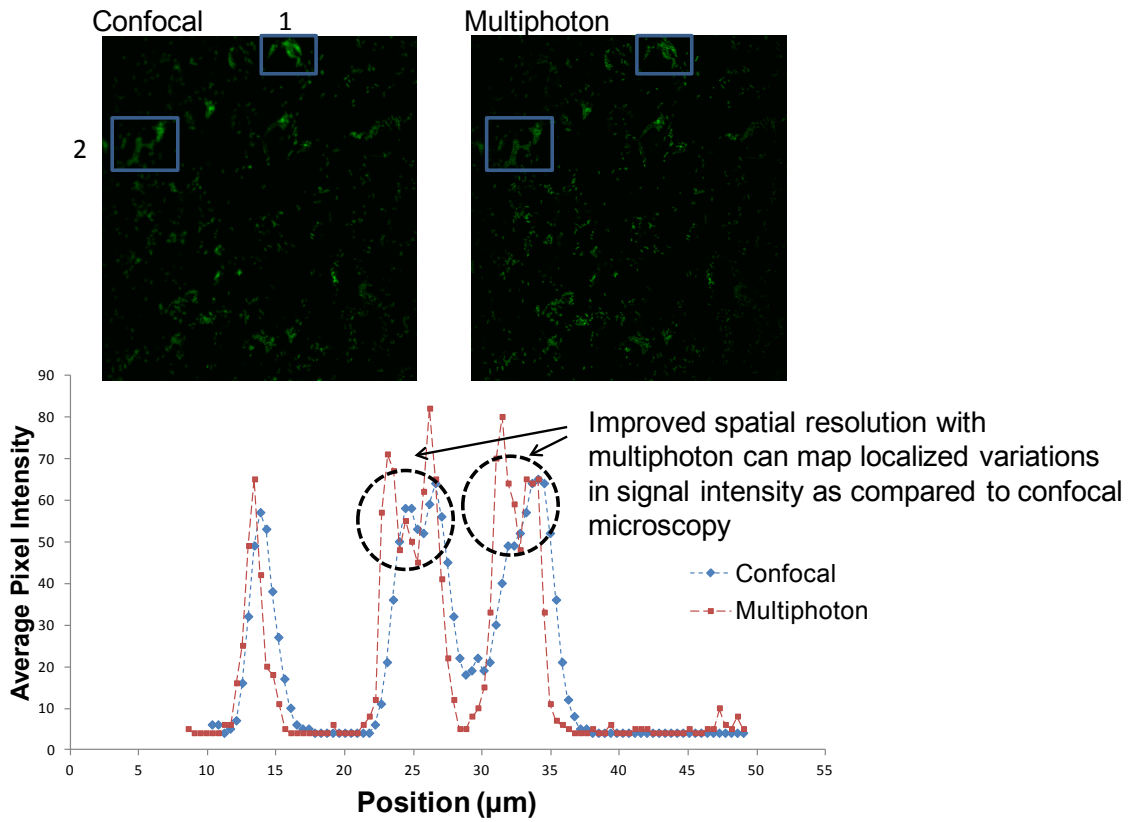
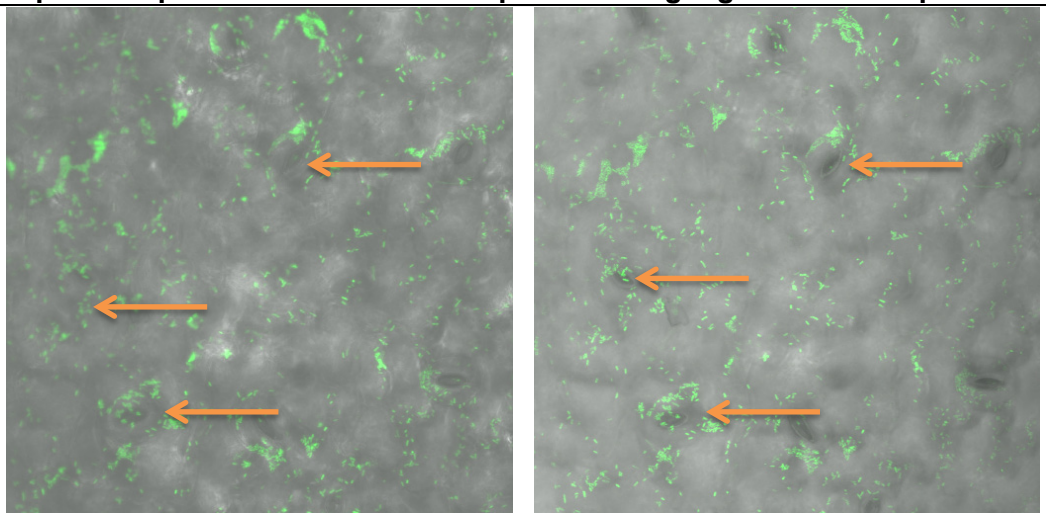


Figures 2(a-b): Comparison of confocal and multiphoton fluorescence imaging data. Improved spatial resolution of multiphoton is highlighted in the representative line scan



Figures 2 (c-d): Comparison of confocal and multiphoton imaging data. The image on the left is a confocal image of an *E. coli O157:H7* surface contaminated lettuce leaf. The right image is a multiphoton image of the same sample.

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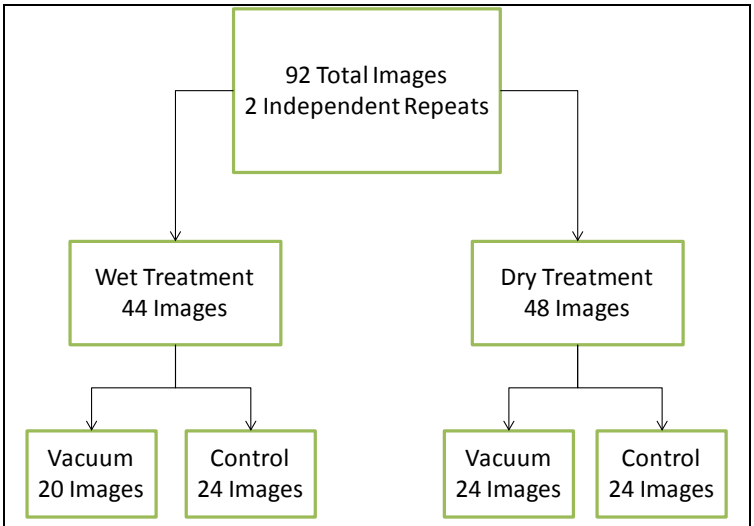

Figure 3. Design of imaging experiments to characterize potential infiltration during vacuum cooling

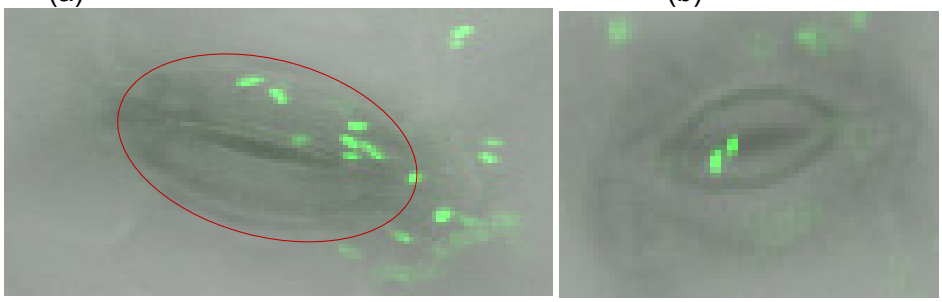

Figure 4: Approach to quantify association of microbes with stomata and to measure internalization of microbes in stomata cavity

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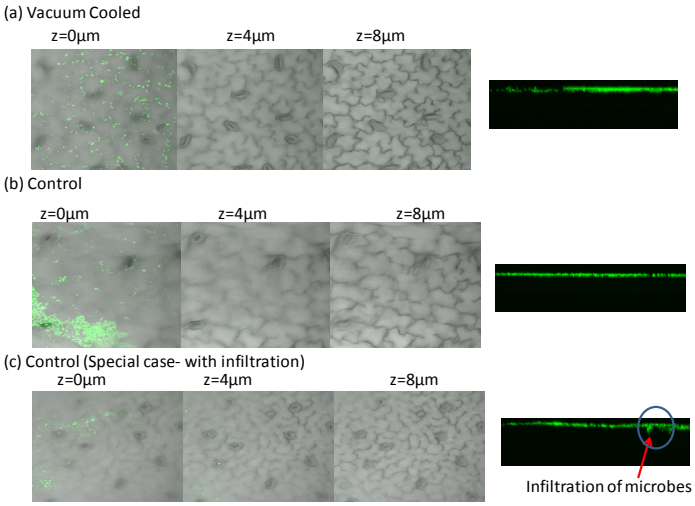

Figure 5(A) Imaging micrograph illustrating no significant increase in internalization after vacuum cooling of lettuce leaves inoculated on abaxial side and maintained under wet conditions (excess moisture on surface)

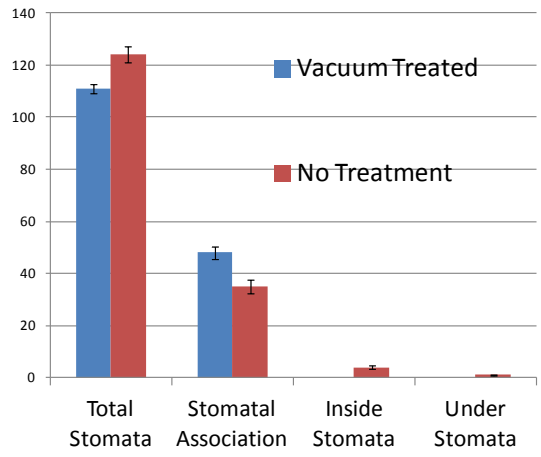

Figure 5(B) Quantification of changes in localization of microbes with vacuum cooling of lettuce leaves inoculated on abaxial surface.

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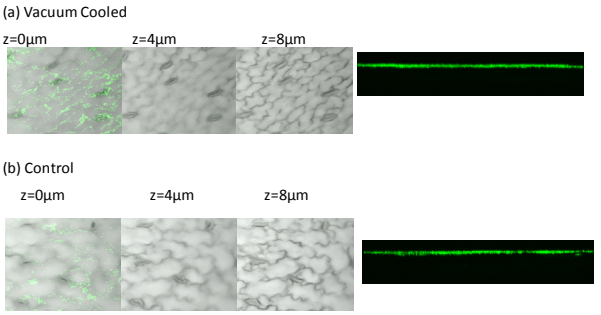

Figure 6(A) Imaging micrograph illustrating no significant increase in internalization after vacuum cooling of lettuce leaves inoculated on abaxial side and dried after inoculation

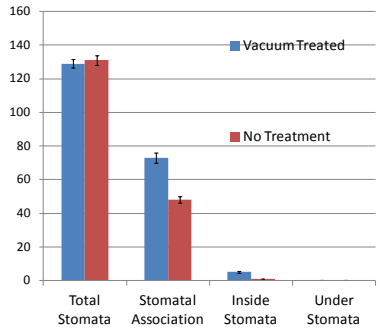

Figure 6(B) Quantification of changes in localization of microbes with vacuum cooling of lettuce leaves inoculated on abaxial surface.

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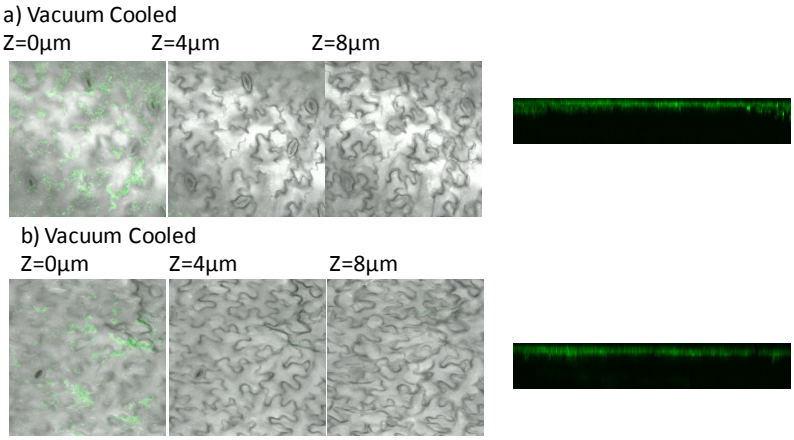

Figure 7(A) Imaging micrograph illustrating no significant increase in internalization after vacuum cooling of lettuce leaves inoculated on adaxial side under high moisture conditions

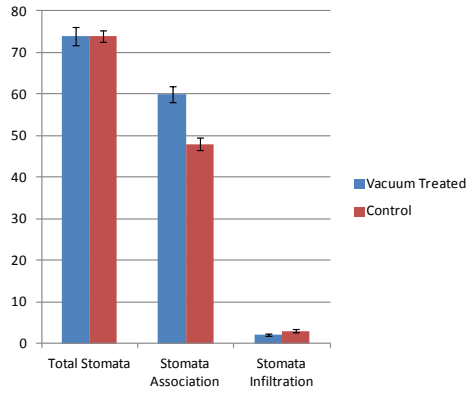

Figure 7(B) Quantification of changes in localization of microbes with vacuum cooling of lettuce leaves inoculated on adaxial surface under high moisture conditions.

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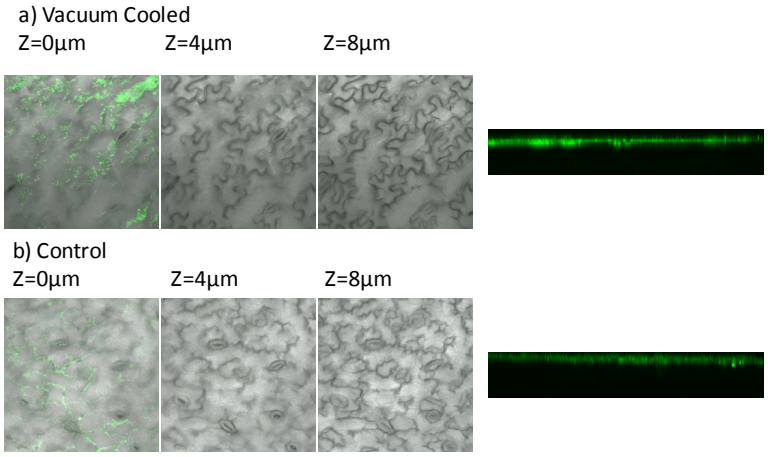

Figure 8(A) Imaging micrograph illustrating no significant increase in internalization after vacuum cooling of lettuce leaves inoculated on adaxial side under low moisture conditions

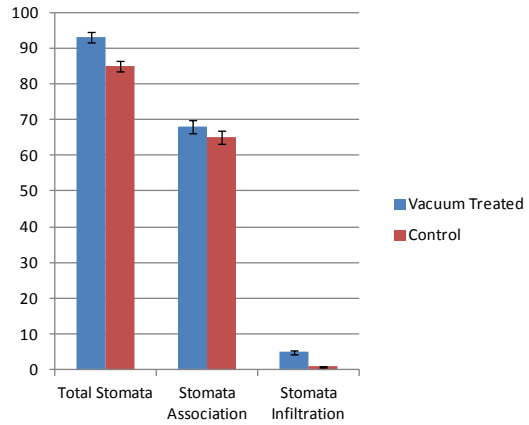

Figure 8(B) Quantification of changes in localization of microbes with vacuum cooling of lettuce leaves inoculated on adaxial surface under low moisture conditions

1. **Li, H.P., M. Tajkarimi, and B.I. Osburn, *Impact of vacuum cooling on Escherichia coli O157 : H7 infiltration into lettuce tissue.* Applied and Environmental Microbiology, 2008. 74(10): p. 3138-3142.**
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