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Quantitative real time measurements of bacteria–bacteriophages interactions in fresh lettuce leaves

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ABSTRACT

Bacteriophages are promising bactericidal agents for controlling growth of pathogenic and spoilage bacteria in fresh produce and in minimally-processed food. However, there is a lack of quantitative understanding of phage–bacteria interactions within a food matrix. To address this unmet need, the interactions of lytic bacteriophages with target model bacteria on the surface and interior (infiltrated) of a fresh produce were characterized using bioluminescence spectroscopy and imaging approaches. The results showed that the phages applied to the surface of lettuce leaves effectively reduced the level of bacterial targets that were present both on the surface and inside of lettuce leaves. However, there were significant differences in the initial rate of decrease of bacteria in the three model systems examined in this study: (a) homogenous well-mixed solution, (b) inoculated on the surface, and (c) infiltrated into the interior of lettuce leaves. The results also demonstrate the application of bioluminescence imaging for non-invasive measurement of interactions between bactericidal agents and target pathogens over an extended period of time in lettuce samples. The number of bacterial cells in lettuce samples before and after phage treatment predicted based on bioluminescence measurements was in agreement with the number calculated by conventional plate counting methods. In summary, the results highlight a non-invasive real-time imaging approach to quantitatively measure interactions of bacteriophages with both the surface inoculated and the infiltrated model bacterial cells in leafy greens.

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1. Introduction

There is a significant need to develop anti-microbial and sanitation methods that are highly specific for targeted pathogens and maintain safety of minimally-processed food (Heaton and Jones, 2008; O'Flaherty et al., 2009). Among various potential solutions, lytic bacteriophages have emerged as an effective biocontrol approach to specifically limit growth of pathogenic organisms in minimally-processed foods (Hagens and Loessner, 2007). Lytic bacteriophages are viruses that are natural predators of bacteria and have specificity for targeting selected strains of bacteria. The high bactericidal activity of lytic bacteriophage is based on rapid amplification of infected phages in bacterial cytoplasm, followed by lysis of the infected bacteria. Thus, the unique advantages of bacteriophages as compared to conventional anti-microbial agents are high specificity for infecting host bacterial cells and rapid amplification (100–10,000 in 30–40 min) upon successful infection of a host bacterium (Abuladze et al., 2008; Campbell, 2003).

Studies have shown significant potential of phages in limiting growth of pathogenic bacteria in fresh produce and minimally processed meat (Bigwood et al., 2008; Leverentz et al., 2001). In 2006, the Food and Drug Administration (FDA) approved a *Listeria monocytogenes*-specific phage preparation (LMP-102) for use as an antimicrobial agent against *L. monocytogenes* contamination of ready-to-eat foods (Abuladze et al., 2008).

Despite the significant potential of this technology, there is limited understanding of phage–bacteria interactions on the surface and interior of a food matrix. Presence of multiple troughs and ridges on the surface of a food matrix and the internalization of pathogens inside a food matrix (Kroupitski et al., 2009) can significantly affect the efficacy of bacteriophages by limiting interaction of phage and target bacterial cells (Abuladze et al., 2008). The role of food matrix in influencing phage–bacterial interactions is highlighted by the fact that the efficacy of phages in controlling target bacteria is significantly influenced by nature of food materials (Abuladze et al., 2008). The overall objective of this study was to establish a real time non-invasive measurement approach to characterize phage bacterial interactions in fresh produce. The non-invasive measurement approaches were based on both bioluminescence imaging and spectroscopy. Bioluminescence spectroscopy was used for real time non-invasive measurement of changes

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in number of microbial cells, while bioluminescence imaging was used for characterizing changes in spatial and temporal distribution of microbes in intact fresh produce following treatment with phages. This combination of imaging and spectroscopy provides a novel approach to evaluate the role of food matrix in influencing interactions of phages with microbes that cannot be directly assayed based on conventional microbial analysis. Further, bioluminescence imaging and spectroscopy approaches also address the critical challenge of non-invasive visualization of microbes in intact fresh leafy greens using fluorescence imaging and spectroscopy methods (using GFP and fluorescent dyes as stains). Sensitivity of fluorescence methods to characterize microbial distribution on a whole leaf samples has been significantly limited due to plant auto fluorescence (Chapman et al., 2005; Tilsner and Oparka, 2010; Zhang et al., 2010). Development of these non-invasive measurement methods will significantly advance the current understanding of phage–bacterial interactions in intact leafy greens that is currently limited to well-mixed model systems (Bigwood et al., 2009; O'Flynn et al., 2004).

For characterizing phage–bacterial interactions in fresh produce, *Escherichia coli* and T4 phage were selected. Lettuce was selected as a model fresh produce. To demonstrate the influence of food matrix on phage–bacterial interactions, three models systems were developed, i.e. phages and bacteria in homogeneous well mixed system; bacteria on surface with surface sprayed phages; internalized bacteria in lettuce leaves with surface sprayed phages. Comparison of these model systems using bioluminescence spectroscopy and imaging will demonstrate the significance of food matrix in influencing efficacy of phages in controlling target bacteria including internalized bacteria. To demonstrate the quantitative ability of bioluminescence measurements, the results of non-invasive measurements were compared with conventional culture-based enumeration methods for quantification of bacterial cell counts (Guenther et al., 2009).

In summary, the results of this study demonstrate (a) a quantitative approach for non-invasive characterization of spatial and temporal dynamics of phage–bacteria interactions in fresh produce; and (b) evaluate the role of food matrix in influencing phage–bacterial interactions and the effectiveness of phages in controlling both surface inoculated and internalized bacteria in fresh produce.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Bacterial strains *E. coli* DH5α-*lux* and DH5α-GFP were gifts from Prof. Glenn M. Young (University of California, Davis). The plasmid pSB401 (Winson et al., 1998) in strain *E. coli* DH5α-*lux* contains a complete lux operon, luxCDABE (bioluminescence genes), from firefly (*Photinus pyralis*) (Dewet et al., 1985). Strain DH5α-GFP constitutively expresses green fluorescent protein. Prior to each experiment, bacteria were grown in LB broth supplemented with 25 μg/ml tetracycline for approximately 6 h with constant shaking (200 rpm) at 37 °C to achieve exponential phase cultures (OD_{600} of 0.6–0.8). In order to demonstrate the stability of the plasmid, we cultured *E. coli* DH5α-*lux* with and without tetracycline for over 48 h in LB media. Our measurements show that the cell density (OD600) and the bioluminescence signal intensity were same (within the statistical variation) in bacterial cells with and without tetracycline for over 48 h of culture. Therefore, during the total duration of our imaging and spectroscopy experiments (24 h) the plasmid is maintained in bacterial cells even in absence of selection marker. Bacteriophage strain Coliphage T4r was purchased from Carolina Inc. (Burlington, NC) and diluted in sterile PBS to prepare phage solution at a concentration of 1×10^8 PFU/ml.

2.2. Correlation between the bioluminescence signal intensity and the bacterial colony forming units

E. coli DH5α-*lux* was propagated as described above and serially diluted in sterile PBS. Bioluminescence signals from bacterial cells in PBS were measured using a plate-reader (Synergy2, Bio Tek Inc., Winooski, VT) at room temperature. In parallel, the serial dilutions of bacterial samples were also cultured on LB agar to determine the number of colony forming units. Correlation between the number of bacterial colony forming units and the bioluminescence signal intensity was determined.

2.3. Surface inoculation model

Romaine lettuce (*Lactuca sativa* var. valmaine) was grown in a greenhouse at UC-Davis. Lettuce leaves were harvested from the first and second whorls of the head (the outer whorls representing the oldest leaves of the plant) 25–35 days after germination and stored at 4 °C for a maximum of 1 day before the onset of the experiment. Lettuce leaves were washed under running water for 30 s and dried at room temperature for 10 min. Leaf explants were carefully selected for uniform age, color, and texture, and cut into 1 cm diameter disks with a cork borer.

E. coli DH5α-*lux* cultured in LB broth supplemented with 25 μg/ml tetracycline for 5–6 h (1×10^8 CFU/ml) was applied to leaf surface using a commercial fine mist sprayer (Scentsational Shoppe Inc., Bergenfield, NJ). Mist sprayer was selected to mimic spreading of bacterial sample over a leaf surface similar to that achieved by irrigation methods in the field conditions. Further, spreading of bacteria over a leaf surface provides a good model to compare the response of surface inoculated and infiltrated bacterial cells to phage treatment. Leaf disks (12 pieces) were placed in petri dishes and the sprayer was pumped manually five times to inoculate the leaf disks with an approximately 100 μl of bacterial culture. The inoculated leaf disks were stored in an air flow cabinet for an additional 30 min to allow for attachment of inoculated bacterial cells to leaf surfaces and to remove excess moisture introduced during spraying. Following incubation and initial drying, inoculated lettuce leaf samples ($n = 6$) were then sprayed with bacteriophages (1×10^8 PFU/ml) using the same spraying method as outlined above to give approximate multiplicities of infection (MOI) of 1. Phage-treated lettuce samples were then removed and sealed in clean petri dishes lined with wetted filter paper (Whatman #1, Maidstone, UK) and incubated at room temperature (23–24 °C) in ambient light. The wetted filter paper was used to maintain humidity conditions so that excessive drying of leaf disk during extended incubation intervals does not influence the bacterial response (VanderGheynst et al., 2008). The phage treated lettuce disks ($n = 6$) and control samples without phage treatment ($n = 6$) (sprayed with the same volume of sterile PBS as used for the treatment samples) were monitored using a plate-reader and a widefield bioluminescence imaging system (IVIS, Xenogen Inc., CA) to measure changes in bioluminescence intensity from bacterial cells on intact lettuce disks. To enumerate bacterial cells inoculated on leaf tissues and to measure decreases in bacterial cell count with phage treatment, lettuce disks were rinsed with PBS prior to phage treatment ($n = 6$) and after 24 h ($n = 6$) of exposure to phages using a laboratory paddle blender (Stomacher® 400 circulator, Seward Inc., Bohemia, NY). Stomacher was used to release bacterial samples from the surface using a paddle blender (Buesing and Gessner, 2002; Fry et al., 1985). Since our leaf disks were small,

this approach provides a non-destructive approach to release bacteria from the surface of leaf disks with extensive agitation. Bacteria recovered from individual lettuce leaf disks were measured using standard plate counting methods. Bacterial cells recovered from the leaves were diluted in sterile PBS and plated on LB agar (Fisher Scientific, Pittsburgh, PA). The number of colony-forming units (CFU) was determined by plate counts after incubation at 37 °C for 24 h.

2.4. Development of infiltration model

Leaf disks (12 pieces) were immersed in 10-ml bacteria suspension (1×10^8 CFU/ml) in a 50 ml tube. The tubes were then placed in a vacuum chamber (Welch-Ilmvac Inc., Niles, IL) and held under vacuum (600 mm Hg) for 10 min at room temperature. The vacuum was quickly released to facilitate infusion of the bacteria into the tissue, as descried previously (VanderGheynst et al., 2008). The vacuum-infiltrated lettuce tissues were subsequently washed three times with a washing interval of 5 min each using sterile PBS to remove unattached bacteria. Immediately after washing, leaf disks ($n = 6$) were placed in petri dishes and bacteriophages (1×10^8 PFU/ml) were sprayed onto the leaf surface to give approximate MOI of 1 (Viazis et al., 2011a,b). Untreated control samples were sprayed with the same volume of PBS as used for the treatment samples. Thus, both the control and treatment leaves were maintained under the same set of surface moisture and humidity conditions throughout the experiment. Both the control and treated samples were then transferred to clean petri dishes and the bioluminescence signal was measured using both a plate-reader and an IVIS bioluminescence imaging system for widefield imaging. To enumerate number of bacterial cells per lettuce disk, lettuce leaf disk samples (prior to and after phage treatment) were immersed into sterile PBS in sterile polyethylene bags and homogenized for 2 min using a semi-automated homogenizer (Homex 6, Bioreba AG Inc., Switzerland) to release infiltrated bacteria cells. This homogenizer mechanically breaks apart the whole leaf disks and releases the infiltrated bacterial cells from inside of lettuce leaves (Barak et al., 2001; Rowhani et al., 1998). Samples were serially diluted in sterile PBS and the number of bacteria cells was enumerated using plate counting methods.

2.5. Bioluminescence spectroscopy

Bioluminescence signal intensity from lettuce leaf samples inoculated or infiltrated with *E. coli-lux* was measured using a plate-reader with a 15 s integration time and a bandpass filter (572–608 nm). The sensitivity of bioluminescence measurements for detecting bacterial concentration using a plate reader was determined based on the ratio of bioluminescent signal intensity from bacterial cells to the background signal intensity from media alone. To avoid any contributions from ambient light fluctuations (small as compared to signal intensity) during spectroscopy measurements, a signal to noise ratio greater than or equal to two was selected as the minimum threshold for detecting bacteria. These measurements were conducted using serial dilutions of bacteria in sterile PBS.

To evaluate the effect of bacteriophage on the viability of *E. coli* DH5α-*lux* present on the surface and inside of lettuce leaves, normalized bioluminescence values were calculated as:

$$\text{Normalized bioluminescence signal intensity } (I_normalized) = (I_{t\text{-sample}} - I_{\text{blank}})/(I_{0\text{-sample}} - I_{\text{blank}})$$

where $I_{t\text{-sample}}$ = relative bioluminescence intensity of bacteria-contaminated lettuce samples after "t" hours treatment; $I_{0\text{-sample}}$ = relative bioluminescence intensity of bacteria-contaminated samples before treatment; I_{blank} = relative bioluminescence intensity of non-contaminated samples.

2.6. Widefield bioluminescence imaging

Bioluminescence intensity from lettuce samples inoculated or infiltrated with bacterial cells was measured using a widefield bioluminescence imaging system equipped with an ICCD camera and a band pass emission filter (RFP (deRed) (558–583 nm) (IVIS 100 Series, Caliper Life Sciences Inc., Hopkinton, MA). The integration time for each image was 1 s. Bioluminescent signals were represented as pseudocolor images, where the colors were correlated with bioluminescence signal intensity (red represented the most intense signal, and blue represented the least intense signal). The pseudocolor images were overlaid on the white light images to characterize spatial distribution of the bioluminescent bacteria on lettuce discs. Phage-treated (6 pieces) and untreated lettuce samples (6 pieces) were kept in sealed clean petri dishes. At 0, 5 and 24 h following phage inoculation, samples were removed from sealed dishes and imaged using the IVIS widefield bioluminescence imaging system. After 3–5 min of widefield bioluminescence imaging, lettuces samples were placed back in clean, sealed petri dishes lined with wetted filter paper.

2.7. High resolution fluorescence microscopy

To validate distribution of *E. coli* cells inside of lettuce leaf disks 24 h after vacuum infiltration process, a multiphoton confocal microscopy was used. For this study, green fluorescence protein (GFP) tagged *E. coli* DH5α was infiltrated into lettuce leaves and incubated for 24 h. After vacuum infiltration and three washes, bacteria-infiltrated leaf disks were sectioned into 100–150 μm slices using an oscillating tissue slicer (EMS 5000, Electron Microscopy Sciences Inc., Hatfield, PA). Multi-photon microscopy (Zeiss LSM 510 (Carl-Zeiss Inc., Thornwood, NY)) was used for high resolution imaging of infiltrated bacterial cells in leaf sections. In this study, multiphoton fluorescence images were obtained using 880 nm laser excitation and a 500–530 nm bandpass emission filter using a 40× oil objective (NA = 1.4). Images were acquired using optical sectioning of a sliced leaf tissue moving from the surface to the interior of a leaf in a z-scan mode with an interval of 1 μm between consecutive sections. Images were also collected from control leaves (without bacterial infiltration) to measure the level of autofluorescence signal under the same set of imaging conditions. Z-projection image was processed using Zeiss LSM image analysis toolbox. To reconstruct the 3-d projection from individual z-scans, 32 projections with a difference angle of 11° and a first angle of 0° were used.

2.8. Statistical analysis

Statistical analysis was carried out using Microsoft® Excel 2007 (Microsoft Inc., Bellevue, WA) and SAS® (version 9.1 SAS Inc., Cary NC)). Student's *t*-test or ANOVA was used to check the statistical significance between the treatments at 95% confidence level. Differences between means were evaluated with Duncan's test and difference at $p < 0.05$ was considered to be significant.

3. Results

3.1. Correlation between bioluminescence intensity and bacterial numbers

E. coli cells with the luxCDBAE constitutively expressed the luciferase enzyme and its substrate synthesis proteins, thus

eliminating the need for addition of exogenous substrate to detect bioluminescence signal. Bioluminescence measurements (Fig. 1) using serial dilution of bacterial cells in solution show a linear relationship between the log of bioluminescence intensity and the log of bacterial concentration (CFU). The calculated regression equation curve between the relative bioluminescence intensity and the bacterial concentration (CFU) was:

$$\log I = 0.55 \log \text{CFU} + 1.22 \quad (r^2 = 0.98) \tag{1}$$

Based on this measurement, the minimum detectable concentration of *E. coli* DH5α-*lux* was approximately 10 CFU/ml. The signal intensity for 10 CFU/ml was at least two times higher than the background bioluminescence intensity of the media. This linear trend between the log of bioluminescence intensity and the log bacterial count (CFU) is in agreement with the results reported by Siragusa et al. (1999) and Kim et al. (2009).

3.2. Efficacy of bacteriophages in reducing levels of target bacteria in a well mixed solution

To characterize the efficacy of phages in reducing level of target model bacterial cells in well mixed solution, bioluminescence signal intensity was measured as a function of time for bacterial cultures with and without treatment of phages (Fig. 2).

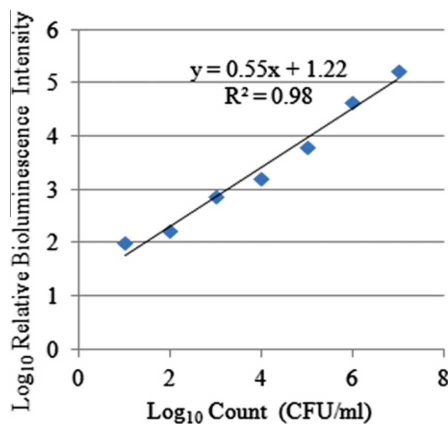

Fig. 1. Correlation between bioluminescent signal intensity and concentration of bacterial cells. Bioluminescence intensity was measured using a plate-reader with a 15 s integration time and a bandpass filter (572–608 nm). Bacterial cell concentration was measured using standard plate counting methods.

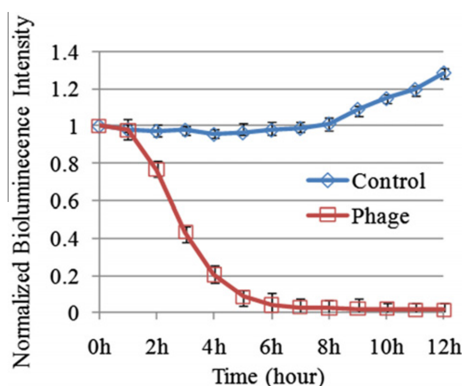

Fig. 2. Decrease in normalized bioluminescence intensity of *E. coli* DH5α-*lux* (8 $\log_{10}$ CFU/ml) upon treatment with T4 bacteriophages. Control samples (*E. coli* DH5α-*lux*) were incubated without bacteriophages. Treatment with phages resulted in approximately 20% ($p = 0.013$) and 90% ($p = 0.009$) decrease in normalized bioluminescence intensity at 2 and 5 h post treatment, respectively.

Table 1
Equations to describe the decrease in normalized bioluminescence intensity as a function of time during the initial 5-h phage treatment in three model systems.

Models of bacteria–bacteriophages interactions	Changes in normalized bioluminescence intensity as a function of time
Homogeneous well mixed solution	$I_normalised = -0.21t + 1.30$ ($r^2 = 0.96$)
Surface inoculation model	$I_normalised = 1.27e^{(-0.3t)}$ ($r^2 = 0.97$)
Infiltration model	$I_normalised = 0.98e^{(-0.005t^{2.9})}$ ($r^2 = 0.98$)

I_normalized = Normalized bioluminescence signal intensity of the sample; *t* = duration of bacteriophage treatment (h).

Bioluminescence signals from untreated (control) bacteria culture remained close to the initial signal intensity levels during the first 8 h of incubation. After 8 h of incubation, a slow increase in bioluminescence signal intensity (approximately 30% increase) was observed during incubation (total time of 12 h) at room temperature. Treatment with phages resulted in an approximately 20% decrease ($p = 0.013$) in bioluminescence intensity in 2 h (compared to the initial baseline of bioluminescence intensity) and a 90% decrease ($p = 0.009$) in bioluminescence intensity in 5 h of treatment with phages. The normalized intensity of bacterial culture showed a linear decrease during the initial period (5 h) of phage treatment (Table 1; $r^2 = 0.96$). Bioluminescence intensity was maintained at reduced levels for an extended period of time (total time period of 12 h) after the initial decrease following treatment with phages. In subsequent experiments, the time period of 5 h was selected for measuring the initial rate of decrease in normalized bioluminescence intensity of bacterial cells on surface and inside of lettuce leaves following treatment with phages.

3.3. Efficacy of bacteriophages in leaf surface inoculation model

Fig. 3 shows a representative widefield image of lettuce leaves inoculated with *E. coli* cells expressing *lux*. To validate that the inoculated model bacterial cells were indeed on the surface of lettuce leaves, lettuce leaves were washed and then imaged using widefield bioluminescence system (Karsi et al., 2008). The widefield imaging results (Fig. 3) show a significant reduction ($p = 0.021$) in bioluminescence signal intensity indicating that most of the surface inoculated bacterial cells can be removed from lettuce leaves with a simple washing method.

Fig. 4a shows the differences in normalized bioluminescence intensity between the phage-treated and control leaf samples during the initial 5 h of incubation. Bioluminescence intensity was measured over the initial 5 h at 1 h intervals. As shown in Fig. 4, the bioluminescence signal in the control leaf samples increased by 30%, while samples treated with T4 bacteriophage (1×10^8 PFU/ml) showed an approximately 80% reduction ($p = 0.037$) in the normalized bioluminescence intensity. Decrease in normalized bioluminescence intensity as a function of time during the first 5 h can be described by an exponential relationship ($r^2 = 0.97$) as shown in Table 1.

In the control sample, an approximately 2.3-fold increase in bioluminescence intensity was observed during a 24 h period. Bacterial cell count predicted based on an experimentally determined correlation between the relative bioluminescence intensity and the bacterial concentration (Eq. (1)) was compared with enumeration of bacterial colonies (Table 2). The results show that the number of bacterial cells predicted based on Eq. (1) are in agreement with the results obtained using the conventional colony counting approach. These results highlight the ability of bioluminescence measurements to quantify changes in levels of bacterial cells in intact fresh produce. Based on these results, there was an approximately 1.5–2 log reduction in bacterial counts before and after treatment (24 h) with T4 bacteriophages.

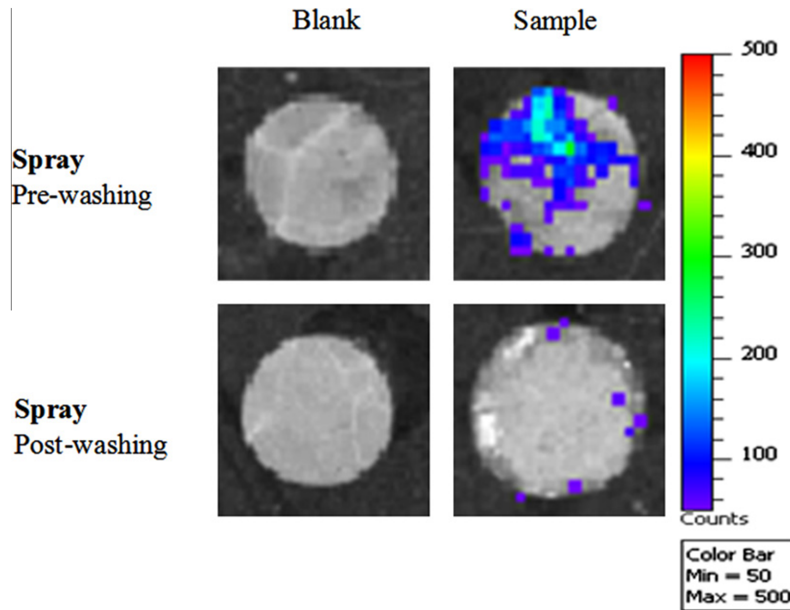

Fig. 3. Wieldfield imaging to validate surface localization of bacterial cells sprayed on fresh lettuce leaves and to compare pre and post washed lettuce leaf samples. Lettuce leaves (surface inoculated model) sprayed with bacterial cells was washed three times to remove surface inoculated bacteria.

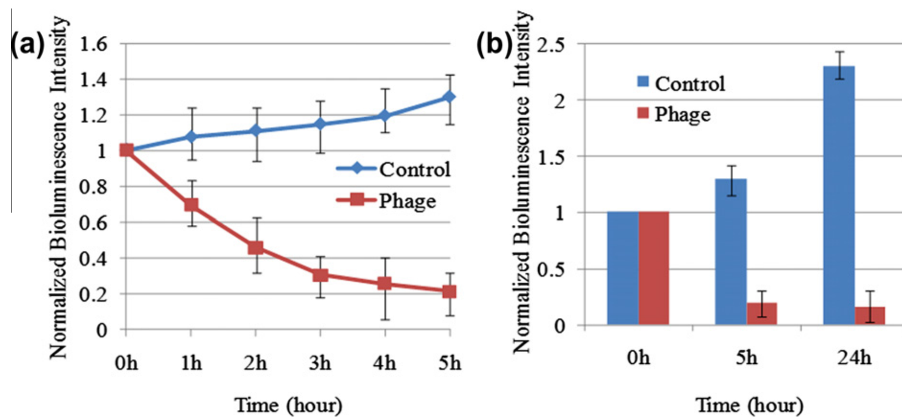

Fig. 4. Normalized bioluminescence intensity to determine the efficacy of bacteriophages in reducing the number of bioluminescent bacterial cells inoculated on surface of lettuce leaves. Control leaves inoculated with bacterial cells were not treated with phages. (a) Decrease in normalized bioluminescence intensity for the initial 5 h of phage treatment measured at intervals of 1 h. The bioluminescence signal in the control leaf samples increased by 30%, while the samples treated with T4 bacteriophage showed an approximately 80% reduction ($p = 0.037$) in the normalized bioluminescence intensity during the initial 5 h of treatment. (b) Decrease in normalized bioluminescence intensity for a 24 h period measured at discrete time points (0, 5 and 24 h). After 24 h, the normalized bioluminescence signal intensity of the control leaf samples increased by approximately 2.3-fold as compared to initial signal intensity while the phage treated samples showed greater than 95% reduction ($p = 0.028$) in the normalized bioluminescence signal intensity.

Table 2
Mean numbers of bacterial cells on the surface of lettuce leaves predicted from bioluminescence intensity (Eq. (1)) and calculated from standard plate counting methods before and after bacteriophages treatment (24 h).

Sample	Relative bioluminescence intensity	Log count by correlation curve ($\log_{10}$ CFU/ml ± SD)	Log count by plate count ($\log_{10}$ CFU/ml ± SD)
0 h	6174 (±420)	4.7 (±0.1)	5.0 (±0.3)
24 h Phage treated sample	985 (±532)	3.2 (±0.6)	3.0 (±0.4)
24 h Control sample	13,891 (±785)	5.3 (±0.9)	5.8 (±0.7)

SD: standard deviation.

Fig. 5 shows the widefield bioluminescence imaging to characterize spatial and temporal distribution of bacterial cells on surface of lettuce leaves. The T4 bacteriophages treated leaf samples with inoculated bacterial cells experienced a decrease of approximately 82% in bioluminescence intensity after 24 h, while the control samples (without phage treatment) had an increase in bioluminescence intensity of 3.4-fold. These imaging results are in agreement with the bioluminescence intensity measurements using a plate reader. In the phage treated samples, bacterial cells are only observed near the cut surface of the disk after 24 h of treatment,

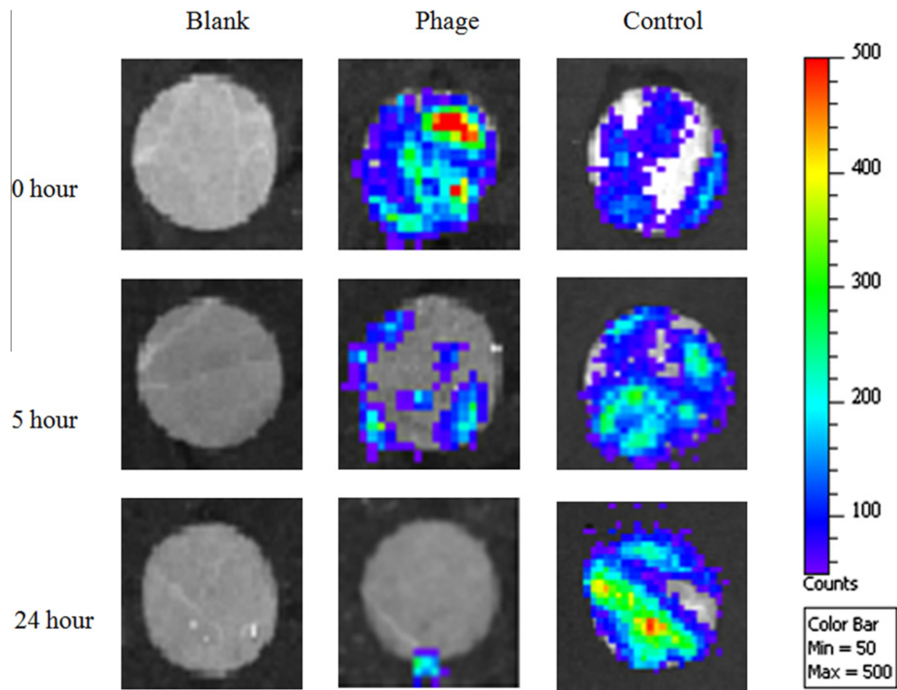

Fig. 5. Widefield imaging to characterize spatial and temporal changes in relative bioluminescence intensity of bacteria inoculated on surface of lettuce leaves upon treatment with phages. Control samples (lettuce leaves inoculated with bacteria) were not treated with phages.

while the control sample shows a significant increase in bioluminescence signal throughout the leaf disk.

3.4. Infiltration of bacteria inside lettuce tissue

Leaves vacuum-infiltrated with model bacteria with and without washing were imaged using a widefield bioluminescence imaging system to characterize differences between the surface inoculated and the infiltrated bacteria in lettuce leaves (Fig. 6). Results show that the bioluminescence intensity only decreased by 18% upon washing (after three washes for 5 min each). This result is in contrast with the results obtained with the surface inoculated bacteria (Fig. 3), in which an 84% decrease ($p = 0.038$) in bioluminescence intensity was observed following washing. These results indicate that with infiltration, a large fraction of bacterial cells were present inside of lettuce tissue and could not be removed with a simple washing procedure. Bioluminescence signal intensity from washed leaves infiltrated with *E. coli* DH5α-lux was monitored over a 24 h period (Fig. 7). In control samples, a rapid, approximate 2-fold increase in the density of infiltrated

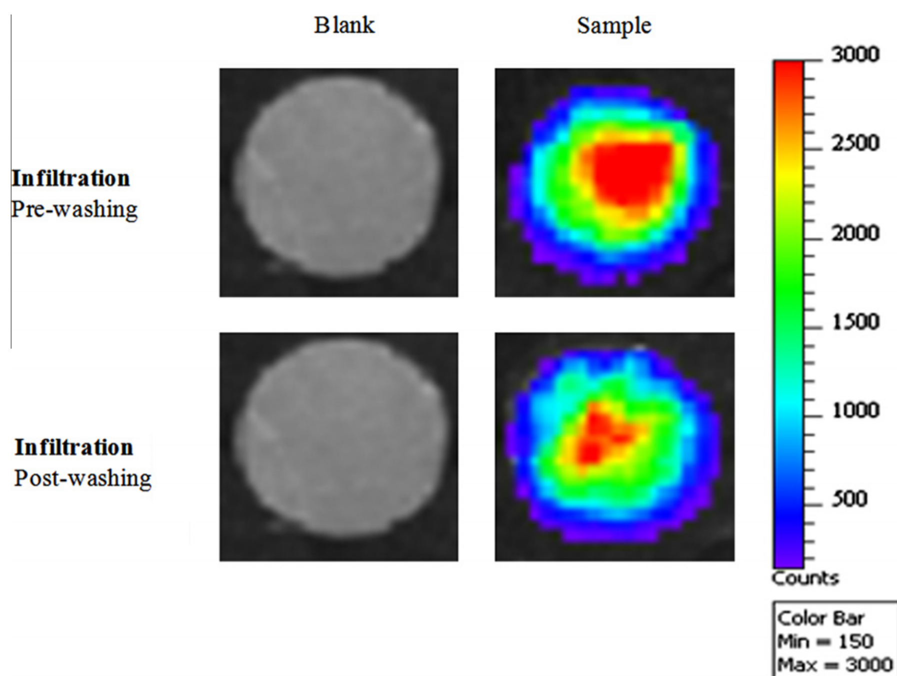

Fig. 6. Widefield imaging to validate infiltration model. Imaging results highlight retention of bacterial cells inside of lettuce leaves upon washing.

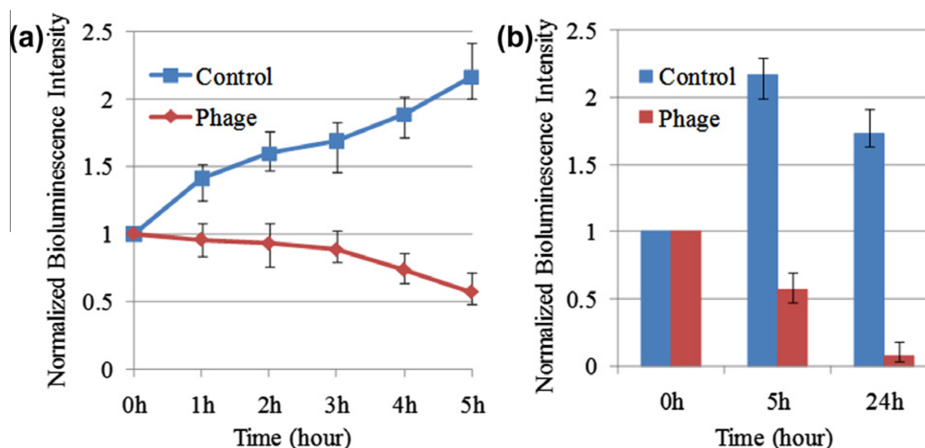

Fig. 7. Normalized bioluminescence intensity to determine efficacy of bacteriophages in reducing the number of bioluminescent bacterial cells infiltrated inside lettuce leaves. Control leaves infiltrated with bacterial cells were not treated with phages. (a) Decrease in normalized bioluminescence intensity for the initial 5 h of phage treatment measured at intervals of 1 h. Normalized bioluminescence signal intensity of the phage-treated samples decreased by = approximately 50% during the first 5 h ($p = 0.045$) while the normalized bioluminescence intensity in the control samples increased by approximate 2-fold. (b) Decrease in normalized bioluminescence intensity for a 24 h period measured at discrete time points (0, 5 and 24 h). Normalized bioluminescence signal intensity of the phage-treated leaf samples decreased by over 90% ($p = 0.015$) after 24 h incubation, while the normalized bioluminescence intensity in the control leaf samples showed a net increase of 1.7-fold.

Table 3
Mean numbers of bacterial cells infiltrated inside lettuce leaves predicted based on bioluminescence intensity (Eq. (1)) and numbers of bacterial cells calculated from standard plate counting methods before and after bacteriophages treatment (24 h).

Sample	Relative bioluminescence intensity	Log count by correlation curve ($\log_{10}$ CFU/ml ± SD)	Log count by plate count ($\log_{10}$ CFU/ml ± SD)
0 h	26,675 (±1237)	5.8 (±0.1)	5.5 (±0.7)
24 h-Phage treated sample	2134 (±1069)	3.3 (±0.5)	3.0 (±0.9)
24 h-Control sample	45,672 (±2877)	6.3 (±0.8)	6.0 (±0.8)

SD: standard deviation.

bacterial cells was observed based on changes in bioluminescence intensity in the first 5 h, followed by a slight decrease in bioluminescence intensity during the next 17 h period. The control samples show a net increase of 1.7-fold in bioluminescence intensity of bacteria over 24 h. Normalized bioluminescence signal intensity of the phage-treated samples decreased by approximately 50% (p = 0.045) during the first 5 h of treatment and over 90% ($p = 0.015$) during 24 h incubation. The decrease in bioluminescence intensity in infiltrated bacteria over the first 5 h of phage treatment was described by an exponential relationship ($r^2 = 0.98$) with a shape factor (power of t) of 2.92 as described in Table 1. It is noted that the decrease in normalized bioluminescence intensity as a function of time in leaf surface inoculation model showed first-order kinetics, which has the shape factor equal to 1.

The number of bacterial cells predicted based on bioluminescence intensity measurements was compared with conventional enumeration methods. The results predicted based on bioluminescence intensity were consistent with the data obtained by plate counting. Both the assays show an approximately 2–2.5 log reduction in infiltrated bacterial cells in lettuce leaves after 24 h of treatment with T4 bacteriophages (Table 3). It is important to note that the initial concentration of bacteria in vacuum-infiltrated lettuce leaves was higher than that achieved by surface inoculation of bacterial cells using mechanical spraying.

Widefield bioluminescence imaging was used for characterization of spatial and temporal distribution of infiltrated model bacterial cells upon treatment with phages in lettuce leaves. During the first 5 h (Fig. 8), the phage treated lettuce leaf samples infiltrated with bacterial cells show a decrease (approximately 37% decrease, $p = 0.023$) in bioluminescence intensity. Upon 24 h of incubation of infiltrated lettuce leaves with phages, a significant decrease (approximately 95%, $p = 0.034$) in bioluminescence intensity was observed. The imaging results are in agreement with the measurements of bioluminescence intensity using a plate reader. Over the same time period, the control sample shows an overall increase in bioluminescence intensity. These results clearly illustrate that phages sprayed onto the surface of lettuce leaves were able to limit growth of bacterial cells infiltrated in intact leaf discs.

Localization of infiltrated bacteria inside a leaf tissue 24 h post infiltration was validated using high resolution multiphoton fluorescence microscopy (Fig. 9). Fig. 9 shows the z-axis projection images calculated based on the stack of individual z section images acquired for control and vacuum infiltrated lettuce leaves. Results in Fig. 9a shows that the infiltrated bacterial cells were present at different depth levels within the leaf tissue 24 h post infiltration. These results demonstrate retention of bacterial cells within the lettuce tissue after infiltration. Taken together, widefield imaging (Fig. 8) and high resolution microscopy (Fig. 9) validate the infiltration of bacterial cells in intact lettuce leaf, and highlight the efficacy of phages applied on the surface in controlling growth of bacterial cells inside a leaf tissue.

4. Discussion

Culture based methods have been conventionally used for quantitative evaluation of interaction between bacteria and phages (Hagens and Loessner, 2007; O'Flaherty et al., 2009). Using culture based methods, studies have quantified both the decrease in bacterial counts upon interaction with phages and also the

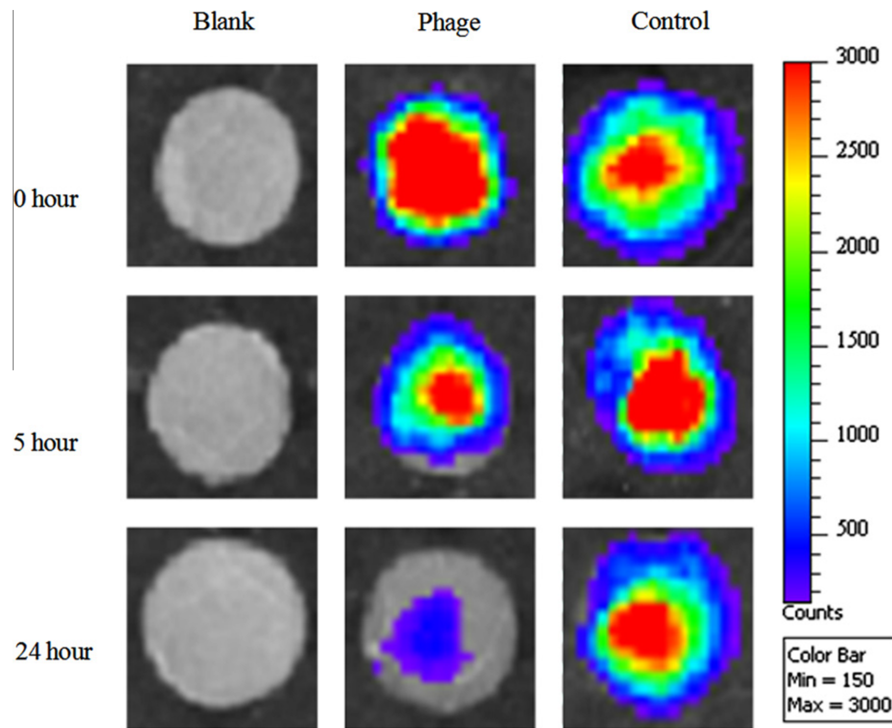

Fig. 8. Widefield imaging to characterize spatial and temporal changes in relative bioluminescence intensity of bacteria infiltrated inside lettuce leaves upon treatment with phages. Control samples (lettuce leaves infiltrated with bacteria) were not treated with phages.

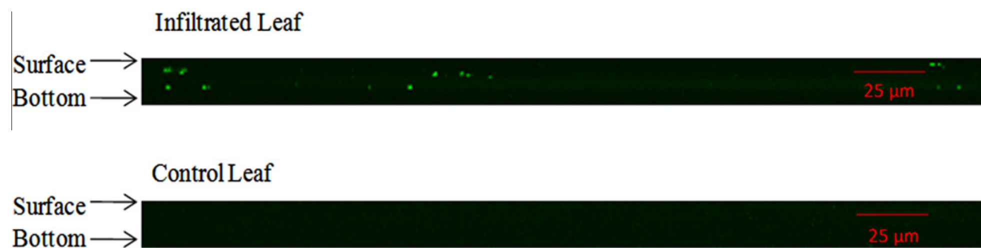

Fig. 9. Multiphoton fluorescence imaging to validate retention of infiltrated bacterial cells *E. coli* DH5a–GFP inside lettuce tissue after 24 h. *z*-Axis projection image show localizations of bacterial cells inside infiltrated leaf at different depth levels within a lettuce leaf sample. Control leaf was not infiltrated with bacterial cells.

increase in phage count (based on PFU-plaque forming units) resulting from replication of infected phages in bacterial cells (Abedon, 2009; Cairns et al., 2009). These culture based methods are the gold standard in the field of microbiology. In this study, we have compared the bacterial counts (CFU) predicted based on bioluminescence intensity measurements with the bacterial cell counts measured using the standard plate assays. The results (Tables 2 and 3) demonstrate that the numbers of bacterial cells predicted based on bioluminescence measurements on the surface and within infiltrated lettuce leaves are in agreement with the measurements based on conventional culture-based method (colony forming units).

This study demonstrates the potential of bioluminescence spectroscopy and imaging for real time non-invasive characterization of phage–bacterial interactions in fresh produce. The unique advantage of bioluminescence spectroscopy and imaging approach is the ability to non-invasively follow the same sample over an extended period of time. This approach can significantly complement the current culture based methods of analysis of phage–bacterial interactions. Non-invasive widefield bioluminescence imaging of bacterial cells in fresh produce has high signal-to-background ratio due to low autoluminescence intensity of plant tissue. This is in contrast to high autofluorescence levels in plant leaves that significantly limit detection of bacterial cells on intact leaf tissues using widefield fluorescence imaging and spectroscopy. The results of this study (Figs. 5 and 8) demonstrate the application of widefield bioluminescence imaging for characterizing efficacy of phages for biocontrol of target bacterial cells. Widefield imaging measurements (Figs. 3 and 6) were also used for characterization of differences in removal of bacterial cells with washing after inoculation on the surface of and infiltration into lettuce leaves. Taken together these results, demonstrate the potential of non-invasive real time bioluminescence measurements to characterize phage–bacterial interactions on fresh produce.

One of the key goals of this study was to evaluate the influence of food matrix on phage–bacterial interactions using non-invasive spectroscopy and imaging. For evaluating the role of food matrix, the phage–bacterial interactions in homogeneous solution were compared with surface inoculated and infiltrated bacterial cells in intact lettuce leaves. In case of homogenous solutions, the rate of decay in normalized bioluminescence intensity in the first 5 h can be described by a linear relationship between the bioluminescence intensity and incubation time. In case of bacterial cells inoculated onto the surface of lettuce leaves, the initial rate of decrease

in bioluminescence intensity in the first 5 h can be described by an exponential decay function with a shape factor of 1. For the case of bacterial cells infiltrated into lettuce leaves, the decay in normalized bioluminescence intensity shows an initial lag period of approximately 3–4 h. This initial rate of decline in the first 5 h can be described using an exponential curve with the shape factor of 2.92. These results demonstrate that the initial rate of decrease in number of bacterial cells was significantly different among the three selected model systems. These measurements highlight the influence of transport barriers in food matrix on phage–bacterial interactions. On the surface of lettuce leaves, mobility of phages on the surface is the key transport barrier. In the case of infiltrated bacterial cells in lettuce leaves, the transport of phages from the surface to the interior of leaf tissue and lateral mobility within a leaf tissue are the key transport barriers.

The results of these measurements also demonstrate that the phages applied to the surface of lettuce leaf were effective in reducing the number of bacterial cells on surface and interior of lettuce leaves by approximately 2–2.5 log after 24 h of treatment. The results of decrease in surface inoculated bacteria with phage treatment (approximately 2 log) are in agreement with the current literature (Viazis et al., 2011a,b). These studies have shown a similar decrease in the number of bacterial cells on surface of lettuce leaves and food contact materials at room temperature using phages at an MOI of 1. To the best of our knowledge, there are no known reports that have demonstrated the potential of phages to reduce infiltrated bacterial cells in intact plant tissues. Thus, our results provide a novel insight into the potential of phages to control internalized pathogens in leafy greens. These novel results are supported by a recent study (Wei et al., 2010) that has shown evidence of internalization of viral particles into intact leaf samples. Thus, the internalization of phages in leaf samples can provide an initial starting point for infecting infiltrated bacteria. This initial infection of bacteria will result in amplification of phages, resulting in rapid decline in number of internalized bacteria. The results shown in Fig. 7 indicate a similar trend. The initial lag period observed in bioluminescence measurements highlight the initial lag time required for the phages to come in contact with bacterial cells inside the leaf sample, followed by a rapid decline in bioluminescence intensity and associated bacterial cell count over a 24 h incubation period.

Multiphoton microscopy used for imaging bacteria-infiltrated leaf sections (Fig. 9) verified that the infiltrated bacterial cells were indeed inside of a lettuce leaf after 24 h post-infiltration. The effectiveness of phages in reducing the number of bacterial cells inside of lettuce leaves highlights the ability of surface-applied phages to permeate into the lettuce leaves. Further studies are required to characterize the depth and rate of permeation of surface-applied phages and other viral particles in leafy green vegetables.

5. Conclusions

In this study non-invasive bioluminescence spectroscopy and imaging was used for characterization of interactions of phages with the surface-inoculated and infiltrated bacteria. The results of these measurements show that the phages applied on the surface of lettuce leaves were effective against bacterial targets that were present both on the surface and inside of lettuce leaves. The number of bacterial cells in lettuce samples predicted based on bioluminescence measurements (using the correlation between bioluminescence intensity and bacterial cells in solution) were in agreement with the numbers calculated based on conventional plate counting methods. This highlights the ability of non-invasive measurements to provide quantitative analysis of bacterial cells and their dynamics upon treatment with biocontrol agents in intact leafy greens. Despite similar overall efficacy of phages (after 24 h) in reducing target bacterial cells on surface and inside of lettuce leaves, the results of the study show significant differences in the initial rate of decrease in the number of bacterial cells on the surface and inside of lettuce leaves. These differences in the initial rate of decrease of bacterial cells highlight the significance of transport barriers for phage–bacteria interactions in the selected model systems. Bioluminescence imaging measurements demonstrated: (a) non-invasive characterization of spatial and temporal changes in bacterial cells on the surface and inside of lettuce leaves upon treatment with phages; (b) validating localization of bacterial cells on the surface and inside of lettuce leaves in surface-inoculated and infiltration models. Overall, the results of this study provides novel insights into efficacy of phages in controlling target bacterial cells both on surface and inside of lettuce leaves and highlight application of non-invasive real time imaging and spectroscopy tools for characterizing interactions of biocontrol agents with model bacterial cells in intact food systems.

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