Inoculation and Persistence of *Salmonella enterica* Serovars in Lettuce Cultivar Salinas

Stephanie Giori
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Made possible with the help of Dr. Paula Oblessuc and Samuel Koehler

Dr. Maeli Melotto
Principal Investigator
Associate Professor, Department of Plant Sciences

Dr. Johan Leveau
Master Advisor
Professor, Department of Plant Pathology

ABSTRACT

Salmonella enterica, a genus of the Enterobacteriaceae family, has been at the forefront of numerous food outbreaks in the United States as well as abroad. Many distinct variations (serovars) of this bacterium exist, and this paper focuses on six serovars that were isolated from recent fruit and vegetable outbreaks. A protocol was developed to screen these *S. enterica* serovars for their persistence in the apoplast of the Salinas lettuce (*Lactuca sativa L.*) cultivar. After considerable troubleshooting and design alterations, a final procedure was established using vacuum inoculation. Preliminary results show potential differences in the apoplast persistence of certain serovars. Additional experiments and protocol adjustments are discussed.

INTRODUCTION

Fruits and vegetables are a key component to a healthy, well-rounded diet. In addition to their high nutrient density and common availability, these foods may be eaten raw, with no prior preparation or cooking required. While this is highly appealing, it also makes fresh produce notoriously susceptible to foodborne outbreaks. *Salmonella enterica* is one of the most common human pathogens found on fresh food. Between 1973 and 1997, 48% of produce-related outbreaks involved a distinct strain (serovar) of *S. enterica* (Sivapalasingam 2004). This has prompted further research into the relationship between plants and this tenacious human pathogen.

Food contamination with S. *enterica* can occur during any stage of the production cycle (Faticia 2011). Alarmingly, the serovar S. Typhimurium can persist in soil and on lettuce and parsley leaves for up to 231 days (Islam 2004). The bacterium's longevity might help explain why it is so commonly associated with food outbreaks. In addition, tissue damage on lettuce leaves has been shown to attract Salmonella spp. more than undamaged leaves, possibly due to the nutrient-rich environment beneath the phyllosphere (Kroupitski 2009). While internalization does not appear to be Salmonella's primary objective, it does occur. The bacterium has been shown to congregate near plant stomata—likely due to their proximity to internal photosynthates—and can gain access through these openings (Kroupitski 2009).

Research has shown that *S. enterica* may suppress plant immunity, allowing for prolonged persistence in the apoplast. Variable flagellar surface antigens and the bacteria's type III secretion system (T3SS) have been identified as important contributors to this phenomenon (Garcia 2014). Since over 2,500 serovars of *Salmonella enterica* exist, it is possible that apoplast persistence might differ between strains (Ellermeier 2006). This paper introduces a protocol to test the persistence of different *S. enterica* serovars in lettuce (*Lactuca sativa L.)* cultivar Salinas using vacuum inoculation.

METHODS

Preparation of Lettuce. 21 seeds of refrigerated Salinas lettuce (*Lactuca sativa L.*) cultivar seed stock were placed on a soaked paper towel in a petri dish and left to sprout in a well-lit area (normally, around 2 days). 18 of these sprouted seeds with similar root sizes were transplanted into Peat Pots, and a tall plastic dome was placed over the plant tray, leaving 1 inch of space for evaporation. Plants were regularly watered and maintained in a growth chamber at 15°C. Seven days after transplanting, 0.05g Peters Excel multipurpose fertilizer/plant was mixed with 30mL of industrial water and added to the tray. A gentle rocking motion was used to ensure equal fertilizer distribution. When the plants reached 2-3 weeks old, they qualified for inoculation.

Preparation of Pre-Inoculum. *Salmonella enterica* isolates from previous vegetable outbreaks were provided by various institutions, and six lettuce cv. Salinas lettuce plants were inoculated with each isolate. Three isolates (control + 2 experimental) were tested each week. The control group, coded as isolate "4", is strain 14028s. This Typhimurium serovar was chosen due to its well-documented growth pattern in similar lettuce studies in this laboratory. To prevent anaerobic growth and to ensure the viability of the isolates being tested, pre-inoculum was prepared one day before inoculation. Each of the following pre-inoculum steps use sterile materials and were carried out in the Biological Safety Cabinet (BSC). **At 10AM**, to ensure an optical density (OD) of 0.4-0.6 by 5pm, two test tubes containing 10mL LSLB media + 1 colony were prepared for each isolate. One was incubated at 28°C/150rpm and the other at 37°/150rpm. A blank was incubated at either of these temperatures. **At 5pm** the same day, the test tubes were brought back to the BSC, shook, and 1mL of each was transferred into sterile cuvettes for measurement with a Spectrophotometer. For each isolate, the test tube that yielded an OD within 0.4-0.6 was selected, and the equation (0.001 OD)(40mL H_2O) = (measured OD)(x mL) was used to determine how much of this isolate needed to be pipetted into 40mL of water to reach an OD of 0.001. Once each isolate amount had been pipetted into a labeled centrifuge tube filled with 40mL of water, each tube was vortexed for 10 seconds and 5µl was pipetted into a 125mL flask filled with 50mL of LSLB media (OD=0.0000001). These flasks, as well as a blank, were placed in an incubator overnight at 28°C/150rpm.

Preparation of Inoculum. These steps were not performed in the BSC. The following day, **at 8AM**, the flasks were gently shook to ensure even measurements. 1mL of each solution was pipetted into a new cuvette and optical densities were measured with a spectrophotometer. The final bacterial concentration used for plant inoculation was 0.002 (10^6). To arrive at this value, the equation (0.002 OD)(300mL H_2O) = (measured OD)(x mL) was used to determine how much inoculum stock needed to be added to 300mL of water. Once this amount was calculated for each isolate, it was pipetted into a clean centrifuge tube and all tubes were centrifuged at 20°C/2800rpm for 20 minutes. After centrifugation, the supernatant was discarded and ~30mL of water from the original 300mL of water already prepared for each isolate was added to the tubes. Each tube was vortexed to re-suspend the bacteria, and the final inoculum was made by re-adding this amount to the original 400mL beaker. 0.01% of Silwet L-77 (30µl for 300mL water) was added to each of the final inoculums to prevent water surface tension during vacuum inoculation. A sterilized magnetic stir bar was also added to each beaker.

Vacuum Inoculation. A flat of fertilized 2-3 week old lettuce plants were selected from the Plant Growth Chamber and brought to the bench. After stirring the inoculum for ~30 seconds to ensure even bacterial distribution, half of it was poured into a 250mL beaker and one plant was inserted, upside-down, into the solution. Aluminum foil was used to keep the soil out of the inoculum. This apparatus was then placed inside a vacuum chamber (Image 1). After replacing the lid and attaching the suction tube, the vacuum pump was turned on for exactly one minute. The suction tube was then removed, allowing the chamber to depressurize and visibly force inoculum into the apoplast of all leaves. Every 3 plants, the inoculum was replaced with the remaining 150mL that had been stirring on the bench. These steps were repeated for each isolate with a new beaker and a new set of 6 plants.

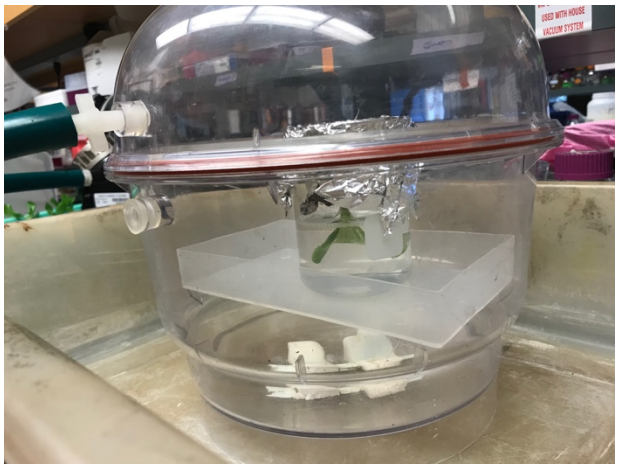

***Image 1*: Lettuce cv. Salinas in vacuum chamber, suspended in 150 mL of inoculum.**

Enumeration of *Salmonella enterica* Bacterium Persistence. On both Day 0 (inoculation day) and Day 10, the tray of inoculated plants was brought into the BSC room and the following

procedure was carried out in the BSC using sterilized equipment. The second leaves of 3 plants, identifiable by the spiral-like growth pattern of lettuce, were carefully snipped with tweezers and sterilized for 1 minute each in 2% bleach, 70% ethanol, and sterile water. These leaves were then blotted dry on paper towels and examined for any injuries that could compromise the experimental results. After drying, a sterile Number 3 cork-borer was used to punch out a total of four 0.5mm-diameter leaf discs from the upper parts of inoculated leaves. These discs were placed in 1.5 mL microfuge tubes with 100µl of sterile water. Plastic pestles attached to a hand-held electric drill were used to grind up the leaf discs until no solid pieces were visible. Each of these microfuge tubes were then vortexed, and 10µl of the first solution was pipetted into 90µl of sterile water in another 1.5 mL microfuge tube to make a 1:10 dilution. This was repeated until reaching a 10^2 dilution, and this entire procedure was repeated for each isolate using new, sterile materials. 20µl of all dilutions were plated on square agar plates to minimize pipetting errors. All plates were left to air dry in the BSC before being incubated at 28°C overnight.

Data Analysis. The following morning **at 9AM,** the dilutions that yielded a single colony forming unit (CFU) range of 10-100 were counted under a light microscope. The bacterial population was estimated by multiplying half the CFU (since we plated 20µl) by the dilution factor. The final bacterial population was evaluated by area (cm^2), so the final number of CFU was multiplied by 1.25 ($A = \pi \times r2$; $A = (3.14 \times 0.252) \times 4$). Statistical analysis was completed by calculating the average (n=6) and standard error using Microsoft Excel. Significance of the difference between two samples was obtained by performing a Student's t-test. Ideally, these experiments should be repeated multiple times to assess the robustness of data between different biological replicates.

RESULTS

Seven wildtype *Salmonella enterica* serovars were used in this study. The behavior of strain 14028s (isolate "4") in lettuce has been well-documented in the laboratory and was chosen as an internal control group.

***Table 1*: Culture Codes and respective Strain Designations for *Salmonella enterica* serovars. OASS = Outbreak-Associated *S. enterica* Strain**

Association	Isolate Code	Organism	Strain Designations
	4	*Salmonella enterica* ser. Typhimurium	14028s
OASS	**8**	*Salmonella enterica* ser. Agona	LJH0517
OASS	**15**	*Salmonella enterica* ser. Enteritidis	LJH0704
OASS	**16**	*Salmonella enterica* ser. Oranienburg	LJH0705
OASS	**17**	*Salmonella enterica* ser. Kottbus	LJH0706
OASS	**11**	*Salmonella enterica* ser. Montevideo	LJH0519
OASS	**14**	*Salmonella enterica* ser. Michigan	LJH0553

To investigate whether certain serovars persist in the apoplast of lettuce cv. Salinas better than others, batches of six, 2-week-old plants were inoculated with isolate. Two-tailed, two-sample, equal variance t-tests were performed using Microsoft Excel (=TTEST(array1, array2, 2, 2)) to compare results from Day 0 and Day 10. Significance levels of 0.05 and 0.01 were evaluated, and asterisks (*) were used to denote whether a Day 10 growth pattern was statistically significant at either level (**: significant difference at $p<0.05$. ***: significant difference at $p<0.01$).

At the beginning of this study, a protocol had not yet been developed for screening *S. enterica* serovar persistence in lettuce cv. Salinas. Thus, syringe infiltration without pre-inoculum was used in the early stages of experimental design. This method has since been abandoned due to leaf damage and possible anaerobic growth of *S. enterica*. Nonetheless, the data has been included to emphasize the need for additional experiments with isolates 14 and 15 (Figures 1 and 2).

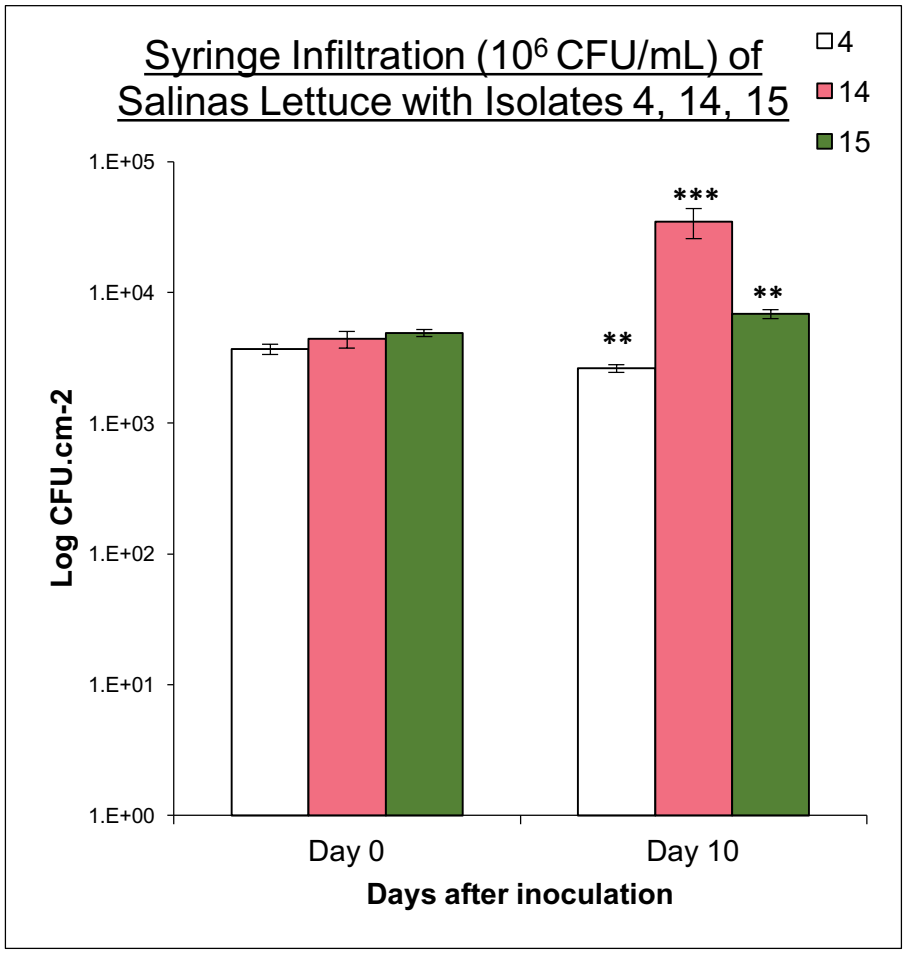

Figure 1: **Persistence of serovars 4, 14, and 15 (mean ± SEM) in inoculated lettuce cv. Salinas plants (n=18) at Days 0 and Day 10. ** significant difference at p<0.05. ***significant difference at p<0.01.**

The Day 0 x Day 10 p-values calculated for isolates 4, 14, and 15 in Figure 1 above were 0.0345, 0.0066, and 0.015, respectively. By Day 10, the concentration of each isolate had changed significantly compared to Day 0. Isolate 14 increased by almost one logarithm, and this difference was significant at the 0.01 significance level. Isolates 4 and 15 exhibited changes at the 0.05 alpha level, with isolate 4 declining slightly and isolate 15 slightly increasing in concentration (Figure 1).

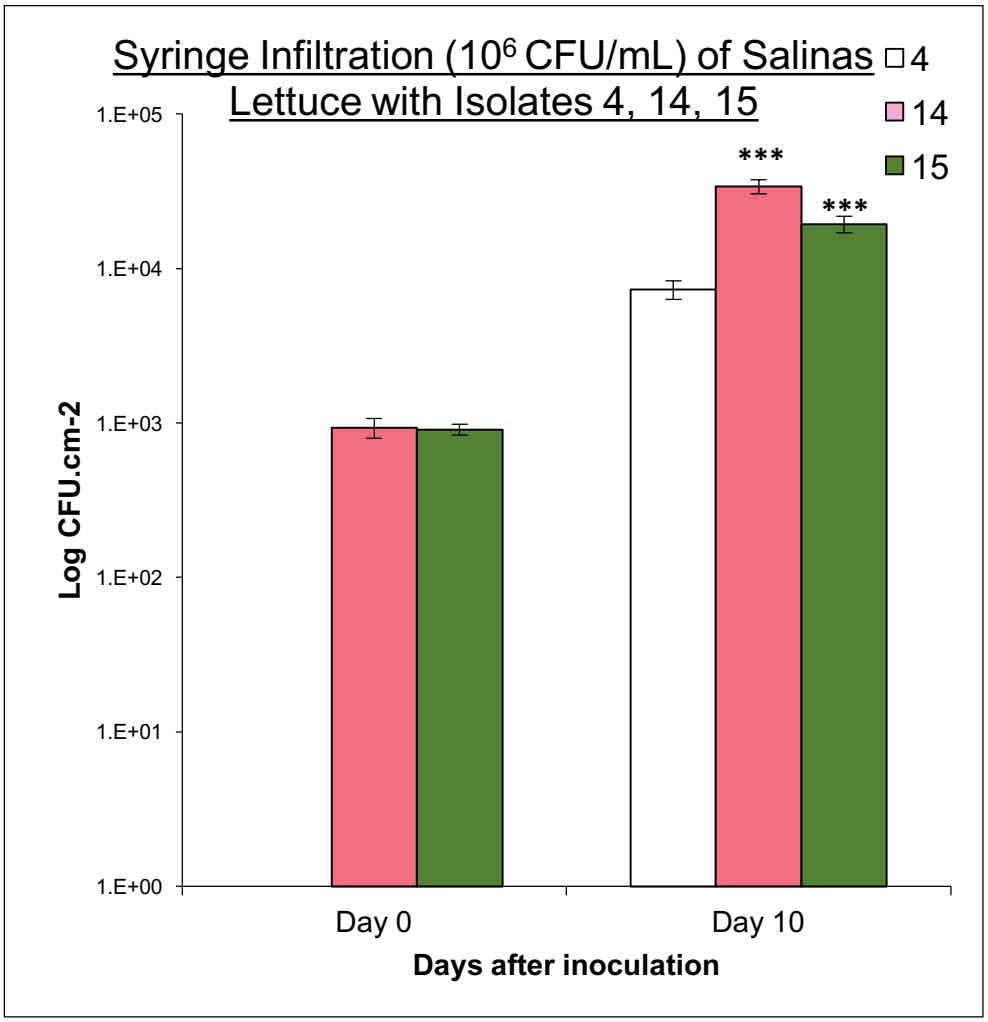

Figure 2: **Persistence of serovars 4, 14, and 15 (mean ± SEM) in inoculated lettuce cv. Salinas plants (n=18) at Days 0 and Day 10. **significant difference at p<0.05. ***significant difference at p<0.01.**

The Day 0, isolate 4 CFU results were contaminated and not included in Figure 2 above. Thus, Day 0 x Day 10 t-tests were only performed for isolates 14 and 15. The p-values for 14 and 15 were 3.0×10^{-6} and 2×10^{-5}, indicating statistically significant increases in these serovar concentrations at alpha level 0.01 (Figure 2).

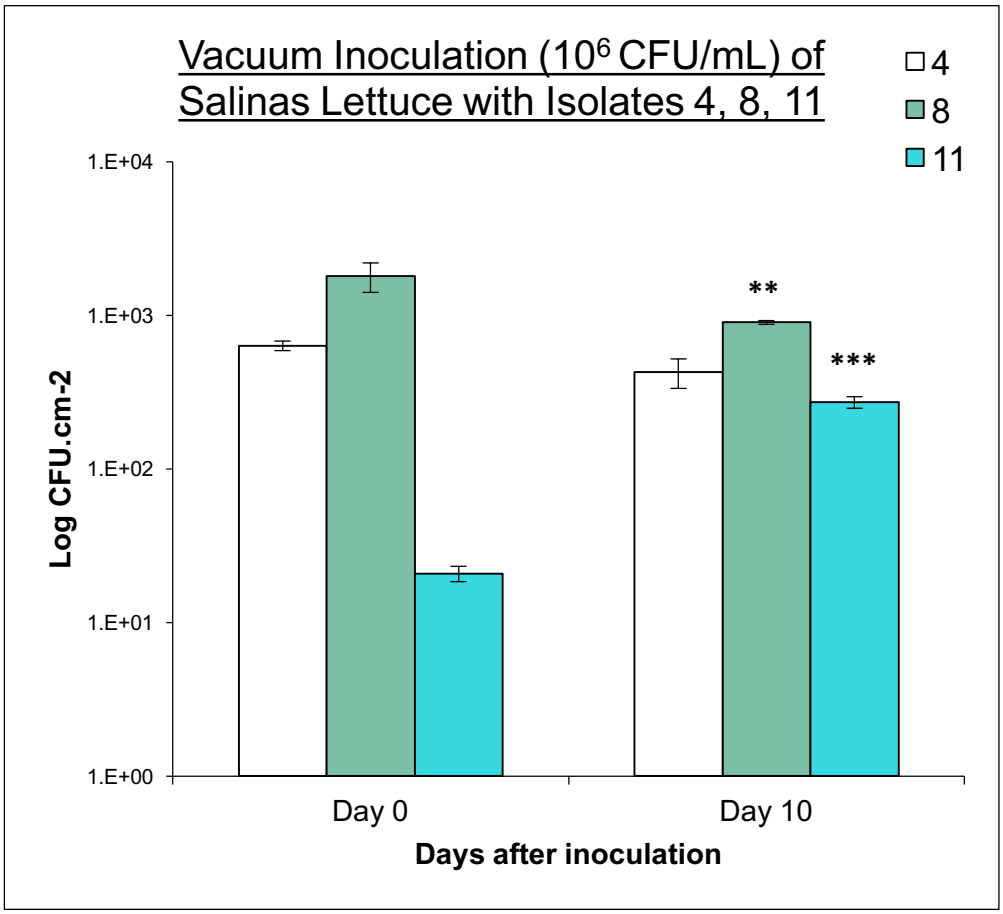

***Figure 3*: Persistence of serovars 4, 8, and 11 (mean ± SEM) in inoculated lettuce cv. Salinas plants (n=18) at Days 0 and Day 10. ** significant difference at $p<0.05$. ***significant difference at $p<0.01$.**

To prevent leaf damage and anaerobic growth, vacuum inoculation with pre-inoculum was adopted as the final protocol (as depicted in the Methods section). The first experiment with this procedure is shown in Figure 3 above. Isolates 4, 8, and 11 were tested, and Day 0 x Day 10 p-values for isolates 4, 8, and 11 were 0.074, 0.042, and 7.01×10^{-7}, respectively. On Day 10, there was a statistically significant difference in isolate 11 at $p<0.01$, as well as isolate 8 at $p<0.05$ (Figure 3).

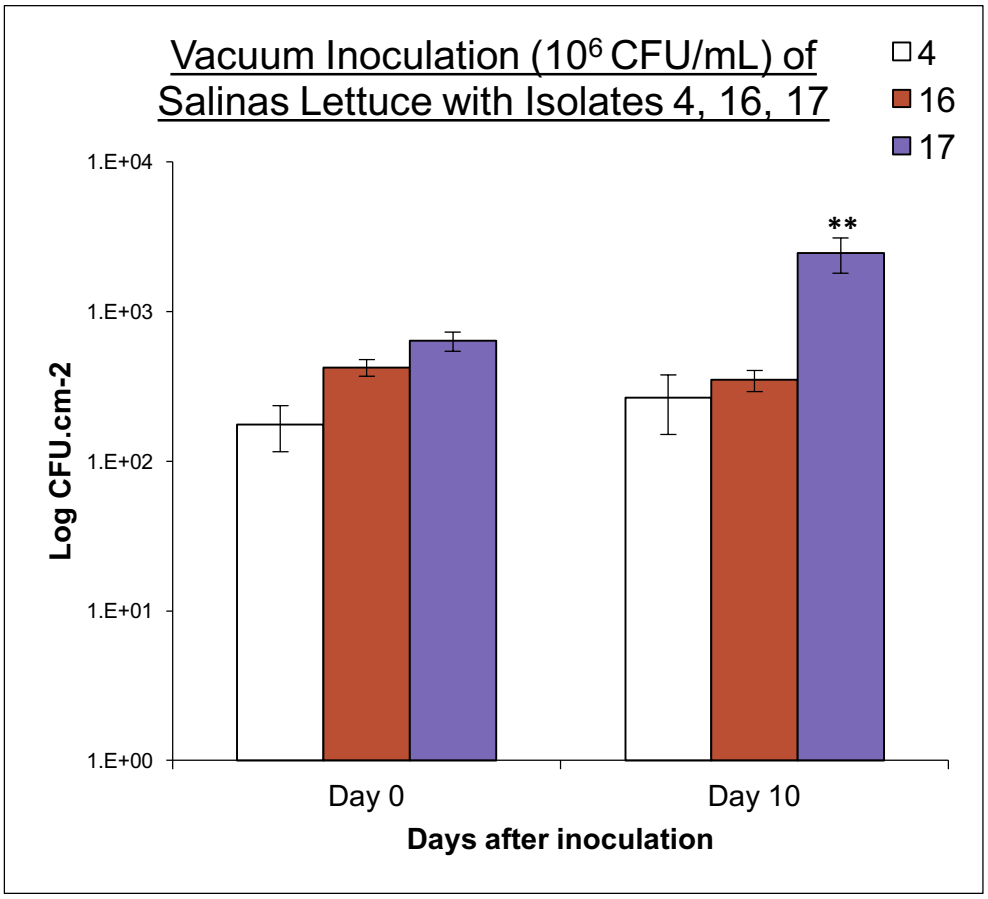

Figure 4: **Persistence of serovars 4, 16, and 17 (mean ± SEM) in inoculated lettuce cv. Salinas plants (n=18) at Days 0 and Day 10. ** significant difference at $p<0.05$**

The Day 0 x Day 10 p-values for isolates 4, 16, and 17 shown in Figure 4 above were 0.50, 0.35, and 0.0189, respectively. Isolate 17 had a statistically significant increase in growth when evaluated at alpha level 0.05, but not at alpha level 0.01. Isolates 4 and 16 persisted in the apoplast on Day 10 but did not exhibit significant changes in growth (Figure 4).

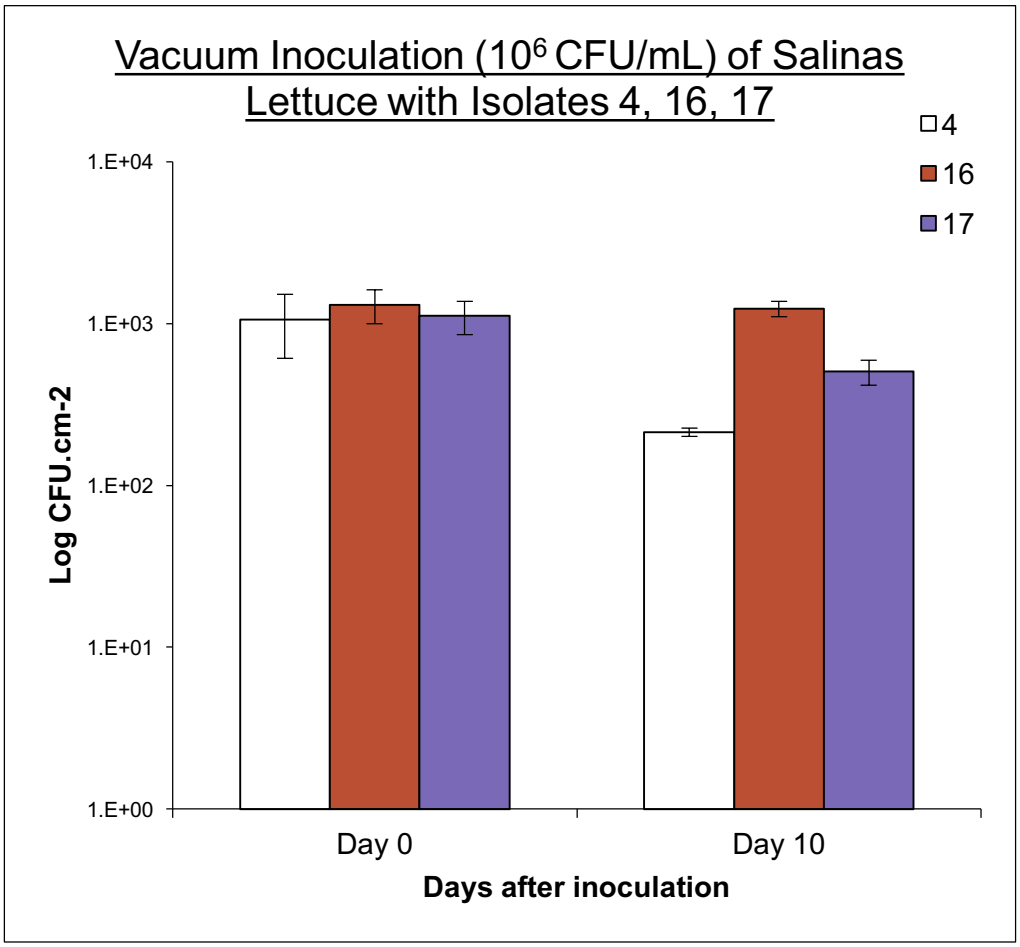

Figure 5: **Persistence of serovars 4, 16, and 17 (mean ± SEM) in inoculated lettuce cv. Salinas plants (n=18) at Days 0 and Day 10.**

Potentially due to Day 10 plating errors, isolate 4 only grew in 2 of the 6 plated squares, and isolate 16 grew in 4. Thus, Day 0 and Day 10 sample sizes differed, and variances for the samples portrayed in Figure 5 above could not be assumed equal. No t-test was performed. Isolate 17, however, did exhibit Day 10 growth and a two tailed, two sample, equal variance t-test was performed. The p-value was 0.05 (Figure 5).

DISCUSSION

The purpose of this study was to develop and test a protocol that could analyze the persistence of *S. enterica* serovars in lettuce (*Lactuca sativa L.*) cultivar Salinas. Figures 1 and 2 above depict results from syringe infiltration, which consisted of filling a blunt syringe with the proper 10^6 inoculum concentration and directly injecting the solution into the second leaf's stomata. While isolate 14 shows significant Day 10 growth in both figures, this inoculation method was ultimately abandoned. The blunt force trauma caused by syringe infiltration may induce wilting or other forms of plant immunity—any of which could alter the experimental results. In addition, the final inoculum was prepared by selecting one colony from a freshly streaked plate and allowing it to grow overnight (28°C/150rpm) in a 125mL flask filled with 50mL of LSLB media. This method was problematic because it did not filter out bacteria that were already dead, and it subjected those that were alive to compete for limited oxygen and nutrients. These bacteria may have switched to an anaerobic lifecycle, which could have altered their growth and behavior in lettuce cv. Salinas leaves. Thus, additional experiments with isolates 14 and 15 using vacuum inoculation and pre-inoculum are necessary before drawing conclusions regarding their persistence.

The first vacuum inoculation incorporating pre-inoculum protocol tested isolates 4, 8 and 11 (Figure 3). Definitive results from this experiment are not valid due to the significant difference in Day 0 bacterial concentrations in the second leaf. Immediately after inoculation, bacterial CFU/cm^2 of isolate 11 differed from isolates 4 and 8 by nearly two logarithms. Isolates 4 and 8 also differed from one another, and this was significant at $p<0.05$. Since each final inoculum contains a bacterial concentration of .002 (10^6)—and plant age, size, seed stock, and growth are uniform—the Day 0 CFU/cm^2 results for each isolate should not be statistically dissimilar. Factors that may contribute to this variance include pipetting or plating errors, counting errors, and the order of inoculation. Perhaps removal of these plants from the 15°C growth chamber induced a stomatal response, hindering inoculation efforts in the last batch of plants.

Vacuum inoculation with isolates 4, 16, and 17 proved promising. On Day 0 of the first experiment (Figure 4), isolate 4 differed significantly ($p<0.05$) from isolates 16 and 17 regarding bacterial CFU/cm^2. However, the Day 0 concentrations for isolates 16 and 17 were very similar. By Day 10, isolates 4 and 16 persisted in the second leaf but did not exhibit significant concentration changes. Isolate 17 increased in concentration by $2x10^3$ (significant at $p<0.05$). A replicate of this experiment was performed (Figure 5) and Day 0 results were fairly unanimous, with all three isolate concentrations hovering around $1x10^3$ CFU/cm^2. On Day 10, however, isolate 17 concentrations were not significantly different, and statistical analysis could not be performed on isolates 4 and 16 due to a lack of data. Since Day 0 results were normal, plating errors and/or leaf damage were likely culprits for the lack of growth on Day 10.

To eliminate bacteria and fungi that might be present on the leaf surface, leaves were regularly sterilized with bleach and ethanol prior to plating. If a leaf was torn, wilted, or otherwise damaged, these chemicals could have entered the tissue and killed the bacteria. In addition, prior to serial dilution, each second leaf must be drilled with a pestle until completely liquefied. Otherwise, bacteria may remain trapped in leaf tissues that are too large to pipette, resulting in lower-than-expected CFUs. To address these issues, caution should be taken during plating to completely grind up the second leaf, and sterilization should be limited for damaged leaves. To prevent leaf damage, plants should be regularly watered and gently handled. Vacuum inoculation may help minimize plant stress and leaf damage compared to syringe infiltration, but as indicated by the data, other factors likely play a role in leaf viability.

CONCLUSION

- Vacuum inoculation with *Salmonella enterica* is an adequate alternative to syringe infiltration
- The persistence of S. *enterica* in the plant apoplast may be serovar-specific
- Numerous biological replicates are recommended to avoid Type I error (ex. concluding that serovar concentration has changed when it has not)

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